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원 저

Preventive mechanisms of Artemisia capillaries extract on ethanol-induced apoptosis in neuroblastoma cell line

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Abstract

茵蔯藥針液이 에탄올로 유발된 신경아세포의 아폽토시스에 대한 보호 효과의 기전

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목적 : 茵蔯이 SK-N-MC 신경아세포주에서 에탄올에 의해 유발된 아폽토시스에 대한 보호작용의 기전을 연구하였다.

방법 : SK-N-MC cell line에서의 세포 보호 기전을 알아보기 위하여 reverse transcription polymerase chain reaction (RT-PCR) 기법을 이용하여 bcl-2, bax 및 caspase-3의 변화를 관찰하였다.

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결과 : RT-PCR을 이용하여 분석한 결과 SK-N-MC neuroblastoma에서 에탄올 처치는 bax, bcl-2 및 caspase-3 mRNA의 발현을 증가시켰다. 인진액의 전처치후 에탄올 처치한 신경아세포에서는 에탄올에 의해서 증가된 bax와 caspase-3 mRNA 발현이 억제되었으나, bcl-2의 발현에는 유의한 증가를 나타내지 않았다.

결론 : 이상의 결과를 통하여 에탄올에 의해서 유발된 신경아세포의 아폽토시스에서 인진이 세포보호 효과가 있음이 확인되었고 그 기전은 bax와 caspase-3의 억제에 기인할 가능성을 시사한다고 할 수 있다.

Key words **: Artemisia capillaries, apoptosis, neuroblastoma, ethanol, bax, bcl-2, caspase-3**

I. INTRODUCTION

Ethanol (EtOH) exposure has been shown to result in alteration in morphology and function of the central nervous system (CNS). Behavioral studies on the effects of ethanol treatment suggest that the hippocampus, which has been particularly identified as one of the targets for neurotoxic effects, is more sensitive than other regions and plays a prominent role in memory and learning processes[1].

But these mechanism of EtOH-induced brain damage in the developing nervous system are still elusive. In recent years, however, the role of EtOH as an inducer of apoptosis has been described in astroglia[2], neuroblastoma[3], hepatocytes[4] and thymocytes[5]. In addition, Ikonomidou et al[6]. have reported that EtOH induced apoptotic neurodegeneration in the developing rat brain in vivo.

Apoptosis, programmed cell death, play an important role in neural tissue homeostasis and is an activate form of death by which the dying neurons up regulate and/or transform certain gene. Two important groups of gene involved in apoptotic cell death are bcl families[7-9] and a class of cysteine proteases known as caspases[10,11]. The gene of bcl families can be separated into two functionally distinct gene; anti-and pro-apoptotic gene. One anti-apoptotic gene, bcl-2 is the firstly cloned gene which is expressed in the CNS, and that can protects cell death in the developing brain[12]. The other pro-apoptotic gene, bax is expressed abundantly and selectively in neurons and that promotes cell death while the bcl-2 blocks cell death[13]. The caspases stand in the crucial step of the apoptotic process and are normally expressed in many mammalian cells[14]. In particular, caspase-3, is the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many proteins[15].

Because of the possibility of the involvement of apoptosis as a process of EtOH-

induced cell death in the CNS, it appears logical that a drug which inhibits apoptosis may be of use in reducing the possible damage of the CNS by EtOH.

Artemisia capillaries (AC) is a famous traditional Oriental medical herb and used mainly as a choleretic, anti-inflammatory, and diuretic agent in the treatment of epidemic hepatitis[16]. It has been used for treatment of various liver disease, including alcoholism, in Korea. Recently, Hu et al[17]. reported that AC induces apoptosis in human hepatoma cell line SMMC-7721.

Several reports suggested that ethanol-induced apoptosis may contribute to the pathophysiology of neurodegenerative disease. However, relatively few studies have been published concerning the prevention of apoptosis by AC. In the present study, to investigate the anti-apoptotic mechanisms of AC on ethanol-induced apoptosis in the neuroblastoma cell line SK-N-MC, we have performed reverse transcription polymerase chain reaction (RT-PCR) analyses.

II. MATERIALS AND METHODS

1. Drugs & Reagents

AC was purchased from Kyung-Dong market (Seoul, Korea). After washing, AC was immersed in cold water for 12 hr, and aqueous extract from AC was made by using rotatory evaporator. Ethanol was obtained from Merck (Darmstadt, Germany).

2. Cell culture

The human neuroblastoma cell line, SK-N-MC cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured by previous reported method[18]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA). These media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL) at 37℃ in 5% CO_2, 95% O_2 in a humidified cell incubator. Cells were plated at a density of 1×10^6 cells in culture dish (Corning Incorporated, NY, USA) and the media was changed once per 2 days. For analysis of the apoptotic effects, EtOH exposed at concentration 100mM for 3hr. For analysis of the protective effects of AC against cell death induced by EtOH, cells pretreated with AC at concentration of 0.1mg/ml and 1mg/ml for 24hr.

3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SK-N-MC cells using RNAzol^{TM}B (TEL-TEST, Friendswood, TX, USA) as per the manufacturer's instructions. Single-strand cDNA was synthesized using a reaction mixture containing 2 ㎍ of mRNA template, and 1㎕ of random hexamers (Promega, Madison, WI, USA) were mixed and heated at 65℃ for 10 min. One ㎕ of AMV reverse transcriptase (Promega), 5㎕ of 10mM dNTP (Promega, Madison, WI, USA), 1㎕ of RNasin (Promega), and 5㎕ of 10x A-

MV RT buffer (Promega) were then added and the final volume was brought up to 50㎕ with dimethyl pyrocarbonate (DEPC)-treated water, and the reaction mixture was then incubated at 42℃ for 1hr. Bax, bcl-2 and caspase-3 primer sequences as each other reported by Wang et al[19]. were used. For human bax, the primers sequences were 5'-GTGCACCAAG-GTGCCGGAAC-3' (a 20-mer sense oligonucleotide starting at position 375) and 5'-TCAGCCCATCTTCTTCCAGA-3' (a 20-mer antisense oligonucleotide starting at position 560). For human bcl-2, the primer sequences were 5'-CGACGACTTCTCCCGCCGCTACCGC-3' (a 25-mer sense oligonucleotide starting at position 334) and 5'-AGATCATCTCTGCCT-GAGTATCTT-3' (a 25-mer antisense oligonucleotide starting at position 628). For caspase-3, the primer sequences were 5'-CTCGGTCTGGTACAGATGTCGATG-3' (a 24-mer sense oligonucleotide starting at position 412) and 5'-GGTTAACCCGGGTAAGAATGTGCA-3' (a 24-mer antisense oligonucleotide starting at position 922). For cyclophilin, the internal control used in the study, the primer sequences were 5'-ACCCCACCGTGTTCTTC-GAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTTGCCAT-GGACAAGATG-3' (a 20-mer sense oligonucleotide starting at position 332). The expected sizes of the PCR products are 205bp (for bax), 318bp (for bcl-2), 533bp (for caspase-3) and 299bp (for cyclophilin). PCR amplification was performed in a reaction volume of 40㎕ containing 1㎕ of each cDNA, 1㎕ of each set of primers at a concentration of 10㎌M, 4㎕ of 10 x RT buffer, and 1㎕ of 2.5 mM dNTP and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For bax, bcl-2 and caspase-3, the PCR procedure was carried out under the following conditions: initial denaturation at 94℃ for 5 min, followed by 40 amplication cycles of denaturation at 94℃ for 30sec, annealing at 58℃ for 30sec, extension at 72℃ for 30sec, and with an additional extension step at 72℃ for 5 min using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA). For cyclophilin, the condition were similar, except that 25 amplification cycles were executed. The final amount of RT-PCR products for each of the mRNA were compared densitometrically using Molecular Analyst™ software version 1.4.1 (Bio-Rad, Hercules, CA, USA).

4. Statistical analysis

Statistical analysis was performed using Student's t-test and results were expressed as mean ± S.E.M. Differences were considered significant for $P < 0.05$.

Ⅲ. RESULTS

1. Effects of EtOH and AC on expression of bax mRNA

RT-PCR analysis of the mRNA level of bax was performed in order to provide an estimation of the relative levels of expression of this gene. Fig. 1 shows the level of mRNA

expression of bax in the SK-N-MC cells. Expression of bax mRNA was increased by exposure to EtOH (2.78 ± 0.62), and this figure was reduced by 0.1 mg/ml and 1 mg/ml of AC pre-treatment before EtOH exposure to 2.34 ± 0.53 and 1.46 ± 0.34, respectively. In this results, EtOH increased bax mRNA expression and AC pre-treatment decreased bax mRNA level concentration-dependent manner.

Fig. 1. Results of RT-PCR analysis of the levels of bax mRNA. The mRNA level of gene was reduced in the EtOH-treated cells compared to those in the control. * means P < 0.05 vs. control group; # means P < 0.05 vs. 100 mM EtOH group.

2. Effects of EtOH and AC on expression of bcl-2 mRNA

RT-PCR analysis of the mRNA level of bcl-2 was performed in order to provide an estimation of the relative levels of expression of this gene. Fig. 2 shows the levels of mRNA expression of bcl-2 in the SK-N-MC cells. In the case of bcl-2, increased expression induced by EtOH treatment (3.31 ± 0.34) was not affected by 0.1mg/ml AC pre-treatment (3.05 ± 0.09) significantly and 1mg/ml of AC pre-treatment decreased bcl-2 to 1.31 ± 0.09. In this results, EtOH increased bcl-2 mRNA expression was decreased by 1mg/ml AC pre-treatment, while 0.1mg/ml did not affect bcl-2 mRNA expression.

Fig. 2. Results of RT-PCR analysis of the levels of bcl-2 mRNA. * means P < 0.05 vs. control group; # means P < 0.05 vs. 100 mM EtOH group.

3. Effects of EtOH and AC on expression of caspase-3 mRNA

RT-PCR analysis of the mRNA level of caspase-3 was performed in order to provide an estimation of the relative levels of expression of this gene. Fig. 3 shows the level of mRNA expression of caspase-3 in the SK-N-MC cells. Expression of caspase-3 mRNA was markedly increased by exposure to EtOH (16.20 ± 1.98) and this figure was reduced by 0.1 mg/ml pre-treatment before EtOH exposure to 7.21 ± 0.38, but 1 mg/ml AC prereatment did not decreased (15.82 ± 2.37). In this results, EtOH increased caspase-3 mRNA expression and 0.1 mg/ml AC pre-treatment decreased caspase-3 mRNA level.

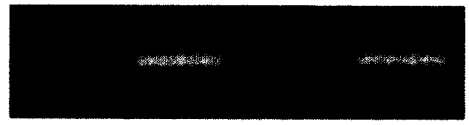

Fig. 3. Results of RT-PCR analysis of the levels of caspase-3 mRNA. The mRNA level of gene was reduced in the EtOH-treated cells compared to those in the control. * means P < 0.05 vs. control group; # means P < 0.05 vs. 100 mM EtOH group.

Ⅳ. DISCUSSION

The purpose of the present study was to find out mechanims of protective effects of AC against EtOH-induced apoptosis in cells of the neuroblastoma cell line SK-N-MC. EtOH intake is associated with various CNS impairments. However, the mechanisms of EtOH-induced brain damage in the developing nervous system are poorly understood. Recently previous studies have demonstrated that EtOH induces apoptotic neurodegeneration in the developing rat brain[6]. It is known that apoptosis, a mode of refulated cell death, is an important mechanism in the developing CNS. Apoptosis is a genetically controlled programmed cell death mechanism serving physiologic and homeostatic functions. It is defined by a number of features, including decrease in cell size, condensation of the cytoplasm, blebbing of the plasma membrane, collapse of the chromatin, fragmentation of DNA into oligonucleosome-length and apoptotic bodies. Ultimately, the apoptotic bodies are phagocytosed and degraded by neighboring cells. It is a normal process during development and a morphologically distinct form of cell death which is involved in the pathogenesis and pathophysiology of several known human disease, such an autoimmune dysfunction, cancer, stroke and neurodegenerative diseases. To date ethanol and apoptosis are known to perform very important roles in physiological phenomena and the pathophysiology of various diseases.

EtOH has been found to induce neuronal apoptosis[2-6], and understanding the molecular events triggering apoptosis is an important step toward the development of effective treatment strategies for such neurological diseases[20]. The bcl-2 family members are characterized by their ability to form complex combinations of heterodimer with bax, and homodimers with itself[7], and are regulators of neuronal apoptosis. When bax, the first proapoptotic homologue, was overexpressed in cells, apoptotic death in response to a death signal was enhanced, earning its designation as a death agonist. When bcl-2 was overexpressed, it heterodimerized with bax and cell death was repressed. Thus, the ratio of bax to bcl-2 serves to determine the susceptibility to apoptosis[8]. Recent studies have provided considerable new information concerning the mechanisms of bcl-2 activity within the apoptosis network. In previous study, bcl-2 overexpression was shown to protect against a variety of adverse conditions, including ischemia, hypoglycemia and oxidative stress[21], and it may be significant that these same conditions have often been hypothesized to be mechanisms contributing to the detrimental effects of EtOH on CNS development. But in our results, increased blc-2 expression by alcohol was not elevated by AC pre-treatment.

One set of molecules that appear to be modulated by the presence of bax is the

caspase[22]. Caspases, a family of cystein proteases, are integral parts of the apoptotic pathway[15,23] and in particular, activated caspase-3 has many cellular targets that, when served and/or activated, produce the morphologic features of apoptosis. Recent reports indicate that caspases may play a role in neuronal cell death during development as well as after neuronal injury. Deaciuc et al[24]. have reported that caspase-3 activity was significantly increased in EtOH-treated rats in vivo. Our results also showed increase of caspase-3 mRNA level in EtOH-treated cell, while cells pre-treated with AC remarkably decreased. Therefore, it was shown that AC inhibits overexpression of bax and caspase-3, apoptotic pathway genes, induced by EtOH.

In the present study, we investigate whether AC influence anti-apoptotic effects on EtOH-induced cell death in neuroblastoma cell line SK-N-MC. Based on the results, we suggested that AC possess protective effects against EtOH-induced apoptosisis in neuroblastoma cells via inhibition of bax and caspase-3 expression levels.

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