Journal of Food and Drug Analysis, Vol. 16, No. 4, 2008, Pages 52-61 藥物食品分析 第十六卷 第四期

Amaranthus spinosus L. Inhibits Spontaneous and Dexamethasone-Induced Apoptosis in Murine Primary Splenocytes

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(Received: October 12, 2007; Accepted: December 5, 2007)

ABSTRACT

Amaranthus spinosus L. (thorny amaranth) is traditionally selected to be an anti-diabetic herb in Taiwan. This study assessed the immuno-modulatory effects of wild *A. spinosus* water extract (WASWE) on spontaneous and dexamethasone (DEX)-induced apoptosis in murine primary splenocytes. The cultured splenocytes treated with WASWE products were harvested and analyzed to assess their apoptotic status according to DNA fragmentation by flow cytometry and agarose gel electrophoresis. The results showed WASWE inhibited the spontaneous and DEX-induced apoptosis of splenocytes. Furthermore, both WASWE and its activated charcoal-treated sample administered at concentration of 1.250 mg/mL inhibited intracellular protein levels of p53 (an apoptotic inducer protein) and p80 (a form of tumor necrosis factor (TNF) receptor), which are involved in apoptosis. The present study suggested the existence of a heat-labile anti-apoptotic component with high molecular weight in WASWE.

Key words: murine primary splenocytes, wild *Amaranthus spinosus* water extract (WASWE), anti-apoptosis, DNA fragmentation, medicinal vegetables

INTRODUCTION

Apoptosis, also known as programmed cell death (PCD), is a process by which cells undergo a form of non-necrotic cellular suicide[(1)]. A key feature of apoptosis is that cells undergoing this programmed form of death are recognized by phagocytes and ingested while still intact, protecting tissues from the potentially harmful consequences of exposure to the contents of the dying cells[(2,3)]. The ladder type DNA fragmentation in size multiples of 180~200 base pairs seems to be the most prominent characteristic in apoptotic cells[(4)]. In the aged, burn injury, and chronic restraint stress, the progressive loss of organ mass could be induced by either decreased cell proliferation or increased apoptotic cell death[(5-7)]. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α were also reported to induce apoptosis in immune cells[(8)]. The glucocorticoid induced apoptosis in thymocytes and was suggested through endogenous endonuclease activation[(9)]. Recently the glucocorticoid dexamethasone (DEX) is found to inhibit plasmacytoid dendritic cell differentiation and increase the apoptotic death[(10)].

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Apoptotic cell death, although it prevents healthy cell damage, is involved in many degenerative diseases such as neurodegenerative diseases, including Alzheimer's and Parkinson's[(11)], and neuromuscular diseases[(12)]. Thus, anti-apoptotic therapy in some cases, especially in normal immune cells, might be beneficial for the corresponding human diseases[(11-12)]. To protect normal cells from damage due to apoptosis, natural food components have been screened to inhibit cell or tissue apoptosis. A novel proanthocyanidin IH636 grape seed extract was found to increase *in vivo* Bcl-X_L expression and prevented acetaminophen-induced programmed and unprogrammed cell death in mouse livers[(13)]. Phenolics including ellagic acid (EA), caffeic acid (CA) and ferulic acid (FA) exhibit anti-apoptotic activity through the Bcl-2 independent mechanism in normal human peripheral blood mononuclear cells[(14)]. The pre-treatment with green tea polyphenols obviously inhibits hepatocellular apoptosis and up-regulates Bcl-2 protein expression in microcystin-LR-treated mice[(15)]. Intraperitoneal administration of caffeic acid phenethyl ester (CAPE), a component of honeybee propolis, reduces diffuse apoptosis in glandular epithelium and stromal cells in endometrial tissues of rats treated with fluoride[(16)]. Different components such as glycoprotein isolated from *Solanum nigrum* L.[(17)] and ginseng saponin[(18)] induce apoptosis in HT-29

cells and human hepatocellular carcinoma cells, recpectively. Food or herb-derived components seem to provide an anti-apoptotic possibility of normal cells.

Amaranthus spinosus L. (thorny amaranth), a plant that grows in the wild fields of Taiwan, serves extensively served as Chinese traditional medicine or wild vegetables to treat diabetes. The water extract from *A. spinosus* has been found to have a low toxicity and to demonstrate an *in vivo* anti-malarial activity in mice[19]. We previously found that wild *A. spinosus* water extract (WASWE) exerted immuno-stimulating effects via activating B lymphocytes and subsequent T cell proliferation *in vitro*[20]. In this study WASWE was further applied to primary murine splenocyte cultures to characterize its anti-apoptotic properties.

MATERIALS AND METHODS

I. *Preparation of WASWE*

The WASWE was prepared as described by Lin *et al.*[20]. Briefly, fresh leaves of *A. spinosus*, which were collected in summer from central areas in Taiwan, were weighed and chopped. Added to the same weight of deionized water, the sample was stirred in a Waring blender to extract the water-soluble constituents. The slurry was then centrifuged at 9,000 ×g (4°C) for 30 min. The supernatant was directly lyophilized to obtain a crude WASWE powder and the final yield was about 4.7% (g powder per 100 g of fresh leaves). Part of the supernatant was heated at 100°C for 30 min and then lyophilized to harvest the heat-treated WASWE powder. Part of the crude WASWE powder was dissolved in deionized water and then slowly mixed with activated charcoal (1∶1, w/w) to absorb small WASWE molecules. After standing at 4°C for 30 min, the mixture was filtered with filter paper. The supernatant was lyophilized to obtain activated charcoal-treated WASWE powder.

II. *Experimental Animals for Primary Splenocytes*

BALB/c mice (female, adult) were from the Laboratory Animal Center, College of Medicine, National Taiwan University and maintained in the Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, and the Department of Food Science and Biotechnology at National Chung Hsing University, College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The mice were housed and fed a standard lab diet (normal chow diet). The animal room was kept on a 12-h-light and 12-h-dark cycle. Constant temperature (25 ± 2°C) and humidity were maintained. The animals (8- to 10-weeks old) were sacrificed using CO_2 inhalation to obtain spleens. The abdominal cavities were opened aseptically and the spleens were removed.

III. *Primary Splenocyte Cultures*

The primary splenocyte culture preparation and assay of *in vitro* apoptosis using WASWE were manipulated as described by Lin *et al.*[20]. Spleens were removed aseptically from adult BALB/c mice. Single spleen cells were prepared by lysing the red blood cells. Splenocytes were isolated from each animal and adjusted to 2×10^6 cells/mL in TCM (a defined commercial serum replacement, Celox Laboratories Inc.) medium [10 mL of TCM, 500 mL of RPMI 1640 medium (Atlanta biologicals, M30115), and 2.5 mL of antibiotic-antimycotic solution (100×) containing 10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of amphotericin B in 0.85% saline (Atlanta Biologicals, B22110)]. Splenocytes (0.5 mL/well) with WASWE (0.5 mL/well; 0, 0.250, 0.500, 1.250, or 2.500 mg/mL in TCM medium, respectively) were plated in 24 well plates. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for various time (0, 6, 12, 24, 48, or 72 h). The cells were collected and assayed for the nuclear DNA cell-cycle phase distribution. The viable cells in each case were counted in haemocytometer using trypan blue exclusion staining.

IV. *Cell Cycle Analysis by Flowcytometry*

To determine the cell-cycle phase distribution of nuclear DNA[21-22], primary splenocytes (2×10^6 cells/mL, 0.5 mL/well) from female BALB/c mice exposed to WASWE (0.5 mL/well) for various times (0, 6, 12, 24, 48, or 72 h) were individually collected into a 1.5 mL microcentrifuge tube. The cells were harvested by centrifugation at 400 ×*g* for 10 min and washed twice in ice cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 μm filtered). The cell pellet was re-suspended in 0.4 mL of PBS, and then fixed with 0.4 mL of cold (-20°C) absolute ethanol for 30 min by thorough and slow mixing. The fixed cells were spun down at 3,000 ×*g* for 10 min and then 10,000 ×*g* for 1 min. The cell pellet was washed with PBS once. Then, the cell pellet was vortexed and incubated with 100 μL of RNase A solution (100 μg/mL PBS) at 37°C for 30 min. The nuclear DNA of splenocytes was labeled with 100 μL of propidium iodide (PI, 10μg/mL PBS, Sigma P4170) at 4°C for 15 min. Finally, the nuclear DNA cell-cycle phase distribution was determined using a FACScan flow cytometer (Becton Deckinson, CA). Cell Quest software (Becton Deckinson, CA, USA) was run for data acquisition and analysis. Fluorescence detector (FL2-A) for PI was equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale, Becton Deckinson). Total 10,000 events were acquired for analysis. Histograms are displayed with DNA content (x-axis, PI-fluoresence) versus cell counts (y-axis). The apoptotic cell content is the percentage of cells with subG_1DNA content.

V. *Analysis of DNA Fragmentation by Agarose Gel Electrophoresis*

To assay the effects of WASWE administration on splenocyte DNA fragmentation due to apoptosis, the DNA fragmentation pattern (DNA ladder) was analyzed by 1.5% agarose gel electrophoresis[21,23]. Splenocytes (6×10^6 cells) incubated without and with WASWE (1.250 mg/mL) or/and dexamethasone ((11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methyl-pregna-1,4-diene-3,20-dione, DEX, 10^{-7} M, an apoptosis-inducing reagent) for various time (0, 18, 24, 48, or 72 h) were individually collected by centrifugation at 200 ×*g* for 10 min and then washed with PBS twice. The cell pellets were treated with 0.5 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% Triton X-100) for 10 min at room temperature. After centrifugation at 10,000 ×*g*, 4°C for 10 min, the supernatant was incubated with proteinase K (100 μg/mL) at 50°C overnight. The DNA in lysed solution was extracted using chloroform/phenol (1:1) and precipitated with equal volume of NaCl (500 mM) and isopropanol at -20°C overnight. DNA was collected by centrifugation at 14,000 ×*g* and 0°C for 30 min. The DNA pellet was repeatedly washed with 70% ethanol and collected by centrifugation at 14,000 ×*g* and 0°C for 10 min. The DNA was dissolved in 100 μL of TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and incubated with 1 μg/mL of RNase A for another 1h at 37°C. Horizontal electrophoresis was performed and DNA (500 ng) was loaded over a 1.5% agarose gel. The bands were visualized using ethidium bromide staining under UV illumination. Photographic negatives were scanned and quantified with a Microcomputer Imaging Device (MCID-M4 3.0; Imaging Research Inc., Ontario, Canada) and associated software[24]. The DNA fragmentation level was expressed as relative density (D) × area (A) of small DNA fragment bands (< 800 bps) to total DNA in each lane.

VI. *Analysis of Possible Pro-apoptotic and Anti-apoptotic Protein Expressions*

Splenocytes were cultured without or with WASWE as indicated and lysed on ice for 30 min with 200 μL of an extraction buffer containing 10 mM Tris (pH 7.2), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM sodium orthovanadate, 100 mg/mL phenylmethylsulfonyl fluoride (PMSF), and 0.225 U/mL aprotinin[25]. After centrifugation at 16,000 ×*g* for 20 min at 4°C, the supernatants were collected and mixed with SDS–PAGE sample buffer containing 2-mercaptoethanol. The protein concentration was determined using BCA (Bicinchonic acid) protein assay reagent kit (Pierce 23227) and the protein concentration from each individual treatment was adjusted to the same. Aliquots of 10 μL from each cell lysate were analyzed by SDS-PAGE electrophoresis in a 12.5% polyacrylamide gel containing 0.1% SDS using a Mini-PROTEIN II cell (Bio-Rad Laboratories). The electrophoresis was carried out at 110 V, 30 mA for about 1.5 h. The gels were then stained with commassie brilliant blue R stain.

VII. *Statistical Analysis*

Data were analyzed by the Windows SAS program (Version 6.12). Data are expressed as mean ± S.D. and analyzed statistically using ANOVA followed, if justified by the statistical probability ($P < 0.05$), by Duncan's New Multiple Range test or unpaired Student's t-test. Differences were considered statistically significant if $P < 0.05$.

RESULTS

I. *Effects of WASWE Administration on Spontaneous Apoptosis of Primary Splenocytes from BALB/c Mice*

To evaluate the effects of WASWE on the spontaneous apoptosis of splenocytes from female BALB/c mice, the splenocytes were co-incubated with various concentrations of WASWE for the period of the 72 h. The apoptosis rate and viable cell number were measured by the FACS and trypan blue methods, respectively. Figure 1 shows the effects of WASWE administration on mouse splenic cell cycle regulation incubated at 37°C for 72 h. Splenocyte

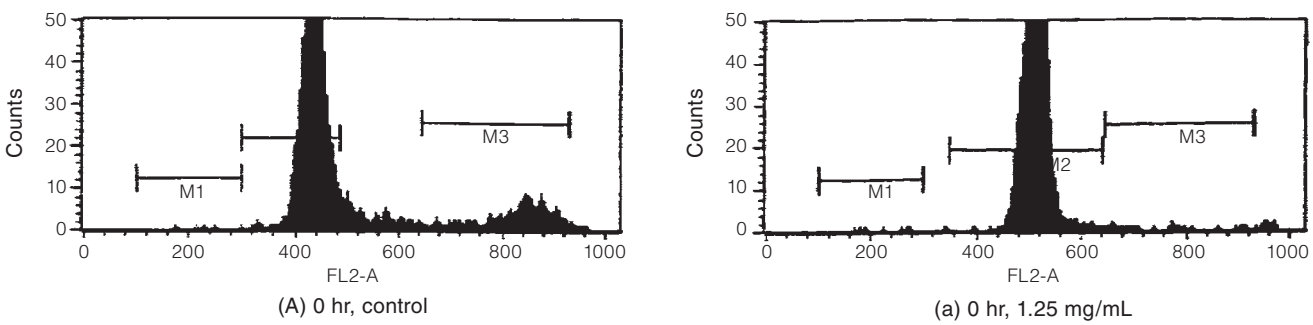

Figure 1. Effects of WASWE administration on mouse splenic cell cycle regulation incubated at 37°C for 72 h. Splenocyte cultures without or with WASWE (1.250 mg/mL) were incubated and harvested at the indicated times 0, 6, 12, 24, 48, and 72 h, and cell cycle phase distribution of nuclear DNA was determined by flow cytometry using PI as DNA-binding fluorochrome. Histograms display DNA content (x-axis, PI-fluorescence) vs. counts (y-axis). M1: apoptotic cells. M2: live cells. M3: proliferating or aggregated cells.

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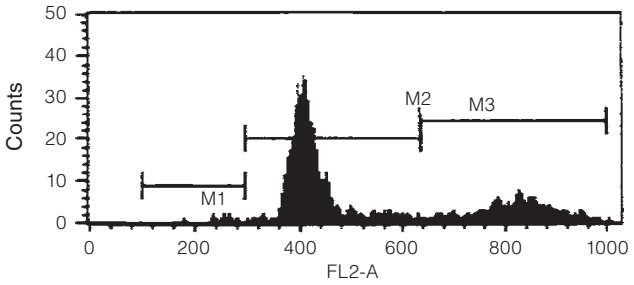
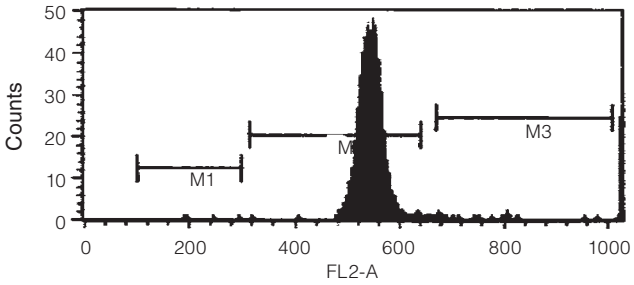
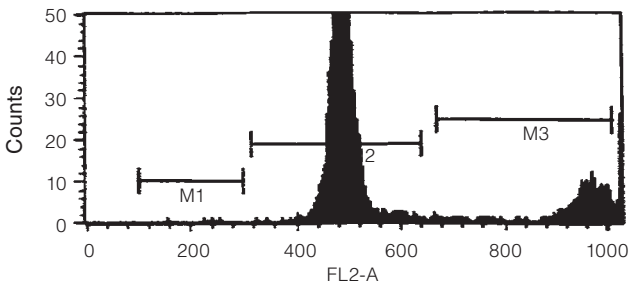


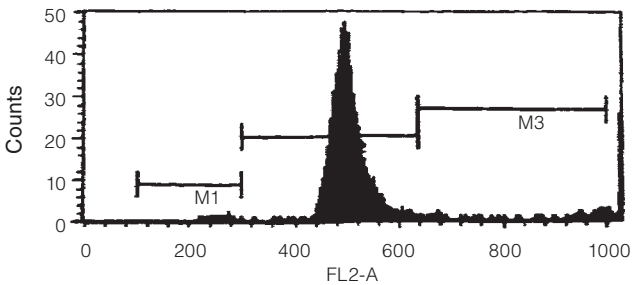
(B) 6 hr, control



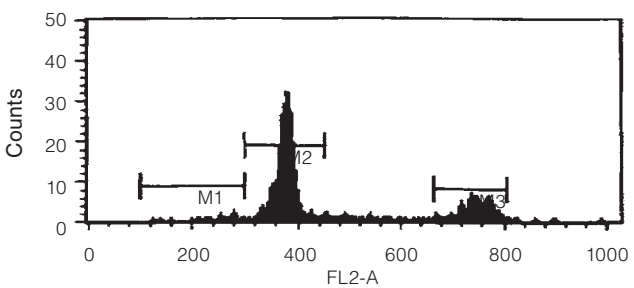
(b) 6 hr, 1.25 mg/mL



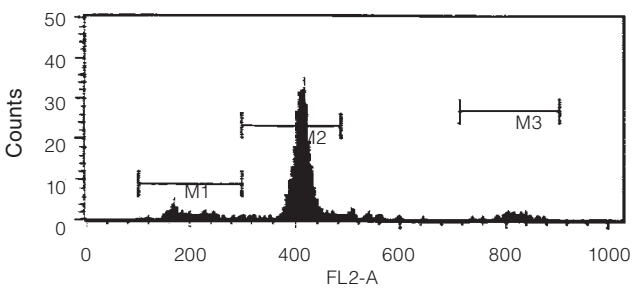
(C) 12 hr, control



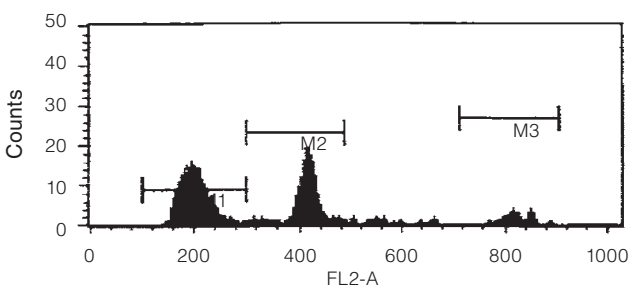
(c) 12 hr, 1.25 mg/mL



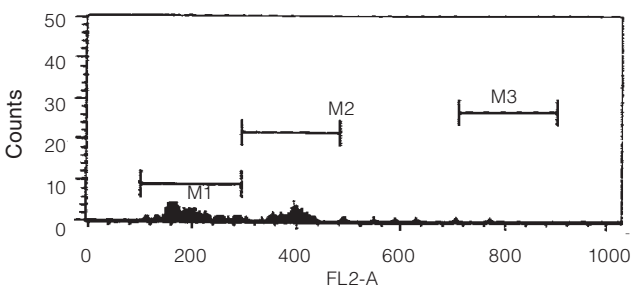
(D) 24 hr, control



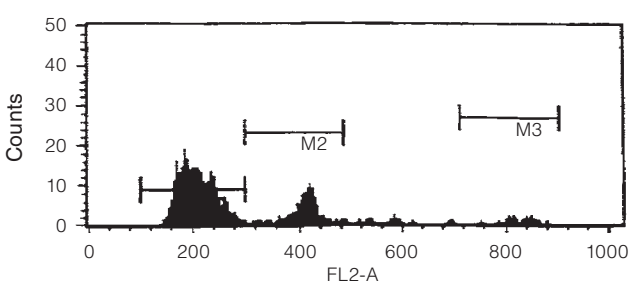
(d) 24 hr, 1.25 mg/mL



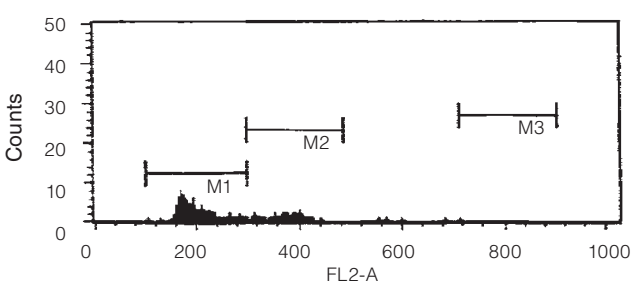
(E) 48 hr, control



(e) 48 hr, 1.25 mg/mL



(F) 72 hr, control



(f) 72 hr, 1.25 mg/mL

Figure 1. Continued

cultures without or with WASWE at the indicated concentration of 1.250 mg/mL were incubated and harvested at the indicated time intervals of 0, 6, 12, 24, 48, and 72 h, and cell cycle phase distribution of nuclear DNA was determined by flow cytometry using PI as DNA-binding fluorochrome. Histograms were displayed with DNA content (x-axis, PI-fluorescence) vs. counts (y-axis). M1, M2, and M3 respectively represent the changes in apoptotic cells, live cells, and proliferating or aggregated cells. The results showed that apoptotic cell counts (M1) increased as the incubation time extended. However, WASWE administration at the indicated concentration inhibited spontaneous apoptosis of primary splenocytes during 72 h incubation. To demonstrate the differences of splenic cell apoptosis after WASWE administration, the spontaneous apoptosis at various incubation time and various WASWE concentrations used was further compared. Figure 2 showed that all concentrations of WASWE administration (0.125, 0.250, 0.625, and 1.250 mg/mL) significantly inhibited the spontaneous primary splenocyte apoptosis in a dose-dependent manner for 72 h *in vitro* incubation. The spontaneous apoptotic control cells reached more than 25% for 72 h incubation, whereas the apoptosis of splenocytes treated with WASWE significantly decreased.

II. *Inter-changes Between Percentage of Spontaneous Apoptotic Cells and Viable Cell Number of Splenocytes Administrated with Various Concentrations of WASWE*

To clarify the correlation between anti-apoptotic and proliferated cells, inter-changes between % of apoptotic cells and viable cell number were depicted as Figure 3 Figure 3(A) showed that apoptotic percentage and viable cell number of splenocytes were affected by WASWE in a dose-dependent manner. The WASWE concentration at the 1.250 mg/mL exerted a maximum activity against spontaneous apoptosis of splenocytes. However, a reverse correlation was shown to exist between apoptotic and viable cell numbers. Real apoptotic cell numbers at the indicated concentrations of WASWE administration were obtained from % of apoptotic cells × viable cell number, and the results were shown in Figure 3(B). The results showed that real apoptotic cell numbers of splenocytes were decreased by WASWE in a dose-dependent manner. The real apoptotic cell numbers were decreased from 20.6×10^4 to 6.68×10^4. The results suggested that WASWE administration indeed inhibit spontaneous apoptosis of splenocyte *in vitro* especially at the indicated concentration of 1.250 mg/mL.

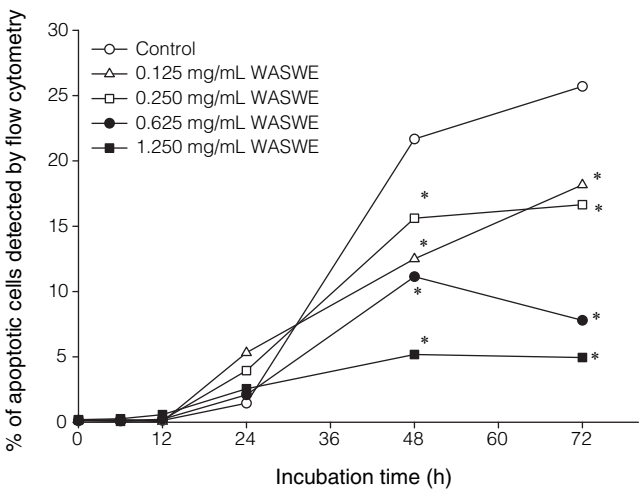


Figure 2. Effects of WASWE administration at the indicated concentrations on spontaneous apoptotsis of mouse splenocytes incubated at 37°C for 72 h. The % of apoptotic cells of each point calculated from cytometry represents the mean value (n=2). The original cell density was 1×10^6 cells/mL. Data are assayed using unpaired Student's *t*-test. Asterisk(*) means significantly different ($P < 0.05$) from the control at the same experimental point through 48 or 72 h incubation.

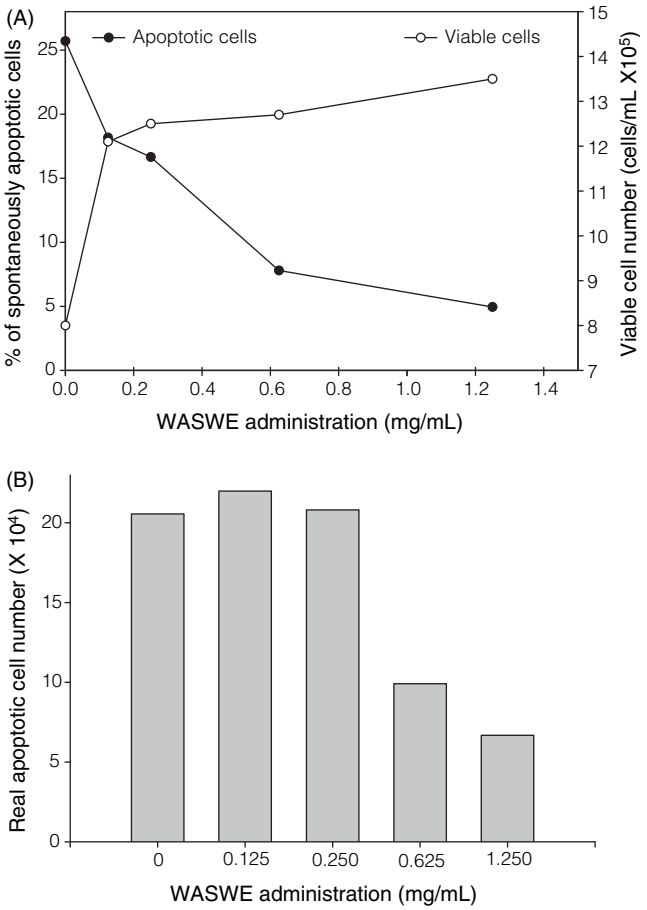


Figure 3. Inter-changes between % of apoptotic cells and viable cell numbers (A) and real apoptotic cell number (B) of splenocytes administrated with different concentrations of WASWE incubated at 37°C for 72 h. The real apoptotic cell number was obtained from % of apoptotic cells × viable cell number. The original cell density was 1×10^6 cells/mL. Each point calculated from flow cytometry and trypan blue exclusion method represents the mean value (n = 2).

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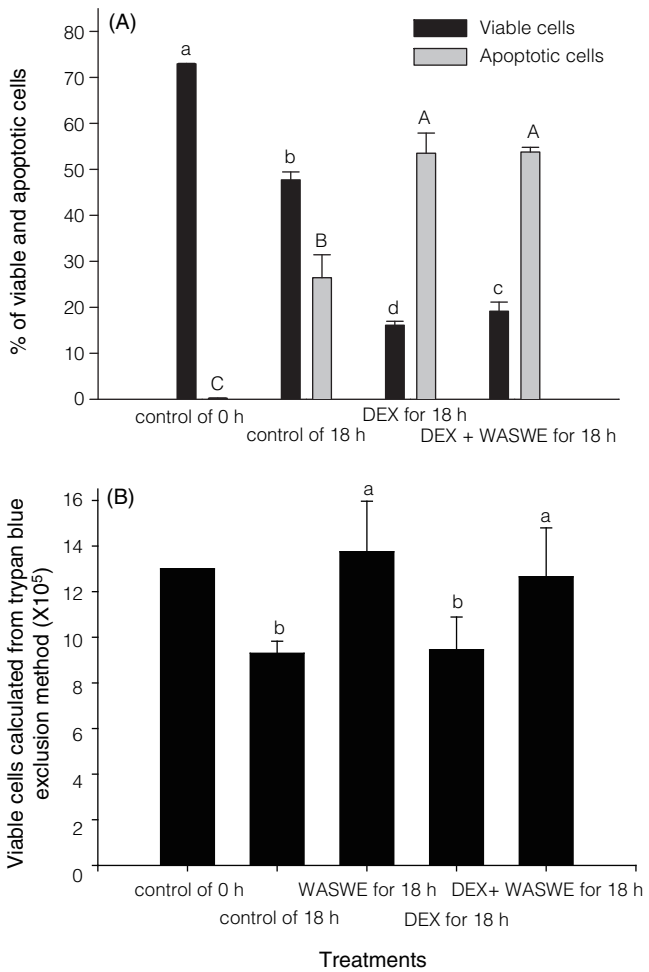

Figure 4. Viable and apoptotic cells in splenocytes cultured in the absence or presence with dexamethasone (DEX, 1×10^{-7} M) and incubated without or with WASWE (1.250 mg/mL) for 18 h and respectively detected by flow cytometry (A) and trypan blue exclusion method (B). The original cell density was 1.3×10^6 cells/mL. Each bar represents the mean value +/– SD (n = 4). Within each graph bars not sharing a minuscule letter or capital letter, respectively, are significantly different ($P < 0.05$) from each other according to Duncan's New Multiple Range test.

III. *Effects of WASWE Administration on Dexamethasone-induced Apoptosis of Primary Murine Splenocytes*

To further elucidate the effects of WASWE administration on glucocorticoid-induced apoptosis, dexamethasone (DEX, 1×10^7 M) in the absence or presence of WASWE (1.250 mg/mL) was administrated to splenocyte cultures for 18 h. Splenocytes were detected by flow cytometry and trypan blue exclusion method. Figure 4 shows viable and apoptotic cells in splenocytes cultured in the absence or presence of DEX and incubated without or with WASWE (1.250 mg/mL) for 18 h. The results showed that antagonistic effects of WASWE and DEX existed between apoptotic (M1) and live cell (M2) rates (Figure 4(A)). The results showed that apoptotic cells (M1) increased, whereas live cells (M2) decreased significantly as the incubation time lengthened to 18 h. DEX (1×10^7 M) treatment for 18 h significantly induced apoptosis and decreased live cells in splenocyte cultures (Figure 4(A)). To avoid the confounding of percentage expression, viable cells were also calculated by trypan blue exclusion method (Figure 4(B)). The results showed that WASWE administration significantly ($P < 0.05$) increased spontaneous and DEX-treated viable cells at the incubation of 18 h.

IV. *Effects of WASWE Administration on Spontaneous or Dexamethasone-induced DNA Fragmentation of Primary Murine Splenocytes*

Spontaneous or dexamethasone-induced splenocyte cultures treated with WASWE at 1.250 mg/mL for 72 h were assayed to further clarify the effects of WASWE administration on DNA fragmentation due to apoptosis. DNA was extracted and electrophoresis was carried out in a 1.5% agarose gel. Figure 5 shows the effects of WASWE administration on DNA fragmentation due to spontaneous and DEX-induced apoptosis. The DNA isolated from spontaneous (control) and DEX-treated splenocytes exhibited a ladder pattern of DNA fragments with size multiples of 180~200 base pair units (bps), as displayed in lanes 2~12 of Figure 5. The DNA ladders from splenocyte cultures

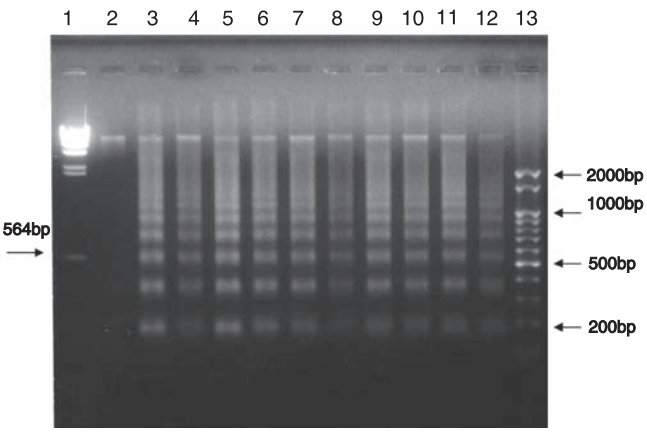

Figure 5. Spontaneous or dexamethasone-induced DNA fragmentation in mouse splenocytes treated with WASWE at the indicated concentration of 1.250 mg/mL for different incubation time. DNA was extracted and electrophoresis was carried out in 1.5% agarose gel. lane 1: λ DNA/Hind III marker, lane 2: spontaneous DNA fragmentation for 0 h incubation, lane 3: spontaneous DNA fragmentation for 18 h incubation, lane 4: DNA fragmentation cultured with WASWE for 18 h, lane 5: DNA fragmentation cultured with dexamethasone (1×10^{-7} M) for 18 h, lane 6: DNA fragmentation cultured with dexamethasone and WASWE for 18 h, lane 7: spontaneous DNA fragmentation for 24 h incubation, lane 8: DNA fragmentation cultured with WASWE for 24 h, lane 9: spontaneous DNA fragmentation for 48 h incubation, lane10: DNA fragmentation cultured with WASWE for 48 h, lane 11: spontaneous DNA fragmentation for 72 h incubation, lane 12: DNA fragmentation cultured with WASWE for 72 h, lane 13: 100 bp DNA ladder marker.

due to apoptosis were obvious for 72 h incubation. Quantitative measurements of DNA fragmentation were further performed using a microcomputer imaging device (MCID). The quantitative apoptotic levels based on DNA fragmentation from 180~200 bps (bottom band 1 in lanes 2~12) of Figure 5 is shown as Table 1. Table 1 shows desitometric profiles of DNA distribution in spontaneous and DEX-treated splenocytes derived from photographic negatives of the gel in Figure 5. There was apparent in general shift in density and area from regions of high to low molecular weight in the treated cells. The DNA fragmentation was negligible in the control of 0 h and merely 1.27% (Figure 5 lane 2). After 18 h incubation, spontaneous DNA fragmentation increased to the level of 4.78% (Figure 5 lane 3). However, WASWE administration lowered the DNA fragmentation level to 1.17% (Figure 5 lane 4). After 24 h incubation, spontaneous DNA fragmentation increased to the level of 2.87% (lane 7); whereas WASWE administration decreased the DNA fragmentation level to 0.09% (lane 8). Even though the primary cells might be killed through physical damage at making of single cells and malnutrition in the media during 48~72 h incubation (Figure 1), WASWE administration provided the protection and slightly inhibited the apoptosis-induced DNA fragmentation of splenocytes (decrease, respectively, from 1.99% to 1.63% and from 1.38% to 0.48%). After exposure for 18 h, DEX (1×10^{-7} M) administration induced 5.21% of apoptosis-induced DNA fragmentation (lane 5). WASWE administration lowered the DEX-induced DNA fragmentation to 3.67% (lane 6). The results indicated that spontaneous or DEX-induced DNA fragmentation due to apoptosis could be inhibited by WASWE administration, especially during the first 18~24 h experimental period of incubation.

Table 1. Quantitative apoptotic levels based on 180-200 bps of DNA laddering fragments from administrated splenocytes of Figure 5

lanes	bands[a]	D × A[b]	DNA fragmentation (%)[c]
Lane 2,	band 1	2	1.27
control of 0 h	total	197	
Lane 3,	band 1	34	4.78
control of 18 h	total	721	
Lane 4,	band 1	6	1.17
WASWE for 18 h	total	537	
Lane 5,	band 1	40	5.21
DEX for 18 h	total	763	
Lane 6,	band 1	24	3.67
DEX+WASWE for 18 h	total	644	
Lane 7,	band 1	18	2.87
control of 24 h	total	631	
Lane 8,	band 1	0	0.09
WASWE for 24 h	total	437	
Lane 9,	band 1	13	1.99
control of 48 h	total	652	
Lane 10,	band 1	9	1.63
WASWE for 48 h	total	580	
Lane 11,	band 1	8	1.38
control of 72 h	total	598	
Lane 12,	band 1	2	0.48
WASWE for 72 h	total	373	

[a]band 1: 180~200 bps; total: all the visualized bands in the same lane.
[b]D × A: density multiplies area in the gel photograph.
[c]DNA fragmentation (%) = ((D×A) of band 1/ (D×A) of all bands in the same lane) × 100.

V. *Effects of WASWE Products on Intracellular Protein Expression of Splenocytes*

To clarify the effects of WASWE administration on the intracellular protein expression of splenocytes, various WASWE products were incubated with splenocytes for 48 h. The intracellular proteins were extracted and subjected to 12.5% SDS-PAGE electrophoresis analysis. The results, as shown in Figure 6, two novel intracellular proteins, with molecular weights of 53 kDa (p53) and 80 kDa (p80), were obviously changed after WASWE administration (Figure 6). On the control lane, rare expressions of p53 and p80 were observed (Figure 6 lane 1). Contrary to our expectation, the expression levels of p53 and p80 increased upon administrations with WASWE, activated charcoal- or heat-treated WASWE for 48 hr at the indicated low concentration of 0.078 mg/mL (Figure 6 lanes 2, 3, and 4). However, the expressions of p53 and p80 decreased after administrations with WASWE and acti-

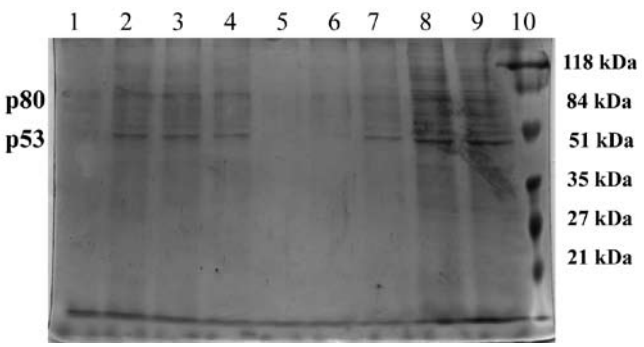

Figure 6. Effects of different WASWE products on intracellular protein expression of splenocytes incubated for 48 h. Original cell density: 5×10^6 cells/mL. Gel: 12.5% SDS-PAGE. lane 1: control, lane 2: WASWE (0.078 mg/mL), lane 3: activated charcoal-treated WASWE (0.078 mg/mL), lane 4: heat-treated WASWE (0.078 mg/mL), lane 5: WASWE (1.250 mg/mL), lane 6: activated charcoal-treated WASWE (1.250 mg/mL), lane 7: heat-treated WASWE (1.250 mg/mL), lane 8: LPS (5μg/mL), lane 9: PHA (5μg/mL), lane10: protein markers.

vated charcoal-treated WASWE at the indicated high concentrations of 1.250 mg/mL (Figure 6 lanes 5, 6, and 7). A biphasic effect on intracellular expression levels of p53 and p80 existed upon administration of extremely low and high concentrations of WASWE.

DISCUSSION

Apoptosis is verified to participate in many degenerative, neurodegenerative, and neuromuscular diseases[11,12]. The present study demonstrated that WASWE administration significantly inhibited spontaneous primary splenocyte apoptosis in a dose-dependent manner *in vitro* (Figure 1 and Figure 2). The results further indicated that DEX-treatment for 18 h significantly induced apoptosis of splenocytes, but decreased viable cells in splenocyte cultures (Figure 4). However, WASWE administration significantly increased spontaneous and DEX-treated viable cells compared to those of control and DEX treatment only at the same incubation time of 18 h (Figure 4(B)). Chronic restraint stress results in significant increase in the expression of pro-apoptotic genes, including Fas, FADD, p53, p21, and BNIP3 (Bcl-2/adenovirus E1B 19 kDa-interacting protein 3) in the spleen from restraint stressed mice[26]. Decrease in Bcl-2 and increase in Fas receptor expression as well as the increased activation of caspase 8, 3, and 9 in splenocytes from aged C57/BL mice were also reported[5]. Forrest *et al.*[26] have suggested that finding a target for anti-apoptotic therapy to protect splenocytes is possible. The anti-apoptotic therapy in some cases such as apoptotic cell death in aging and stress might be beneficial and possible. This study suggests that WASWE exhibited *in vitro* anti-apoptotic potential in situations of spontaneous or glucocorticoid DEX-treated stress. It might be further applied to treat particular diseases due to unnecessary apoptosis in splenocytes. However, the effective concentration (1.25 mg/mL) used in this study was still too high. The WASWE should be further purified and unraveled its immuno-modulatory mechanism against splenocytes' apoptosis.

The ladder type DNA fragmentation in size multiples of 180~200 bps due to nuclease activation is the most prominent characteristic in apoptotic cells[4]. This report showed the effects of WASWE administration on DNA fragmentation due to spontaneous and DEX-induced apoptosis in primary murine splenocytes. Our results displayed the DNA ladders in the agarose gel. The shift in density and area from regions of high to low molecular weight in the treated cell lanes was obvious (Figure 5). Quantitative measurement of DNA fragments of 180~200 bps indicated that spontaneous or DEX-induced DNA fragmentation due to apoptosis could be inhibited by WASWE administration, especially during the first 18~24 h of experimental incubation period (Table 1). The results suggest that WASWE might have direct or indirect inhibitory activity on nucleases (DNases).

The present study showed that two novel proteins, p53 and p80, in splenocytes were obviously regulated by WASWE (Figure 6). The p53 expression inhibits the progression of the cell cycle or induces apoptosis[27,28]. The p80 protein expressed in myeloid cells is one form of the tumor necrosis factor (TNF) receptors. The TNF-receptor (p80) is involved in TNF-induced DNA fragmentation, one of the characteristic events at the early stage of apoptosis[29]. The results in this study demonstrated that WASWE products at the indicated concentration of 0.078 mg/mL increased intracellular protein levels of p53 and p80 (Figure 6 lanes 2, 3, and 4). The results suggested that WASWE at the lower concentration induced apoptosis of splenocytes. However, at the higher concentration (1.250 mg/mL) WASWE inhibited intracellular protein expressions of p53 and p80 (Figure 6 lanes 5, 6, and 7), suggesting that the higher concentration of WASWE inhibits apoptosis via down-regulating the expressions of p53 and p80. Obviously, the biphasic effects on intracellular protein levels of p53 and p80 existed upon treatment of extremely low and high concentrations of WASWE. The biphasic effects seem to be universal in immunologic experiments. A recent report indicates that low and high levels of α-tocopherol exert opposite effects on IL-2 expression in activated splenocytes, possibly through modulating PPAR-γ, IκBα, and apoptotic pathway[24]. WASWE has been demonstrated immuno-stimulating effects via activating B lymphocytes and subsequent T cell proliferation *in vitro* at concentration of 1.250 mg/mL[20]. However, WASWE at the lower administration concentration has little stimulation on splenocyte proliferation. This study further demonstrated that WASWE at the lower administration concentration may cause apoptosis of splenocytes *in vitro*. However, the regulatory mechanisms of WASWE remain to be further clarified. The present study provides preliminary evidence and suggests that the regulatory mechanisms of WASWE against apoptosis might be via inhibiting intracellular protein levels of p53 and p80. However, the specific cellular protein of p53 and p80 should be further characterized by immunoblotting analysis. Unfortunately, we could not determine other apoptosis-related intracellular proteins, including Bcl-2, Bcl-X_L, and Bcl-Xs, using western blotting method (data not shown). More apoptotic signals including intracellular ROS, caspase 9, 8, caspase 3, Bax/Bcl-2 ratios, or poly (ADP-ribose) polymerase in treated cells should be further clarified[17].

Activated charcoal can absorb lower molecular weight components such as dyes[30], phenols[31], phenolic compounds[32], and different drugs as well as toxic agents[33], including histamine[34], acetaminophen[35]. Furthermore, the activated charcoal also adsorbs lignin and tannin colours[36], and the natural female sex hormones 17β-estradiol, estriol and estrone[37]. Thus, these compounds described above in WASWE were possibly eliminated by the activated charcoal treatment.

However, heat may destroy most proteins. In this study WASWE were respectively treated with activated charcoal or heat in order to characterize its anti-apoptotic components. The results from this study indicated that activated charcoal-treated WASWE at the higher concentration fully inhibited the productions of intracellular p53 and p80 (Figure 6 lane 5 versus 6). However, the productions of p53 and p80 could not be fully inhibited by heat-treated WASWE (Figure 6 lane 5 versus 7). The results suggest the anti-apoptotic effective components in WASWE may be a heat-labile component, rather than a low molecular weight pigment. Glycoprotein of *Rhus verniciflua* Stokes (RVS glycoprotein) has been isolated and protects splenocyte from apoptosis induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA)[4]. We have found a novel protein with a molecular weight of 313 kDa from WASWE, which directly activates primary B-cell proliferation and subsequent T cell proliferation *in vitro*[20]. We assumed the anti-apoptotic component in WASWE is the 313 kDa protein. However, more information on the properties concerning the anti-apoptotic components in WASWE remains to be elucidated. As the novel protein in WASWE directly activates primary B-cell proliferation and subsequent T cell proliferation *in vitro*[20], this study further suggests that WASWE might inhibit the apoptosis of both B and T lymphocytes in splenocytes (Figure 3(B)). However, the real anti-apoptotic cell types affected by WASWE should be further clarified.

CONCLUSIONS

The present study showed that the WASWE indeed inhibited the spontaneous and DEX-induced apoptosis of murine primary splenocytes. The effective component in WASWE is suggested to be a high molecular weight, and heat-labile component. This is a potentially valuable substance for future nutraceutical or immuno-pharmacological use.

ACKNOWLEDGEMENTS

This study was supported by a research grant NSC91-2313-B-005-091 from the National Science Council, Taipei, Taiwan, Republic of China.

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