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Involvement of endoplasmic reticulum stress in adenosine-induced human hepatoma HepG2 cell apoptosis

LING-FEI WU[1], YAN-QING YE[1], GUAN-YOU HUANG[2], HONG-BIAO LI[1], GUO-PING LI[1], ZE-JIN PU[1], BI-LIU WEI[1] and JIA-LIN FENG[3]

Departments of [1]Gastroenterology, [2]Obstetrics and Gynecology, and [3]Information, Second Affiliated Hospital, Shantou University Medical College, Shantou 515041, P.R. China

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Abstract. Endoplasmic reticulum stress (ERS)-mediated cell apoptosis has been implicated in the development of multiple diseases such as cancer, neurodegenerative diseases and ischemic reperfusion damage. Previous studies have demonstrated the adenosine-induced apoptosis in several tumor cell lines. However, the role of ERS in adenosine-induced human hepatoma HepG2 cell apoptosis remains unclear. The present study was designed to determine whether ERS is involved in adenosine-induced HepG2 cell apoptosis. The MTT assay was used to determine proliferation, and DAPI staining of cell nuclei was performed to determine cell apoptosis. The translocation of CHOP and caspase-3 was observed by immunofluorescence analysis, and the protein expression of CHOP, caspase-4 and caspase-3 was detected by Western blotting. The MTT assay demonstrated that adenosine inhibited HepG2 cell proliferation in a dose-dependent manner. DAPI staining of cell nuclei and cell cycle analysis verified cell apoptosis. The immunofluorescence assay demonstrated that adenosine induced the translocation of CHOP and of caspase-3 from the cytoplasm to the nucleus. Western blotting confirmed that CHOP, caspase-4 and caspase-3 were up-regulated in HepG2 cells after treatment with adenosine. However, JNK protein expression was not altered. These results show that ERS is involved in the adenosine-induced HepG2 cell apoptosis.

Introduction

Recent studies have shown that apoptosis plays an important role in tumor chemotherapy. Apoptosis is an organized programmed and energy-dependent cell death. The apoptotic process includes chromatin margination, nuclear condensation and fragmentation, followed by the formation of apoptotic bodies (1). Apoptosis is different from necrosis, and does not involve the inflammation response (2). Up to now, three predominant apoptotic pathways, namely the death receptor-mediated extrinsic pathway, the mitochondria-mediated intrinsic pathway, and the endoplasmic reticulum stress (ERS)-mediated pathway have been elucidated (3). Several drugs, such as DDP and 5-Fu, which can induce tumor cell apoptosis have been used in the clinical setting (4,5).

Adenosine, a metabolite of ATP, regulates cardiovascular and immunity functions under normal states (6). However, a high concentration of adenosine has been verified to exert cytotoxicity in several cell lines (7,8). Apoptosis has been reported in several adenosine-induced tumor cell lines (9-11), and in our previous studies, apoptosis occurred in adenosine-induced HepG2 cell lines (12,13). However, the mechanisms of adenosine's effects are still unclear.

The endoplasmic reticulum (ER) is a subcellular organelle where protein folding and modification and calcium storage occur (14). ERS-induced apoptosis has been implicated in several diseases such as diabetes mellitus, Parkinson's disease (15) and has been demonstrated in some tumor cell lines such as PC-12 neuronal cells (16) and lung carcinoma A-549 cells (17). Some drugs, ischemia-reperfusion, hypoxia and glucose starvation can disturb ER function and may lead to ERS (18-20). ERS initiates the unfolded protein response (UPR), which alleviates protein overload in the ER (21). There are three effects of the UPR activation: i) a decrease in the total protein synthesis (22); ii) an increase in the expression of chaperone proteins, such as the glucose-regulated protein 78 (GRP78/BiP), which can complex with the unfolded proteins causing their translocation from the ER to the cytoplasm (23); and iii) an increase in the degradation of the unfolded proteins. Activation of the UPR induces an adaptive response in which the cell attempts to overcome the accumulation of misfolded proteins and the ERS (21). In certain cases, however, the activation of the UPR may be insufficient to overcome ERS, ultimately leading to apoptosis (24). In mammals, there are three ER-resident transmembrane proteins, PERK, ATF6 and IRE1, which can sense the apoptotic signals (25). When PERK, ATF6 and IRE1 are active, they up-regulate the C/EBP homologous protein transcription factor (CHOP) expression. CHOP regulates apoptosis-related gene expression and induces

Correspondence to: Dr Ling-Fei Wu, Department of Gastroenterology, Second Affiliated Hospital, Shantou University Medical College, Shantou 515041, P.R. China
E-mail: lingfeiwu@21cn.com

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cell apoptosis (26). ERS-mediated apoptosis through the JNK or caspase-4 pathways have also been reported (27-29).

In previous studies, we and other scholars demonstrated that adenosine-induced HepG2 cell apoptosis was related to adenosine transfer into cells that did not occur through the adenosine receptors (12,13,29). Furthermore, we observed that activation of caspase-3 is indispensable, while that of caspases 8 or 9 is not obvious in the early stage of apoptosis (12,13). We speculated that ERS might be involved in the adenosine-induced HepG2 cell apoptosis. To verify this hypothesis, we investigated the effects of adenosine on ERS-related protein expression in HepG2 cells. The results demonstrate that adenosine dose-dependently inhibits HepG2 cell proliferation through cell apoptosis, and that ERS-related protein expression, such as that of CHOP, caspase-4 and caspase-3 was up-regulated, while JNK expression was not altered. Taken together, our results demonstrate that ERS is involved in the adenosine-induced HepG2 cell apoptosis.

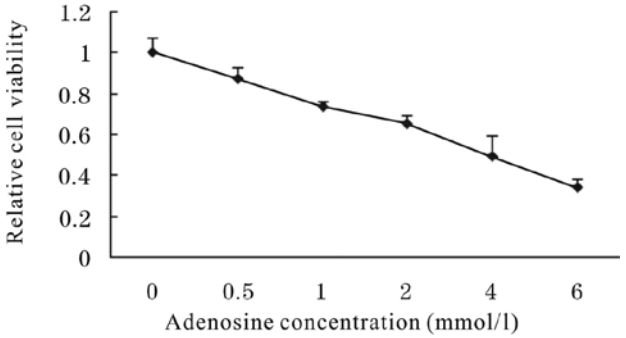

Figure 1. Dose-dependent effect of adenosine on the inhibition of HepG2 cell proliferation. HepG2 cells were exposed to different concentrations of adenosine (range, 0-6.0 mmol/l). After incubation for 36 h, the viability of HepG2 cells was evaluated by the MTT assay. The data are presented as the means ± SD. Independent experiments were repeated three times.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Invitrogen Corporation (Carlsbad, CA). Adenosine was purchased from Tree Tech (China). 4',6-Diamidino-2-phenylindol dihydrochloride (DAPI) and propidium iodide (PI) were obtained from Sigma. Primary antibodies against β-actin, caspase-4, caspase-3, JNK and CHOP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-mouse secondary antibody was purchased from KPL (USA). The anti-rabbit secondary antibody was purchased from Sigma. Alexa Fluor 555-labeled donkey anti-rabbit IgG was purchased from Invitrogen Corporation. Avidin-biotin-horseradish peroxidase complex was purchased from Vector Laboratories, Inc. (USA). Enhanced chemiluminescence detection reagent (ECL kit) was purchased from Pierce (Thermo Scientific, USA). Other materials such as SDS and NaCl were obtained form Shanghai Sangon Biological Engineering Technology Services Co., Ltd. (China). For the experiments, 10 mmol/l adenosine was dissolved in DMEM; 2% bovine serum albumin (BSA), 4% paraformaldehyde and 0.2% Triton X-100 were dissolved in the phosphate-buffered saline (PBS).

Cell culture and treatment. Human hepatoma HepG2 cells were used in this study. Cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5% CO_2, 95% humidity at 37˚C. Cells were detached by 0.25% trypsin/0.02% EDTA. For the immunofluorescence assay and DAPI staining, cells were cultured on coverslips in DMEM for 24 h before addition of adenosine.

MTT assay. The viability assays of HepG2 cells was evaluated with the MTT assay. Briefly, ~$5x10^3$ HepG2 cells were seeded in a well with 200 μl medium cultured in 96-well plates. After the cells were attached, the medium was replaced with different concentrations of adenosine (0 to 6 mmol/l) in culture medium. After culturing for 36 h, the cells were incubated with 20 μl MTT (5 g/l) for another 4 h in a 5% CO_2 incubator. The medium was then removed and 150 μl DMSO were added to each well, followed by thermal agitation for 5 min protected from light. The absorbance of each well was measured with a microtiter plate reader (KHB Labsystems Wellscan K3, Finland) at 492 nm.

Apoptotic nuclei staining. DAPI is a fluorescent dye which specifically conjugates to ds-DNA and is thus used to visualize nuclear morphological features; the nuclei of apoptotic cells demonstrate condensation and fragmentation. Cells were fixed in 4% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. DAPI (500 ng/ml) staining was performed at room temperature for 10 min. The morphology of the nuclei was viewed and captured with a fluorescence microscope (Olympus BX51, Japan).

Flow cytometry. HepG2 cells were harvested and washed three times in cold PBS. The cells were fixed in 70% ethanol at -20˚C overnight and then washed in PBS to remove the ethanol. The cells were treated with RNase (50 μg/ml) and stained with PI (50 μg/ml) for 30 min at 37˚C protected from light. Cellular fluorescence was measured by FCM analysis by a FACScan apparatus (Becton-Dickinson, Heidelberg, Germany).

Immunofluorescence microscopy. For immunofluorescence analysis, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.2% Triton X-100 for 10 min at room temperature, then blocked with 2% BSA for 30 min. Subsequently, the cells were incubated with primary antibodies against caspase-3 (1:100) or CHOP (1:100) or with PBS (blank group) at 4˚C overnight. The next day, HepG2 cells were incubated with the Alexa Fluor 555-labeled donkey anti-rabbit IgG (1:100) for 60 min at 37˚C. The cell nuclei were stained with DAPI for 10 min. The cells were viewed and captured with a fluorescence microscope (Olympus BX51).

Western blot analysis. Cells were washed with PBS, harvested and lysed in RIPA buffer containing 1% sodium deoxycholine, 0.1% SDS, 1% Triton X-100, 10 mM Tris-HCl (pH 8.0), 0.14 M NaCl and 0.5% NP-40. Equal amounts of protein were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Pall Corporation, NY). The membranes were blocked with 5% non-fat milk for 30 min at room temperature,

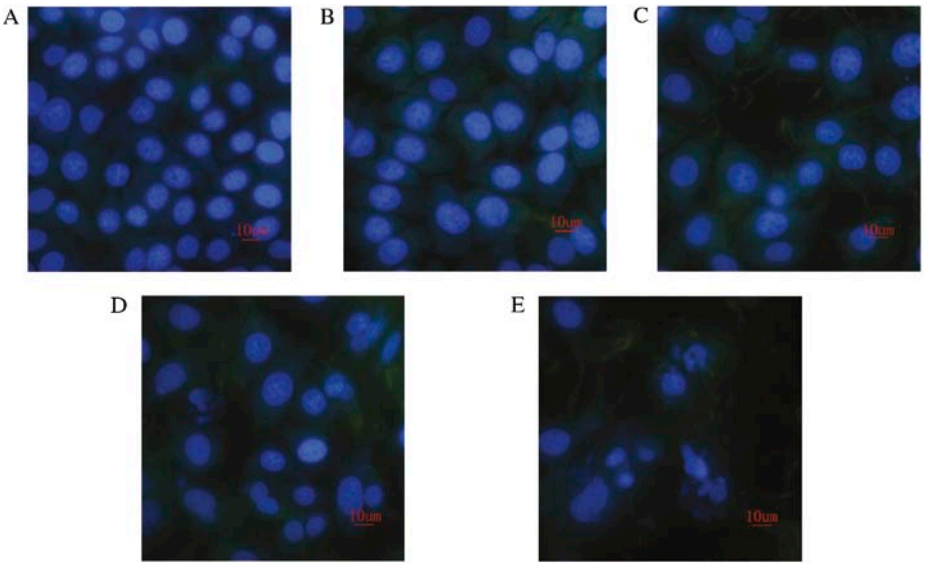

Figure 2. Adenosine induces apoptosis in HepG2 cells in a dose-dependent manner. HepG2 cells were exposed to different concentrations of adenosine (range, 0-4.0 mmol/l). After incubation for 36 h, the nuclei stained by DAPI were observed under a fluorescence microscope. (A) Control (0 mmol/l); (B) 0.5 mmol/l; (C) 1.0 mmol/l; (D) 2.0 mmol/l; (E) 4.0 mmol/l.

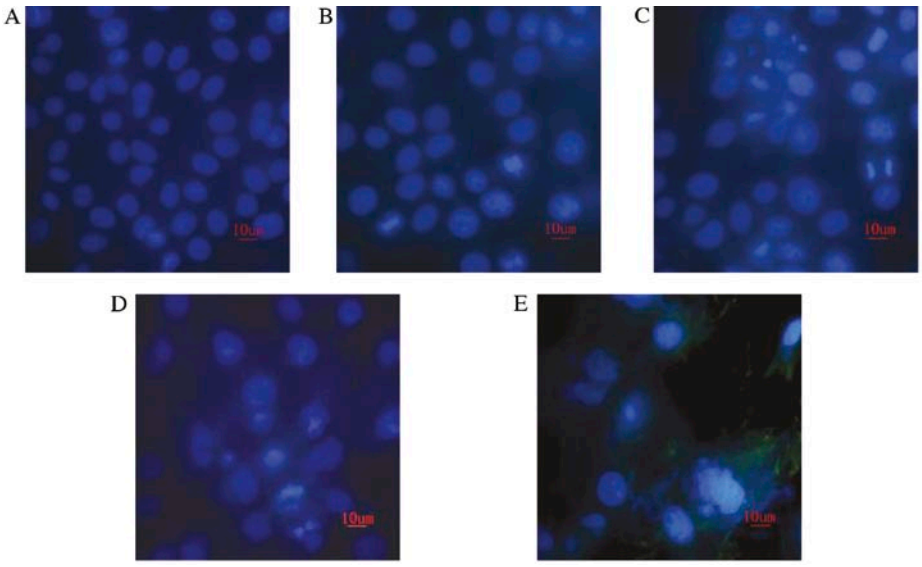

Figure 3. Adenosine induces HepG2 cell apoptosis in a time-dependent manner. HepG2 cells were treated with 2.0 mmol/l adenosine for different times (range, 0-48 h). The nuclei stained by DAPI were observed by a fluorescence microscope. (A) Control; (B) 12; (C) 24; (D) 36; (E) 48 h.

followed by incubation with primary antibodies against β-actin (1:2000), caspase-4 (1:500), caspase-3 (1:1000), JNK (1:1000) or CHOP (1:500), at 4°C overnight. The next day, the membranes were incubated with a biotin-conjugated secondary antibody for 60 min at room temperature, followed by incubation with an avidin-biotin-horseradish peroxidase complex at room temperature for 30 min. The bands were visualized by an ECL detection system. The band intensity was measured with the Quantity One software (Bio-Rad, USA).

Statistical analysis. Data are presented as the means ± SD of at least three independent experiments. Results were analyzed with the unpaired Student's t-test or one way ANOVA for different groups in SPSS 16.0. A $p<0.05$ was considered to indicate statistical significant differences.

Results

Adenosine inhibits HepG2 cell proliferation. To confirm that adenosine is cytotoxic to HepG2 cells, we used the MTT assay to evaluate the relative cell viability. HepG2 cells were exposed to different concentrations of adenosine for 36 h. The number of viable cells in the cell groups treated with 0.5, 1.0, 2.0, 4.0, 6.0 mmol/l adenosine decreased by 13.48±0.12, 27.92±0.25, 35.21±0.42, 51.46±0.24 and 71.42±0.58%, respectively (Fig. 1, $p<0.05$). These results demonstrate that adenosine exerts cytotoxicity to HepG2 cells in a dose-dependent manner.

Adenosine induces HepG2 cell apoptosis as assessed by DAPI staining and the FCM assay. Cell death can be divided into necrosis and apoptosis based on morphological changes.

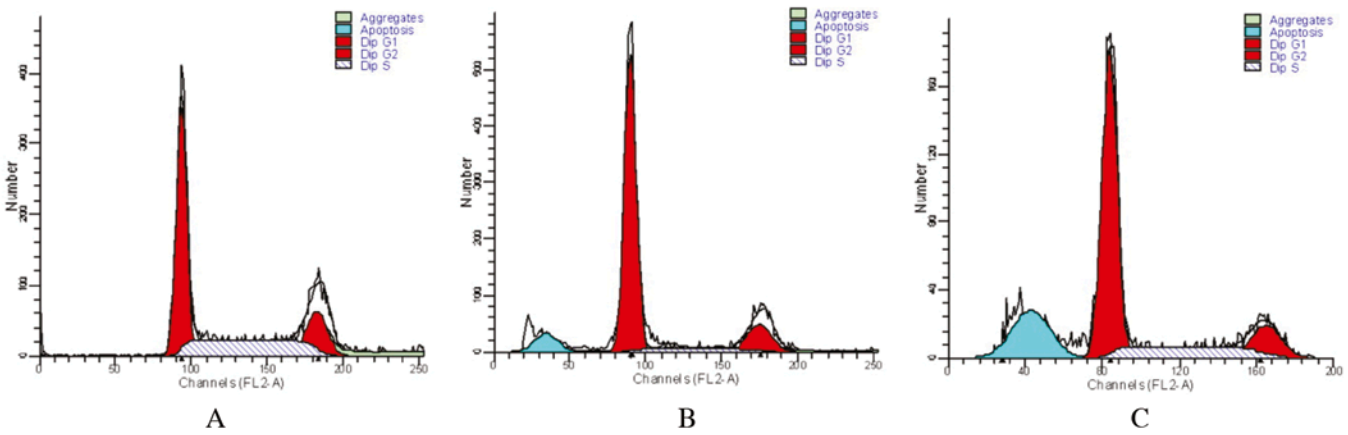

Figure 4. Effects of adenosine on HepG2 cell cycle progression assessed by the FCM assay. Cell cycle phases were determined by propidium iodine staining, followed by flow cytometric analysis. (A) Control group; (B) 2.0 mmol/l adenosine for 12 h; (C) 2.0 mmol/l adenosine for 24 h. The percentages of cells in the sub-G1, G0-G1, S and G2-M phases were analyzed by the WinMDI 2.9 software. The percentages of apoptotic cells were 0.73±0.02%, 7.85±0.12% and 20.71±0.24%, respectively.

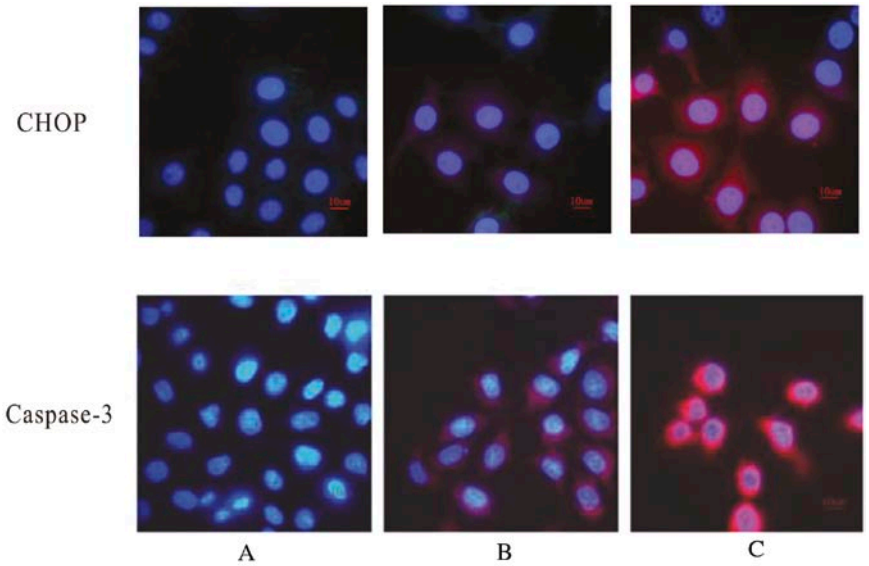

Figure 5. Effects of adenosine on CHOP and caspase-3 protein expression and their translocation determined by the immunofluorescence assay. HepG2 cells were exposed to normal medium or to 2.0 mmol/l adenosine for 36 h. Caspase-3 subcellular localization was observed under a fluorescence microscope. (A) Blank group (normal culture medium with the primary antibody replaced by PBS); (B) Control group (normal culture medium); (C) the 2.0 mmol/l adenosine treatment group.

The morphologic hallmarks of apoptosis include chromatin margination, nuclear condensation and fragmentation. DAPI, a nuclear morphology indicator, nuclear factor and early indicator of cell death (30), was used to evaluate the size and roundness of the nucleus. We examined the cell nuclei morphological features after treatment with different concentrations (0-4.0 mmol/l) of adenosine for 36 h or after treatment with 2.0 mmol/l adenosine for different time-points (0-48 h). Normal nuclei were uniform in the control group (Figs. 2A and 3A), while with increases in the adenosine concentration or with prolongation of the treatment time, the nuclei became condensed and rounded and shrinkage occured (Figs. 2B and C, and 3B and C). Nuclei fragmentation was obvious at higher doses or at later time-points (Figs. 2D and E, and 3D and E). These results demonstrate the occurrence of cell apoptosis. To obtain further evidence for apoptosis, the cell cycles were analyzed by FCM and the sub-G1 DNA content was used to indicate the cell percentage of apoptosis. The results showed that the percentages of apoptotic cells at 0, 12 and 24 h were 0.73±0.02, 7.85±0.12 and 20.71±0.24%, respectively (Fig. 4; $p<0.05$).

Effects of adenosine on CHOP and caspase-3 sub-cellular localization. The immunofluorescence assay was used to explore the sub-cellular localization of CHOP and caspase-3. The results showed that CHOP and caspase-3 were diffused in the cytoplasm mainly in HepG2 cells of the control group (Fig. 5B); adenosine caused CHOP and caspase-3 translocation from the cytoplasm to the nucleus (Fig. 5C).

Effects of adenosine on GRP78, CHOP, JNK, caspase-4 and caspase-3 protein expression. To verify whether adenosine-

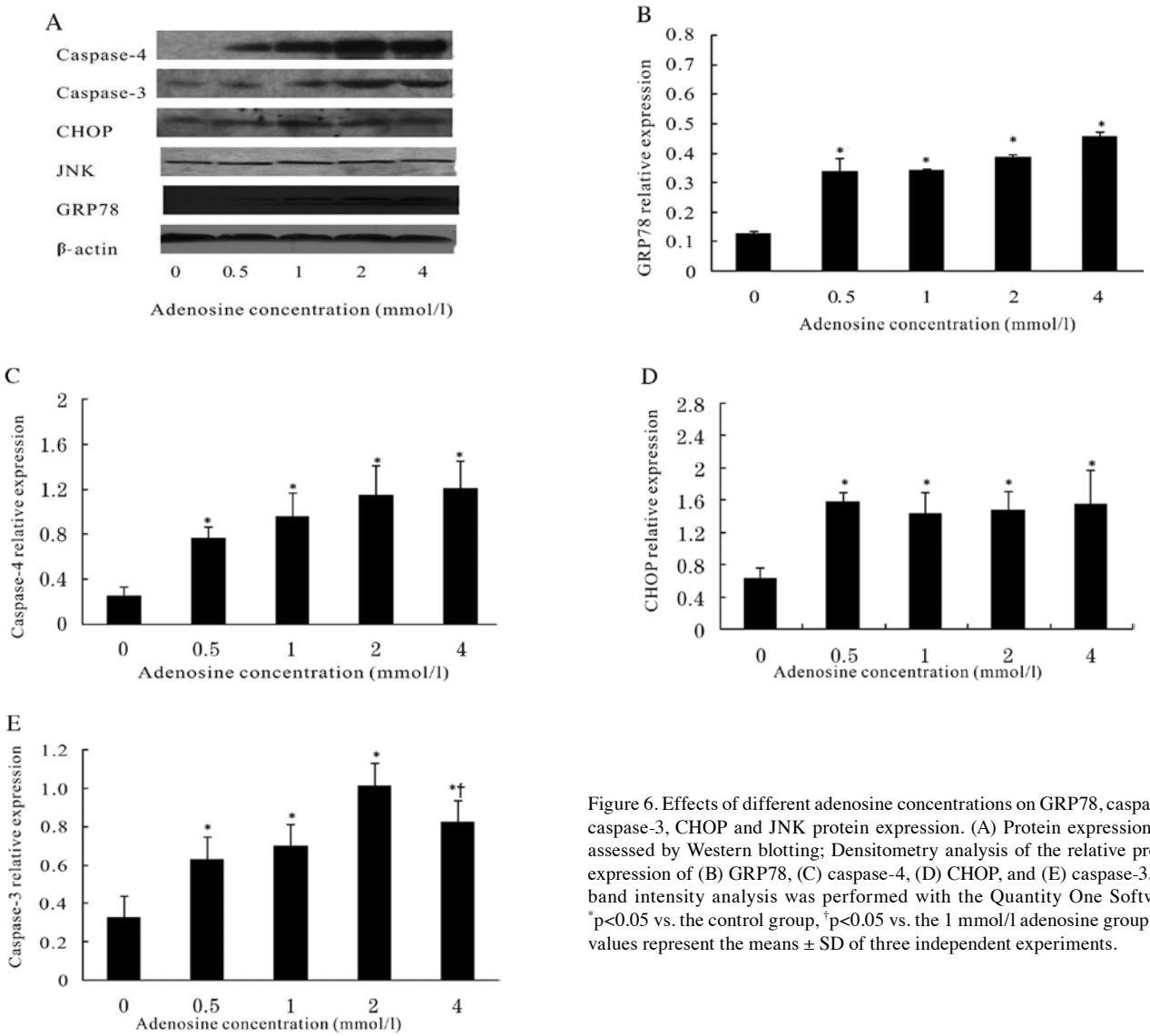

Figure 6. Effects of different adenosine concentrations on GRP78, caspase-4, caspase-3, CHOP and JNK protein expression. (A) Protein expression was assessed by Western blotting; Densitometry analysis of the relative protein expression of (B) GRP78, (C) caspase-4, (D) CHOP, and (E) caspase-3. The band intensity analysis was performed with the Quantity One Software; $^{*}p<0.05$ vs. the control group, $^{\dagger}p<0.05$ vs. the 1 mmol/l adenosine group. The values represent the means ± SD of three independent experiments.

induced cell apoptosis was related to CHOP, GRP78, JNK, caspase-4 and caspase-3, Western blotting was used to detect their protein expression. As showed in Fig. 6A, CHOP, GRP78, caspase-4 and caspase-3 protein expression increased in a dose-dependent manner, compared to the control group (Fig. 6B, C, D and E; $p<0.05$). However, JNK protein expression did not differ between the control and adenosine treatment groups ($p>0.05$).

Discussion

Induction of cancer cell apoptosis is the major strategy for the development of chemotherapy drugs (31). In previous studies, adenosine-induced apoptosis in several tumor cell lines has been demonstrated. The mitochondrial pathway has been shown to be involved in adenosine-induced cell apoptosis in human colonic cancer Caco-2 cells (11) and human breast cancer cells (32). The death receptor pathway has been reported to be involved in 8-chloro-adenosine-induced cell apoptosis in BEL-7402 human hepatoma cells (33-35). However, the role of ERS in the adenosine-induced cell apoptosis has not been elucidated.

Adenosine-induced HepG2 cell apoptosis has been demonstrated in our previous studies (12,13) and by others (10,29). The typical morphological hallmarks of apoptosis include nuclear condensation, nuclear fragmentation and the formation of apoptotic bodies in the absence of an inflammatory reaction (35). In the present study, we verified that adenosine inhibited HepG2 cell proliferation in a dose-dependent manner by means of the MTT assay. Our results also demonstrate that the nuclei of HepG2 cells displayed apoptotic morphological changes with the increases in the adenosine concentration or with prolongation of the treatment time (Figs. 2 and 3). FCM analysis further confirmed that the percentage of sub-G1 phase cells increased after adenosine treatment in a time-dependent manner (Fig. 4). These results support that adenosine inhibits HepG2 cell proliferation through inducing apoptosis, which is consistent with previous studies (12,29).

GRP78 (referred as immunoglobulin heavy chain binding protein, BiP), is a family member of the molecular chaperones and Ca^{2+}-binding stress proteins located in the ER (36). GRP78 was reported as an ERS hallmark in previous studies (17,37). Under normal conditions, the majority of the GRP78 protein is bound to three ER-localized protein sensors, PERK, ATF6

and IRE1. Free GRP78 is kept at low concentrations. When the ERS occurs, the unfolded proteins combine with free GRP78. The depletion of free GRP78 is key for the dissociation of GRP78 from its transducer clients, thus leading to their activation. On the other hand, cells can up-regulate the expression of GRP78 to decrease the dissociation and activation. It has been reported that the expression of GRP78 increases in ERS-induced apoptosis (17,20). In the present study, we also observed the expression of GRP78 increased (Fig. 6A and B), which supports the occurrence of ERS after treatment of HepG2 cells with adenosine.

CHOP (CCAAT/enhancer-binding protein homologous protein, also known as growth arrest and DNA damage-inducible protein) is a transcription factor which is up-regulated and plays a critical role in ERS-mediated apoptosis (17,38). The fact that overexpression of CHOP results in growth arrest and apoptosis has been demonstrated in a rat model (39) and in HL-60 cells (40). Translocation of CHOP from the cytoplasm to the nucleus could regulate the expression of ERS-related genes (17,43). Furthermore, it has been demonstrated that CHOP gene inhibition by drugs or knockout can protect cells from apoptosis mediated by ERS (41,42). Previous studies have demonstrated that CHOP-regulated genes, such as DR5 (44), TRB3 (45) and bcl-2 (46), are involved in the cytoplasm to the nucleus was also observed (Fig. 5) and CHOP protein expression was up-regulated in a dose-dependent manner (Fig. 6A and D). In our previous study, adenosine down-regulated bcl-2 expression (13). However, the mechanism by which CHOP regulates its downstream target genes and leads to cell apoptosis needs further study.

Cascade activation of caspases, such as caspase-3, caspase-8 and caspase-9 play an important role in apoptosis (47-49). Caspase-12, an ER-resident caspase, has been shown to be involved in ERS-mediated apoptosis (50,51). However, human mature caspase-12 protein can not be produced because the gene is interrupted by a frame shift and a premature stop codon. Thus, human caspase-12 does not execute a function in ERS-induced apoptosis (52). Caspase-4, another caspase-1 family member, has the same function as caspase-12 in ERS-induced apoptosis in humans (53). Up-regulation of caspase-4 has been demonstrated in ERS-mediated apoptosis in human bronchial epithelial cells (54), human neuroblastoma SKN-SH cells and human esophageal squamous carcinoma EC109 and EC9706 cells (55). Caspase-3 is believed to be the main executioner caspase and is up-regulated in most apoptotic processes. As an executioner, caspase-3 can be activated by other activated caspases such as caspase-4, -8 and -9, and subsequently cleaves some specific substrates, such as PARP (56). In the present study, caspase-4 was obviously increased after treatment with adenosine. Furthermore, caspase-3 was up-regulated in a dose-dependent manner and its expression peaked at 2.0 mmol/l adenosine, which shows that caspase-4 may activate caspase-3. However, the caspase-3 expression decreased after treatment with 4.0 mmol/l adenosine. Whether this observation is related to caspase-3 being cleaved at the active segment, as reported in our previous studies (13), or to the increase in necrotic cells leading to a caspase-3 decrease need to be further investigated.

Activation of JNK pathway is a common phenomenon in stress-induced apoptosis in response to stress stimuli (57). To delineate the role of JNK in adenosine-mediated apoptosis, we detected the JNK expression by Western blotting. However, the results demonstrate that adenosine has no effects on JNK expression in HepG2 cells.

In summary, the present study demonstrated that GRP78, CHOP, caspase-4 and caspase-3 were up-regulated after adenosine treatment. The ERS pathway may be involved in the adenosine-induced HepG2 cell apoptosis.

Acknowledgements

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