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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

Zhonghai Li [a,b,*], Jingze Wang [a,*]

[a] *State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, 25 Beisihuan Xi Road, 100080 Beijing, China*

[b] *Chinese Human Genome Research Center, 250 Bibo Road, 201203 Shanghai, China*

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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

* Corresponding authors. Present address: 12 Binnenhaven, 6709 PD, Wageningen, The Netherlands. Tel.: +31 317 482 324; fax: +31 317 484 821 (Zhonghai Li). 25 Beisihuan Xi Road, 100080 Beijing, China. Tel.: +86 10 6255 1668; fax: +86 10 6256 5689 (Jingze Wang).

E-mail addresses: lizhhai@gmail.com (Z. Li), wangjz@ioz.ac.cn (J. Wang).

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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.