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Cell Biology International 30 (2006) 940–946

Cell Biology International

www.elsevier.com/locate/cellbi

A forskolin derivative, FSK88, induces apoptosis in human gastric cancer BGC823 cells through caspase activation involving regulation of Bcl-2 family gene expression, dissipation of mitochondrial membrane potential and cytochrome *c* release

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Received 8 May 2006; revised 25 May 2006; accepted 26 June 2006

Abstract

FSK88, a forskolin derivative, was extracted and purified from cultured tropical plant roots, *Coleus forskohlii*. Our previous studies have demonstrated that FSK88 can inhibit HL-60 cell proliferation and induce the differentiation of HL-60 cells to monocyte macrophages. In this study, we showed that FSK88 can induce apoptotic death of human gastric cancer BGC823 cells in a dose- and time-dependent manner. Results showed that FSK88-induced apoptosis was accompanied by the mitochondrial release of cytochrome *c* and activation of caspase-3 in BGC823 cells. Furthermore, treatment with caspase-3 inhibitor (z-DEVD-fmk) was capable of preventing the FSK88-induced caspase-3 activity and apoptosis. FSK88-induced apoptosis in human gastric cancer BGC823 cells was also accompanied by the up-regulation of Bax, Bad and down-regulation of Bcl-2. Theses results clearly demonstrated that the induction of apoptosis by FSK88 involved multiple cellular and molecular pathways and strongly suggest that pro- and anti-apoptotic Bcl-2 family genes, mitochondrial membrane potential ($\Delta\psi_m$), cytochrome *c*, and caspase-3, participate in the FSK88-induced apoptotic process in human gastric cancer BGC823 cells.

Keywords: FSK88; Apoptosis; Bcl-2; Cytochrome *c*; Caspases

1. Introduction

Gastric cancer is the second leading cause of cancer death in the world and particularly prevalent in certain countries including China (Bamias and Pavlidis, 1998; Hohenberger and Gretschel, 2003; Lu et al., 2005). However, its pathogenesis is not completely understood and there are few effective therapies in gastric cancer prevention and treatment (Hampton and Orrenius, 1997). The prognosis of gastric cancer is poor, with a five-year survival of 15%–20% (Thompson et al., 1993). Cancer of the stomach is a disease for which treatment and attitudes vary in different regions of the world. That an organ cancer should show varying causative factors in different parts of the world is not unusual; however, with gastric cancer, it is not only the incidence of the disease, but also the approach of early diagnosis and treatment that varies greatly between the western and eastern hemispheres (Hohenberger and Gretschel, 2003). Currently, inducing cancer cells into apoptosis is one of the important therapeutic intervention approaches in cancer (Ferreira et al., 2002; Hengartner, 2000; Kasibhatlal and Tseng, 2003; Lowe and Lin, 2000; Tamm et al., 2001a,b), therefore, it is crucial to reveal the molecular mechanism of apoptosis in gastric cancer cells. In this study, BGC823 cells, a human gastric cancer cell line, was exploited to investigate

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doi:10.1016/j.cellbi.2006.06.015

the effects of FSK88 on the human gastric cancer cells and the underlying molecular mechanisms.

It has been reported that forskolin, a diterpene from the roots of the herb *Coleus forskohlii*, is capable of preventing tumor colonization and metastasis (Agarwal and Parks, 1983) and inhibiting growth and inducing apoptosis of myeloid and lymphoid cells (Gutzkow et al., 2002; Moon and Lerner, 2003; Taetle and Li-en, 1984). Recently, it has been found that forskolin, as a potent PP2A activator (Feschenko et al., 2002; Neviani et al., 2005), induced marked apoptosis, reduced proliferation, impaired colony formation, inhibited tuomorigenesis, and restored differentiation of BCR/ABL − transformed cells regardless of their degree of sensitivity to imatinib (Neviani et al., 2005).

Our previous studies have demonstrated that FSK88, a forskolin derivative, can inhibit HL-60 cell proliferation and induce the differentiation of HL-60 cells to monocyte macrophages, as shown by enhancing nitroblautetrazolium (NBT) reduction ability and increasing alpha-naphthyl acetate esterase (ANAE) activity. At the same time, FSK88 could decrease the membrane lipid fluidity of the UMR106 cells but had little effect on the normal osteoblastic cells, which possibly indicates that FSK88 could inhibit the proliferation and stimulate the differentiation of cancer cells (Wang et al., 1999). In the present study, we have confirmed that FSK88 induces apoptosis in gastric cancer BGC823 cells and demonstrated that dissipation $\Delta\psi_m$, cytochrome *c* release and caspase activation are involved in FSK88-induced apoptosis.

2. Materials and methods

2.1. Materials and cell culture

The human gastric cancer BGC823 cells were obtained from Beijing Institute for Cancer Research. Cells were cultured in RPMI1640 medium (Gibco BRL) supplemented with heat inactivated 10% fetal bovine serum made by Hyclone (Logan, UT, USA), 100 μg/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml amphotericin B. The cells were grown in a humidified incubator at 37 °C under a 5% CO_2/95% air atmosphere. For each experiment, 2×10^5 cells were seeded in 1 ml of fresh medium per well in a 96-well plate. When cell confluence reached 80%, the cells were treated with or without FSK88 for the indicated time.

FSK88 was extracted and purified (HPLC pure, ≥98%) by the State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, dissolved in pure grade ethanol. Caspase colorimetric assay kits and caspase-3 inhibitor (z-DEVD-fmk) were purchased from Sigma (St. Louis, MO, USA). The cytochrome *c* kits were purchased from R&D Systems (Minneapolis, MN, USA). All chemicals were of the highest pure grade available.

2.2. MTT cell viability assay

An MTT (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) test was performed on cultures 0–72 h after initial plating as described by Mosmann (1983). The MTT test is based on the ability of viable cells to produce formazan from the cleavage of the tetrazolium salt by functional mitochondria. Briefly, 200 μl aliquots of a suspension of exponentially growing cell were seeded in 96-well plates (Flow Laboratories, Inc., USA) and incubated for 12 h. Ten microlitres aliquots of FSK88 at various concentrations were added. After exposure to FSK88 for 0–72 h, 20 μl MTT solution (5 mg/ml in distilled water) was added to each well and the plates were incubated at 37 °C for another 4 h .After the incubation, 200 μl dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) was added to each well to dissolve the formazan. Absorbance was read at 550 nm using a spectrophotometric microplate reader (Labsystems, Finland). The experiments were performed in triplicate and repeated three times.

2.3. Quantitative measurement of apoptosis

Apoptosis was measured by direct determination of nucleosomal DNA fragmentation with the cell death detection ELISA (Roche, Mannheim, Germany), which measures the amount of histone-associated DNA fragments present in the cytosol of apoptotic cells. 1×10^6 BGC823 cells were treated with or without 10 μM FSK88 for the indicated periods of time. Cells were harvested by centrifugation and lysed in 0.5 ml of the lysis buffer provided with the kit. Two microlitres of the extract was used, and the ELISA was performed as instructed by the manufacturer. The data are expressed in photometric units. Each unit corresponds to ∼12,500 apoptotic cells. The optical density was read on a spectrofluorometer at a wavelength of 405 nm. The experiments were performed in triplicate and repeated three times.

2.4. Caspase activity assay

After treatment, cells were washed twice with ice-cold PBS and harvested as described previously (Ortiz et al., 2001). The catalytic activity of caspases was measured with their fluorogenic substrate. Briefly, 10 mg of total protein, as determined by the Bio-Rad protein assay, was incubated, respectively, with 200 mM fluorogenic peptide substrates Ac-DEVD-MCA (for caspase-3), Ac-IETD-MC A (for caspase-8), and Ac-LEHD-FCA (for caspase-9) in a 50 ml assay buffer at 37 °C for 2 h. The release of AMC was measured with a spectrofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The release of 7-amido-4-trifluoromethycoumarin was monitored at 360/530 nm. The experiments were performed in triplicate and repeated three times.

2.5. Assay of mitochondrial membrane potential ($\Delta\psi_m$)

Alterations in the $\Delta\psi_m$ were analyzed by fluorescence spectrophotometer using the mitochondrial membrane potential sensitive dye rhodamine 123, which accumulates eletrophoretically in mitochondria as a direct function of the membrane potential and is released upon membrane depolarization (Emaus et al., 1986), as described by Palmeira et al. (1996) with slight modification. Briefly, after incubation with or without 10 μM FSK88 for 0–72 h, cells were harvested, washed twice with ice-cold PBS, re-suspended in DMEM without FBS, and incubated with 2 μM rhodamine 123 at 37 °C for 10 min. Stained cells were then washed three times with ice-cold PBS, harvested by centrifugation, and re-suspended in 2 ml PBS. The fluorescence intensity of each cell suspensions was measured at excitation wavelength 480 nm and emission wavelength 530 nm in a fluorescence spectrophotometer. The fluorescence intensity was measured using an arbitrary unit representing the $\Delta\psi_m$. The experiments were performed in triplicate and repeated three times.

2.6. Cytochrome c release

BGC823 cells were seeded in 2 ml fresh medium at an initial density of 1.5×10^6 cells/ml and incubated up to 0–72 h with or without 10 μM FSK88. After the incubation, the cells were harvested by centrifugation and washed three times with cold PBS. The cells were re-suspended in 200 μl lysis buffer (200 mM mannitol; 150 mM sucrose; 0.05 mM EGTA; 0.01 mM $MgCl_2$; 20 mM HEPES, pH 7.5; 0.5 mg/ml BSA) and lysed by the addition of 0.02% digitonin, incubated for 1 h at room temperature with gentle mixing. The cytosolic fraction was obtained from 10,000 × g centrifugation for 15 min and was collected for cytochrome *c* assay in 1× RD5P calibrator diluent (cytochrome *c* immunoassay kit, R&D Systems, MN, USA). Adding 200 μl of substrate solution to each well, incubated at room temperature for 30 min, and adding 50 μl stop solution to each well. The absorbance was measured at 450 nm (reference wavelength is 570 nm) and measurements were performed in triplicate. The experiments were repeated three times.

Table 1
Primers used for RT-PCR analysis

Gene	Gene bank accession no.	Primers (5′–3′)		Product size (bp)
		Sense	Anti-sense	
Bcl-2	MI 4745	GGCAAATGACCAGCAGA	TGGCAGGATAGCAGCAC	345
Bcl-xL	Z23115	GAGGCAGGCGACGAGTT	TTCCCATAGAGTTCCACAAAA	317
Bax	L22474	GCTTCAGGGTTTCATCCA	TGTCCACGGCGGCAAT	176
Bad	AF031523	CCAGAGTTTGAGCCGAGTG	CTGTGCTGCCCAGAGGTT	312
β-Actin	NM_00110l	TGGCATCCACGAAACTA	AAAGGGTGTAACGCAACTA	345

2.7. RT-PCR analysis for Bcl-2 family gene expression

Total RNA from BGC823 cells was prepared with TRIzol (Invitrogen) according to the manufacturer's instructions. Two micrograms of total RNA from each sample was subjected to reverse transcription using Superscript first strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. PCR reactions were then carried out by mixing 5 μl of cDNA, 5 μl of 10× PCR buffer, 1 μl of 10 mM dNTP, 2 μl of 25 mM specific gene primer pair, 2 μl of 25 mM β-actin primer pair, 35 μl of H_2O, and 1 μl of 5 U/μl *Taq* DNA polymerase (Invitrogen), and amplified for 25 cycles. The primers used in the PCR reaction are presented in Table 1. Each cycle consisted of denaturing for 45 s at 94 °C, annealing for 30 s at 60 °C, and polymerization for 1 min at 72 °C. The PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and photographed under UV light.

2.8. Statistical analysis

Values are expressed as mean ± SD Student's *t*-test was used for statistical evaluation and $P < 0.05$ was considered significant.

3. Results

3.1. FSK88 affects BGC823 cell viability in a dose- and time-dependent manner

First we assessed dose- (0.1–30 μM) and time-dependent (12–72 h) effects of FSK88 on BGC823 cell viability using MTT assay. As shown in Fig. 1, the inhibitory effect of FSK88 on BGC823 cells was in a dose- and time-dependent manner. In initial dose-response study, FSK88 treatment for 72 h at 0.1 μM and 30 μM doses resulted in $3.7 \pm 0.8\%$ ($p > 0.05$) and $87.3 \pm 5.7\%$ ($p < 0.001$) cell viability decrease in a dose-dependent manner compared with that of control. In a time-response study, 10 μM dose of FSK88 for 12 h and 72 h resulted in $7.5 \pm 0.6\%$ ($p > 0.05$) and $75.3 \pm 6.1\%$ ($p < 0.01$) cell viability decrease compared with that of control.

3.2. FSK88 induces time-dependent apoptosis in BGC823 cells

The effect of FSK88 on BGC823 cell apoptosis was measured by direct determination of nucleosomal DNA fragmentation, which measures the amount of histone-associated DNA fragments present in the cytosol of apoptotic cells. As shown in Fig. 2, FSK88-induced a moderate to strong apoptotic death in a time-dependent manner, 10 μM dose of FSK88 for 12 h and 72 h resulted in $3.8 \pm 0.2\%$ ($p > 0.05$) and $47.4 \pm 4.9\%$ ($p < 0.01$) apoptosis increase compared with that of control cells.

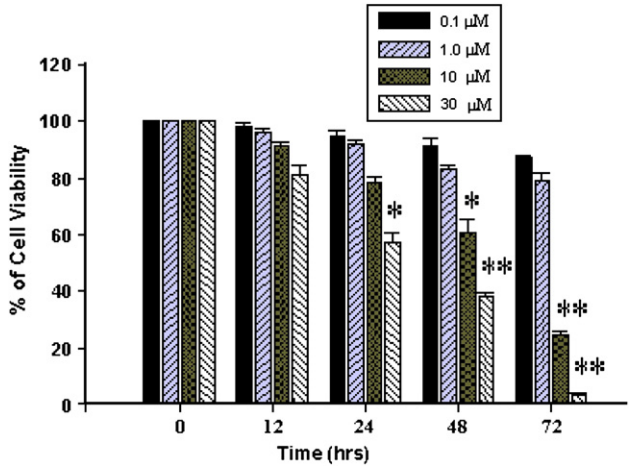

Fig. 1. The dose- and time-dependent effect of FSK88 on cell viability of human gastric cancer BGC823 cells. BGC823 cells were incubated with or without 0.1 μM, 1 μM, 10 μM, or 30 μM of FSK88 for 12 h, 24 h, 48 h, and 72 h, respectively. At the end of the incubation, the cell viability was determined by MTT methods. The cell viability is expressed as the percentage of MTT reduction to control conditions (mean ± SD, $n = 3$), $^*P < 0.01$, $^{**}P < 0.001$ compared with untreated cells by Student's *t*-test.

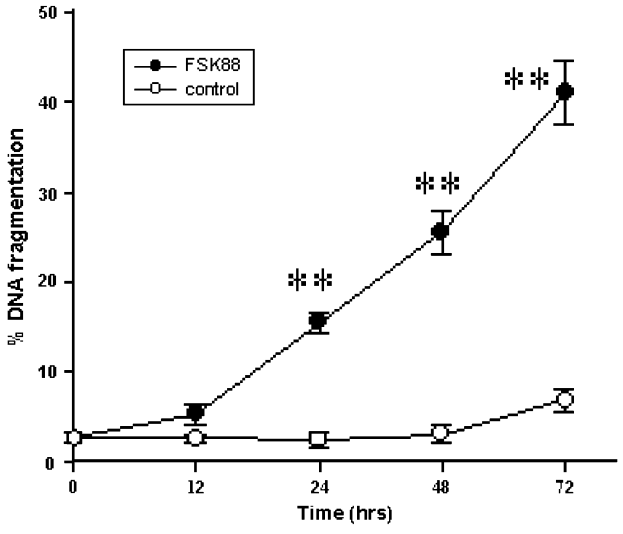

Fig. 2. Percentage of apoptotic cells determined by DNA fragmentation. BGC823 cells were treated without (○) or with (●) 10 μM of FSK88 for the indicated time. At the end of treatment, cells were lysed for quantitative measurement of apoptosis using the percentage of DNA fragmentation as an index. Data are expressed as mean ± SD of three individual determinations at each time point, $^*P < 0.01$ and $^{**}P < 0.001$ compared with untreated cells by Student's *t*-test.

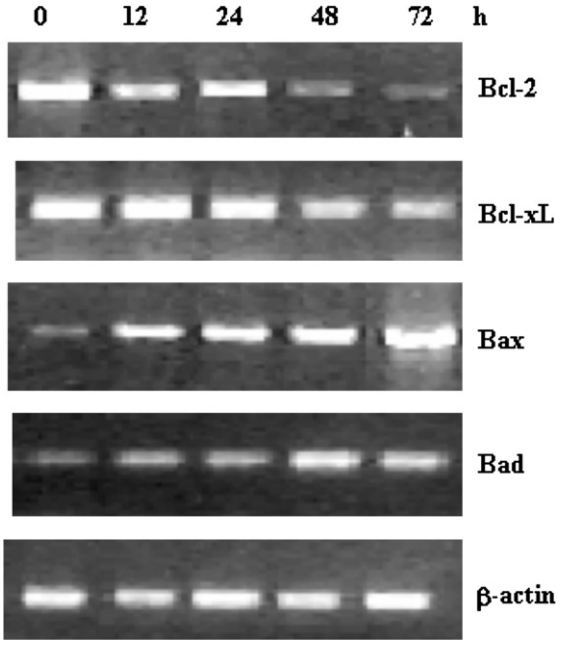

Fig. 3. RT-PCR assay of the effect of FSK88 on Bcl-2 family gene expression in BGC823 cells. The cells were treated with 10 μM FSK88 for the indicated periods of time (in hours), and at the end of treatment total RNA was prepared with TRIzol and Bcl-2 family gene expression was analyzed using RT-PCR. β-actin was used as a control for the amplification. The experiment was performed three times with very similar outcomes, and a representative result is shown.

3.3. Regulation of apoptosis-related Bcl-2 family gene expression in FSK88-treated BGC823 cells

To investigate the molecular mechanism of FSK88-induced apoptosis in BGC823 cells, the gene expression of apoptosis-related Bcl-2 family with or without 10 μM FSK88 was examined. Bax and Bad, two pro-apoptotic factors, were up-regulated in a time-dependent manner whereas Bcl-2, an anti-apoptotic factor, was down-regulated in a time-dependent manner, but the expression of Bcl-xL, another anti-apoptosis factor, changed slightly. Therefore, the ratios of Bcl-2:Bax and Bcl-xL:Bax all decreased in a time-dependent manner (Fig. 3).

3.4. FSK88 disrupts mitochondrial membrance potential and induces cytochrome c release in BGC823 cells

Mitochondrial intergrity or the loss of $\Delta\psi_m$ has been linked to the initiation and activation of some apoptotic cascades (Green and Reed, 1998; Li et al., 1997). To determine whether FSK88-induced apoptosis in BGC823 cells involves mitochondrial disruption, we examined the depolarization of mitochondrial membrane spectrophotometrically. BGC823 cells were treated with or without 10 μM FSK88 for 12 h and 72 h, and at the end of treatments, cells were processed and stained with rhodamine 123. Fig. 4A shows that FSK88 induced a significant time-dependent decrease of $\Delta\psi_m$ following FSK88 treatment accounting for $54.2 \pm 7.9\%$ ($P < 0.01$) and $63.2 \pm 4.7\%$ ($P < 0.001$) at 48 h and 72 h of treatments, respectively, compared with control cells.

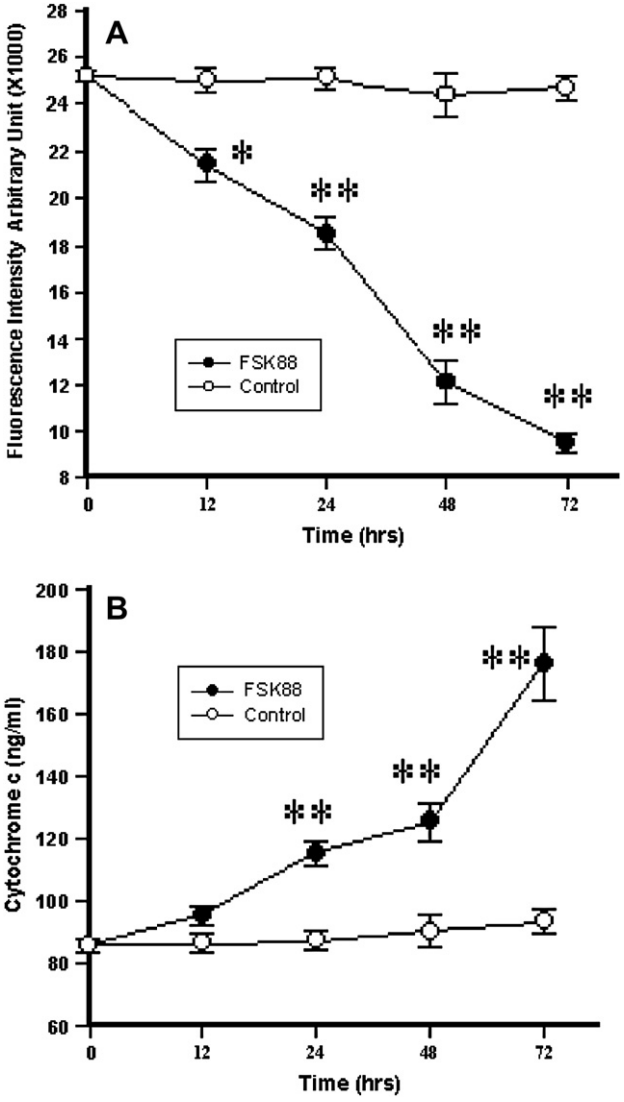

Fig. 4. Measurement of mitochondrial membrane potential and cytochrome *c* release in human gastric cancer BGC823 cells during the FSK88 treatment. (A) BGC823 cells were treated without (○) or with (●) 10 μM of FSK88 for different periods of time and then cells were incubated with 10 μM rhodamine 123 for 10 min. At the end of incubation, the mitochondrial membrane potential was measured spectrophotometrically. (B) BGC823 cells were incubated without (○) or with (●) 10 μM of FSK88 for the indicated time. Cytochrome *c* release was determined by immunoassay. The data are expressed as mean ± SD of three determinations, $^*P < 0.05$ and $^{**}P < 0.01$ compared with untreated cells by Student's *t*-test.

Cytochrome *c* release from mitochondrial inter-membranous space into cytosol has been shown to be a key event in the activation of caspase-9 which subsequently initiates a caspase cascade involving caspase-3 (Reed, 1997; Slee et al., 1999), in order to define an upstream event in FSK88-induced apoptosis in BGC823 cells, we also investigated cytochrome *c* release in cytosolic fraction following FSK88 treatment of cells. Immunoassay of cytosolic fraction of FSK88 (10 μM) treated cells showed an increase in the level of cytochrome *c* at 48 h and 72 h treatment duration compared with control

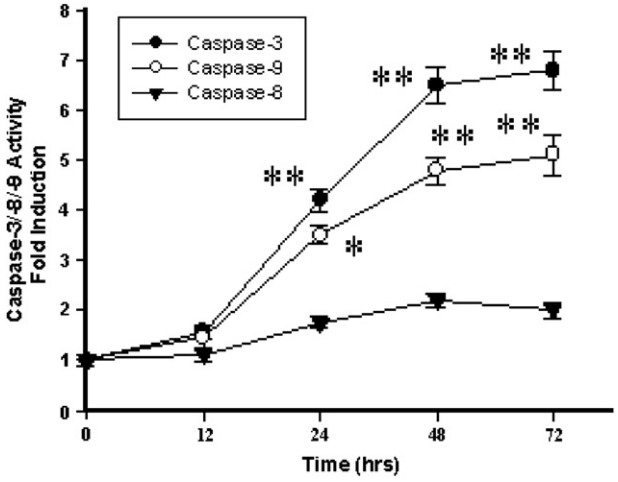

Fig. 5. Time course of caspase enzymatic activities. BGC823 cells were treated with 10 μM FSK88 for the indicated times. Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AFC were used as substrates of caspase-3, caspase-8, and caspase-9, respectively. Caspase activities were assayed as described in Section 2. Data are expressed as mean ± SD of three individual determinations at each time point, $*P < 0.05$ and $**P < 0.01$ compared with untreated cells by Student's *t*-test.

cells (Fig. 4B). The results showing an increase in the level of cytosolic cytochrome *c* suggested that its release from mitochondria to the cytosol might be an important event in FSK88-induced apoptosis in BGC823 cells.

These observations suggest an involvement of cytochrome *c* release from mitochondria as well as a possible disruption of mitochondria in FSK88-induced apoptosis in BGC823 cells.

3.5. FSK88-induced apoptosis is largely mediated via caspase-3 activation

Activation of caspase-3 is a central mechanism of apoptosis in the mitochondria-dependent and death receptor-dependent pathway (Vaux and Korsmeyer, 1999). In the present study, we examined whether caspase-3 activation is involved in the apoptotic process triggered by FSK88.

To investigate whether or not initiator and effector caspases are involved in the apoptotic progression, we measured the activity of caspases-3, -9 and -8 using the synthetic peptide substrates, Ac-DEVD-AMC, AC-LEHD-AFC, and AC-IETD-AMC, respectively. As shown in Fig. 5, caspase-3 activity was detectable after 12 h and then markedly increased after 48 h of FSK88 treatment. The kinetics of caspase-9 activation was similar to that of caspase-3, whereas caspase-8 activity was barely changed during these time periods. Activities of caspase-3, -9 and -8 increased 6.7-, 4.9- and 1.8-fold, respectively, after 72 h FSK88 treatment. To further investigate the function of caspase-3 in FSK88-induced apoptosis, BGC823 cells were pretreated with 50 μM caspase inhibitors (z-DEVD-fmk) for 2 h, and then induced to undergo apoptosis by treatment with FSK88. The results clearly showed that administration of caspase-3 inhibitor significantly affected the cell viability and caspase-3 activity (Fig. 6).

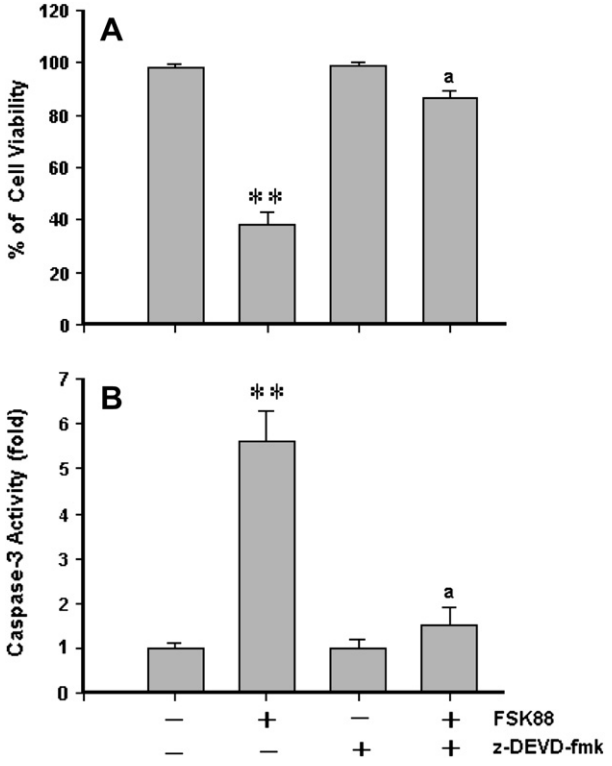

Fig. 6. Inhibition of caspase-3 activity (B) and attenuation of FSK88-induced cell death (A) by caspase-3 inhibitor (z-DEVD-fmk). Human gastric cancer BGC823 cells were pre-treated with 50 μM specific caspase-3 inhibitor 2 h prior to 72 h treatment of 10 μM FSK88. After incubation, cell viability and caspase-3 activity were examined as described in Section 2. All values are mean ± SD of three determinations. $**P < 0.001$ compared with the respective FSK88 and inhibitor free control and $^{a}P < 0.001$ comparison between the absence and presence of z-DEVD-fmk in the same FSK88 treatment group.

4. Discussion

The central finding of the present study is that FSK88, a forskolin derivative, induces apoptotic death of human gastric cancer BGC823 cells through caspase activation involving dissipation of $\Delta\psi_m$ and cytochrome *c* release from mitochondria into the cytosol.

It has been suggested that disruption of $\Delta\psi_m$ plays a pivotal role in initiation of apoptotic induction and is also linked to the release of cytochrome *c* from mitochondria to the cytosol (Green and Reed, 1998; Li et al., 1997; Marchetti et al., 1996). The mitochondria pathway is controlled and regulated by the Bcl-2 family proteins (Michael et al., 2006; Narita et al., 1998; Reed, 1998; Schendel et al., 1997), which are comprised of pro- and anti-apoptotic members (Verma et al., 2006). The anti-apoptotic subfamily mainly comprises Bcl-2 and Bcl-xL. The multi-domain pro-apoptotic subfamily consists of Bax and Bak and the BH3 domain-only protein include Bad and Bim (Rosse et al., 1998). Pro- and anti-apoptotic Bcl-2 family members converge on mitochondria in response to the apoptotic signal and compete to regulate the release of cytochrome *c* (Hengartner, 2000; Schendel et al., 1997; Narita et al., 1998). Several studies have shown that over-expression of Bcl-2 and Bcl-xL prevents the mitochondrial release of cytochrome *c*,

thereby inhibiting the activation of caspase cascade and apoptosis (Kluck et al., 1997; Yang et al., 1997; Chao and Stanley, 1998; Tsujimoto, 2003). Previous reports have documented that the ratio of pro- and anti-apoptotic proteins determined, at least in part, the susceptibility of cells to the death signal (Tsujimoto, 2003).

It is known that released cytochrome *c* binds to Apaf-1 and participates in the activation of caspase-9 (Kluck et al., 1997; Yang et al., 1997; Zhou et al., 1997). The activated caspase-9 activates caspase-3, resulting in the onset of apoptosis. The increases in caspase-3 and caspase-9 are synchronized with the increase in Bax expression and the decrease in Bcl-2 (Tanabe et al., 1998). Our data (Fig. 3) showed that pro-apoptotic Bax and Bad gene expression was up-regulated and anti-apoptotic Bcl-2 gene expression was down-regulated in a time-dependent manner. The time-dependent decrease in the ratios of Bcl-2:Bax and Bcl-xL:Bax showed that the Bcl-2 family genes participated in FSK88-induced apoptosis in BGC823 cells. At the same time, $\Delta\psi_m$ significantly decreased and cytosolic cytochrome *c* greatly increased in a time-dependent manner treated with 10 μM FSK88 (Fig. 4).

As shown in Fig. 5, caspase-9 activity increased about 4.9-fold and caspase-3 activity increased about 6.7-fold after treatment with 10 μM FSK88 for 72 h. Moreover, human gastric cancer BGC823 cells were pre-incubated with specific caspase-3 inhibitor (z-DEVD-fmk) before treatment of FSK88 and analyzed the cell viability and caspase-3 activity by MTT assay and spectrophotometry analysis, respectively. Results showed that pre-incubation of cells with z-DEVD-fmk effectively inhibited the caspase-3 activity and prevented the FSK88-induced cell death (Fig. 6). All these data suggested that FSK88-induced BGC823 cell apoptosis is mainly through caspase activation involving dissipation of $\Delta\psi_m$ and cytochrome *c* release from mitochondria into the cytosol.

In conclusion, our study demonstrates that FSK88 is a potential inducer of apoptosis in human BGC823 cells. It is most likely that mitochondrial pathways are involved in FSK88-induced apoptosis in human gastric cancer BGC823 cells. The treatment of human gastric cancer BGC823 cells with FSK88 activates a cell death pathway that regulates the mitochondrial membrane permeability by down-regulation of Bcl-2 and up-regulation of Bax and Bad, triggering cytochrome *c* release from mitochondria into cytosol. In addition, FSK88 could induce caspase-9 and caspase-3 activation, consequently leading to apoptotic changes, such as DNA fragmentation.

Acknowledgements

This work was supported by grants from National Major Program For New Drug Research of China (No. 96-906-05-93) and President of Chinese Academy of Sciences special grants (No. 676). We would also like to thank Ms G. J. Li and Mr Q. F. Xue for their excellent technical assistance.

References

Agarwal KC, Parks Jr RE. Forskolin: a potential antimetastatic agent. Int J Cancer 1983;32:801–4.

Bamias A, Pavlidis N. Systemic chemotherapy in gastric cancer: where do we stand today? Oncologist 1998;3:171–7.

Chao Debra T, Stanley J. Bcl-2 family: regulators of cell death. Annu Rev Immunol 1998;16:395–419.

Emaus RK, Grunwald R, Lemasters JJ. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. Biochim Biophys Acta 1986;850(3):436–48.

Ferreira CG, Epping M, Kruyt FAE, Giaccone G. Apoptosis: target of cancer therapy. Clin Cancer Res 2002;8:2024–34.

Feschenko MS, Stevenson E, Nairn AC, Sweadner KJ. A novel cAMP-stimulated pathway in protein phosphatase 2A activation. J Pharmacol Exp Ther 2002;302:111–8.

Green GR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.

Gutzkow KB, Naderi S, Blomhoff HK. Forskolin-mediated G1 arrest in acute lymphoblastic leukaemia cells: phosphorylated pRB sequesters E2Fs. J Cell Sci 2002;115:1073–82.

Hampton MB, Orrenius S. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett 1997;414:552–6.

Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770–6.

Hohenberger P, Gretschel S. Gastric cancer. Lancet 2003;362:305–15.

Kasibhatlal S, Tseng B. Why target apoptosis in cancer treatment? Mol Cancer Ther 2003;2:573–80.

Kluck RM, Wetzel EB, Green DR, Newmeyer D. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997;275:1132–6.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmed M, Alnemri ES, et al. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997;91:479–89.

Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis 2000;21:485–95.

Lu WL, Pan KF, Zhang L, Lin DX, Miao XP, You WC. Genetic polymorphisms of interleukin (IL)-1B, IL-1RN, IL-8, IL-10 and tumor necrosis factor and risk of gastric cancer in a Chinese population. Carcinogenesis 2005;26(3):631–6.

Marchetti P, Castedo M, Susin SA. Mitochondrial permeability transition is central coordinating event of apoptosis. J Exp Med 1996;184:1155–60.

Michael C, Victoria DGM, Mari N, Guo W, Stanley K, Scott AA, et al. Mitochondria primed by death signals determines cellular addiction to antiapoptotic Bcl-2 family members. Cancer Cell 2006;9(5):351–65.

Moon EY, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood 2003;101:4122–30.

Mosmann T. Rapid colorimetric assay for cellular growth and survivals: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55–63.

Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, et al. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. Proc Natl Acad Sci USA 1998;95:14681–6.

Neviani P, Santhanam R, Trotta R, Notari M, Blaser BW, Liu S, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL regulated SET protein. Cancer Cell 2005;8:355–68.

Ortiz MA, Lopez-Hernandez FJ, Bayon Y, Pfahl M, Piedrafita FJ. Retinoid-related molecules induce cytochrome *c* release and apoptosis through activation of c-Jun NH2-terminal kinase/p38 mitogen-activated protein kinases. Cancer Res 2001;61:8504–12.

Palmeira CM, Moreno AJM, Madeira VMC, Wallace KB. Continuous monitoring of mitochondrial membrane potential in hepatocyte cell suspensions. J Pharmacol Toxicol Methods 1996;35(1):35–43.

Reed JC. Cytochrome *c*: can't live with it–can't live without it. Cell 1997; 91:559–62.

Reed JC. Bcl-2 family proteins. Oncogene 1998;17:3225–36.

Rosse T, Olivier R, Monney L, Rager M, Conus S, Fellay I, et al. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome *c*. Nature 1998;391:496–9.

Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M, Reed J. Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci USA 1997;94:5113–8.

Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, et al. Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8 and -10 in a caspase-9-dependent manner. J Cell Biol 1999;144:281–92.

Taetle R, Li-en S. Further studies on mechanisms of abnormal prostaglandin response by chronic myelogenous leukaemia granulocyte/macrophage progenitors. Leuk Res 1984;8:833–42.

Tamm I, Dorken B, Hartmann G. Antisense therapy in oncology: new hope for an old idea? Lancet 2001a;358:489–97.

Tamm I, Schriever F, Dorken B. Apoptosis: implications of basic research for clinical oncology. Lancet Oncol 2001b;2:33–42.

Tanabe H, Eguchi Y, Shimizu S, Martinou JC, Tsujimoto Y. Death-signaling cascade in mouse cerebellar granule neurons. Eur J Neurosci 1998;10:1403–11.

Thompson GB, van Heerden JA, Sarr MG. Adenocarcinoma of the stomach: are we making progress? Lancet 1993;342:713–8.

Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol 2003;19:158–67.

Vaux DL, Korsmeyer SJ. Cell death in development. Cell 1999;96: 245–54.

Verma YK, Gangenahalli GU, Singh VK, Gupta P, Chandra R, Sharma RK, et al. Cell death regulation by B-cell lymphoma protein. Apoptosis 2006;11(4):459–71.

Wang JZ, Tan WQ, Zhao Q. Effect of RFP134 on membrane lipid fluidity in UMR106 cells. Dev Reprod Biol 1999;1:23–7.

Yang J, Liu XS, Bhalla K, Kim CN, Ibrado AM, Cai JY, et al. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. Science 1997;275:1129–32.

Zhou H, Hanzel WJ, Liu X, Lutschg A, Wang X. Apf-1, a human protein homologous to *C. elegans* CED-4, participates cytochrome *c*-dependent activation of caspase-3. Cell 1997;90:405–13.