P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wcjd@public.bta.net.cn www.wjgnet.com

World J Gastroenterol 2002;8(2):217-223
World Journal of Gastroenterology
 ISSN 1007-9327

• GASTRIC CANCER •

JTE-522-induced apoptosis in human gastric adenocarinoma cell line AGS cells by caspase activation accompanying cytochrome C release, membrane translocation of Bax and loss of mitochondrial membrane potential

Hong-Liang Li, Dan-Dan Chen, Xiao-Hong Li, Hai-Wei Zhang, Jun-Hua Lü, Xian-Da Ren, Cun-Chuan Wang

Hong-Liang Li, Xiao-Hong Li, Jun-Hua Lü, Xian-Da Ren, Department of Pharmacology, Jinan University Pharmacy College, Guangzhou 510632, Guangdong Province, China
Dan-Dan Chen, Department of Cardiology, First Affiliated Hospital, Zhongshan University, Guangzhou 510089, Guangdong Province, China
Hai-Wei Zhang, Department of Pathology, Jinan University Medical College, Guangzhou 510632, Guangdong Province, China
Cun-Chuan Wang, Department of laparoscopic surgery, First Affiliated Hospital, Jinan University Medical College, Guangzhou 510632, Guangdong Province, China
Supported by National Natural Science Foundation of China, No. 39770300, 30070873, and the Overseas Chinese Affairs Office of the State Council Foundation, No. 98-33
Correspondence to: Prof. Xian-Da Ren, Department of Pharmacology, Jinan University Pharmacy College, Guangzhou 510632, Guangdong Province, China. tsam@jnu.edu.cn
Telephone: +86-20-8522-0261
Received 2001-11-15 **Accepted** 2001-12-08

Abstract

AIM: **To investigate the role of the mitochondrial pathway in JTE-522-induced apoptosis and to investigate the relationship between cytochrome C release, caspase activity and loss of mitochondrial membrane potential (△Ψm).**

METHODS: **Cell culture, cell counting, ELISA assay, TUNEL, flow cymetry, Western blot and fluorometric assay were employed to investigate the effect of JTE-522 on cell proliferation and apoptosis in AGS cells and related molecular mechanism.**

RESULTS: **JTE-522 inhibited the growth of AGS cells and induced the apoptosis. Caspases 8 and 9 were activated during apoptosis as judged by the appearance of cleavage products from procaspase and the caspase activities to cleave specific fluorogenic substrates. To elucidate whether the activation of caspases 8 and 9 was required for the apoptosis induction, we examined the effect of caspase-specific inhibitors on apoptosis. The results showed that caspase inhibitors significantly inhibited the apoptosis induced by JTE-522. In addition, the membrane translocation of Bax and cytosolic release of cytochrome C accompanying with the decrease of the uptake of Rhodamin 123, were detected at an early stage of apoptosis. Furthermore, Bax translocation, cytochrome C release, and caspase 9 activation were blocked by Z-VAD.fmk and Z-IETD-CHO.**

CONCLUSION: **The present data indicate a crucial association between activation of caspases 8, 9, cytochrome C release, membrane translocation of Bax, loss of △Ψm and JTE-522-induced apoptosis in AGS cells.**

Li HL, Chen DD, Li XH, Zhang HW, Lü JH, Ren XD, Wang CC. JTE-522-induced apoptosis in human gastric adenocarinoma cell line AGS cells by caspase activation accompanying cytochrome C release, membarane translocation of Bax and loss of mitochondrial membrane potential. *World J Gastroenterol* 2002;8(2):217-223

INTRODUCTION

Previous studies have showed that non-steroidal anti-inflammatory drugs (NSAIDs) can induce apoptosis in several cell type, including human colorectal tumor cell lines[1,2], human breast cancer cells[3], human lung cancer cells[4], B cell chronic lymphocytic leukemia cells[5] and myeloid leukemia cell line[6]. JTE-522 is a novel NSAIDs, which is a specific inhibitior of cyclooxygenase-2 with significant anti-inflammatory and analgestic properties[7]. In a previous study, we have demonstrated that JTE-522 induced apoptosis in human gastric adenocarcinoma AGS cells, but the detail mechanism is not clear.

Caspase, a family of cysteine protease, is the common executor of apoptosis induced by various stimuli[8,9]. Caspase 8 and caspase 9 are recognized as upstream apoptosis initiators, which activate downstream caspases 3, 6 and 7[10]. The apoptotic action of NSAIDs was inhibited by the caspase inhibitor Z-VAD.fmk, demonstrating the involvement of caspases[5]. However, the pathway leading to caspase activation remains unknown. Caspases play a central role in the execution of apoptosis. The two most well-studied pathways of caspase activation include the surface death receptor pathway and the mitochondrion-initiated pathway. In the mitochondrion pathway, cytochrome C and other apoptogenic proteins (e.g apoptosis-inducing factor) are released from the intermembrane space to the cytosol[12]. Once released, cytochrome C binds to apoptotic protease-activating factor-1 (Apaf-1) and induces activation of caspase 9[12]. Recently, it was shown that bax, a proapoptotic member of bcl-2 family, promoted apoptosis by triggering the release of cytochrome C from mitochondria[13-18]. In the present study, our aims are to investigate the role of the mitochondrial pathway in JTE-522-induced apoptosis and to investigate the relationship between cytochrome C release, caspase activity and loss of mitochondrial membrane potential (△Ψm).

MATERIALS AND METHODS

Cell line and reagents

AGS, a gastric adenocarcinoma cell line, was provided by Cancer Institute, Sun-Yet Sen University of Medical Sciences (China). The cells were grown in RPMI-1640 medium and supplemented with 10% fetal calf serum, penicillin G (100kU/L) and kanamycin($0.1g \cdot L^{-1}$) at 37℃ in a 5% CO_2. Antibodies used in this study were obtained from Santa Cruz. All other chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Determination of cell proliferation rate

AGS cells (1×10^5) were seeded in 12-well plates and cultured for 24h. The cultures were divided into two groups: the girst group (control) was cultured in the RPMI1640 medium, and the second was cultured in the continuous presence of 1mmol/L JTE-522. The cells were then harvested every 24h by trypsinization and cell numbers counted with a hemocytometer. Three cultures were used for experiments at each time point.

Detection of apoptotic DNA fragmentation

AGS cells were grown in 96-well culture plates. The cells were incubated with various doses of JTE-522 for 6h. Apoptotic DNA fragmentation was determined using a commercially obtained enzyme-linked immunosorbent assay (ELISA) kit from Sigma. This assay was based on a quantitative sandwich enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments. Briefly, the cells were incubated in 200μL of lysis buffer provided in the kit, the lysates were centrifuged, and 20μL of the supernatant containing cytoplasmic histone-associated DNA fragments were reacted overnight at 4℃ in streptavidin-coated microtitrator wells with 80μL of the immunoreagent mixture containing biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody. After washing, the immunocomplex-bound peroxidase was probed with 2,2'-azino-di[3-ethylbenzthiazoline sulfonate] for spectrophotometric detection at 405nm.

TUENL

TUNEL assay was performed using the apoptosis detection system. Cells were fixed by 4% paraformaldehyde in PBS overnight at 4℃. The samples were washed three times with PBS and were permeabilized by 0.2% Triton X-100 in PBS for 15min on ice. After washing twice, cells were equilibrated at room temperature for 15 to 30min in equilibration buffer (200mmol/L potassium cacodylate, 0.2mmol/L dithiothreitol, $0.25g\cdot L^{-1}$ bovine serum albumin, and 2.5mmol/L cobalt chloride in 25mmol/L Tris-HCL, PH 6.6) and then incubated in the presence of 5μmol/L pluorescein-12-dUTP, 10μmol/L dATP, 100μmol/L ethylenediaminetetraacetic acid (EDTA), and terminal deoxynucleotidyl transferase at 37℃ for 1.5h in dark. The tailing reaction was terminated by 2×standard saline citrate (SSC). The samples were washed three times with PBS and were analyzed by fluorescence microscopy. At least 1000 cells were counted, and the percentage of TUNEL-positive cells was determined.

Ladder detection assay

After induction of apoptosis, cells (5×10^6/sample, both attached and detached cells) were lyzed with 150μL hypotonic lysis buffer (10mmol/L EDTA, 0.5% Triton X-100 in 1mmol/L Tris-HCL, Ph7.4) for 15min on ice and were precipitated with 2.5% polyethylene glycol and 1mol/L NaCl for 15min at 4%. After centrifugation at 16000g for 10min at room temperature, the supernatant was incubated in the presence of proteinase K ($0.3g\cdot L^{-1}$) at 37℃ for one hour and precipetated with isopropanol at -20℃. After centrifugation, each pellet was dissolved in 10μL of Tris-EDTA (pH 7.6) and electrophoresed on a 1.5% agarose gel containing ethidium bromide. Ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

Cytosolic release of cytochrome C and membrane translocation of Bax

The analysis was performed according to the method of Euguchi *et al*[19], with minor modifications. Briefly, cells were collected and suspended in mitochondria isolation buffer [20mmol/L Hepes-KOH, PH 7.5; 210mmol/L sucrose; 70mmol/L mannitol; 1mmol/L EDTA; 1mmol/L dithiothreitol (DTT); 1.5mmol/L $MgCl_2$, 10 mmol/L KCl_2; and protease inhibitor cocktail supplemented with 10μmol/L digitonin]. The suspensions were incubated at 37℃ for 5min and centrifuged at 15000g for 15min. The supernatants and pellets were collected for Western blotting according to the method described by Tsuchida *et al*[20].

Caspase activity assay

Activities of caspase 8 and caspase 9 were determined by using a fluorometric assay kit according to the method described by Gao *et al*[20]. Briefly, 5×10^5 cells were collected and lysed in 50μL lysis buffer and incubated with fluorochromic caspase substrate, Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (IETD-AFC) and Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (LEHD-AFC) for caspase 8 and caspase 9, respectively. After incubation at 37.5℃ for 1h, the fluorescence was measured by a spectrofluorphotometer with excitation at 400nm and emission at 550nm.

Mitochondrial membrane potential analysis

Rhodamine 123, a cationic fluorescent dye whose mitochordrial fluorescence intensity decreased quantitatively in response to dissipation of $\triangle\Psi m$, was used to evaluate perturbations in $\triangle\Psi m$. Cells were incubated with 5μmol/L rhodamin 123 for 30min, and then were collected, washed, and stained with 10μmol/L PI. The uptake of rhodamin 123 was measured by flow cytometry.

Statistics analysis

The data shown were mean value of at least three different experiments and expressed as mean±SD. Student's test was used to compare data. A *P* value of less than 0.05 was considered as statistically significant.

RESULTS

Effect of JTE-522 on cell proliferation and apoptosis in AGS cells

In order to understand the potential role of JTE-522 in the induction of apoptosis and its possible contribution to AGS cell loss in human gastric adenocarcinoma, the inhibitory effect of JTE-522 on cell proliferation was studied in AGS cells. As shown in Figure 1.

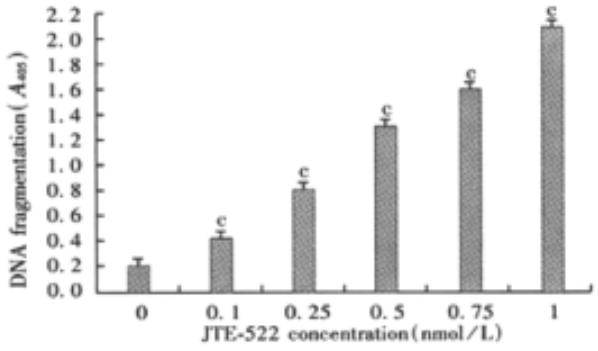

Figure 1 Effect of JTE-522 on AGS cell proliferation. Cells were incubated in the absence or presence of 1mmol/LJTE-522 and cell numbers determined at the time replicates at each time point ($^cP<0.01$).

The cells were cultured in the presence or absence of JTE-522 and cell numbers determined over three days. AGS cell proliferation was markedly inhibited by 1 mmol/L JTE-522. In the absence of JTE-522, the number of control cells doubled approximately every 24h in RPMI1640 medium supplemented with 10% fetal calf serum. The effect of JTE-522 at concentrations from 0.1mmol/L to 1mmol/L on DNA fragmentation in AGS cells was shown in Figure 2.

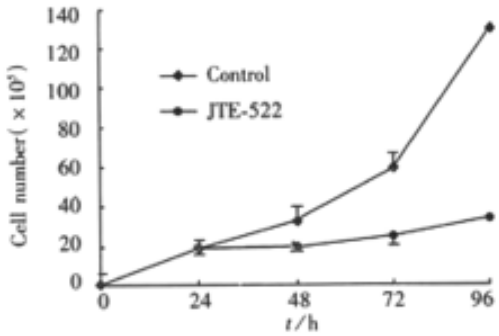

Figure 2 Effect of JTE-522 on DNA fragmentation in AGS cells. Cytoplasmic histone-associated DNA fragments were determined using a commercial ELISA kit. Results were representative of four independent determinations.

JTE-522 was found to significantly induce DNA fragmentation after the onset of incubation and this effect was in a dose-dependent manner. The same results were obtained by TUNEL assay. The number of TUNEL-positive cells was dramatically increased after treatment with JTE-522 for 48h, that is, the percentage of TUNEL-positive cells increased from 19.3±1.7% to 59.8±2.6% (Figure 3).

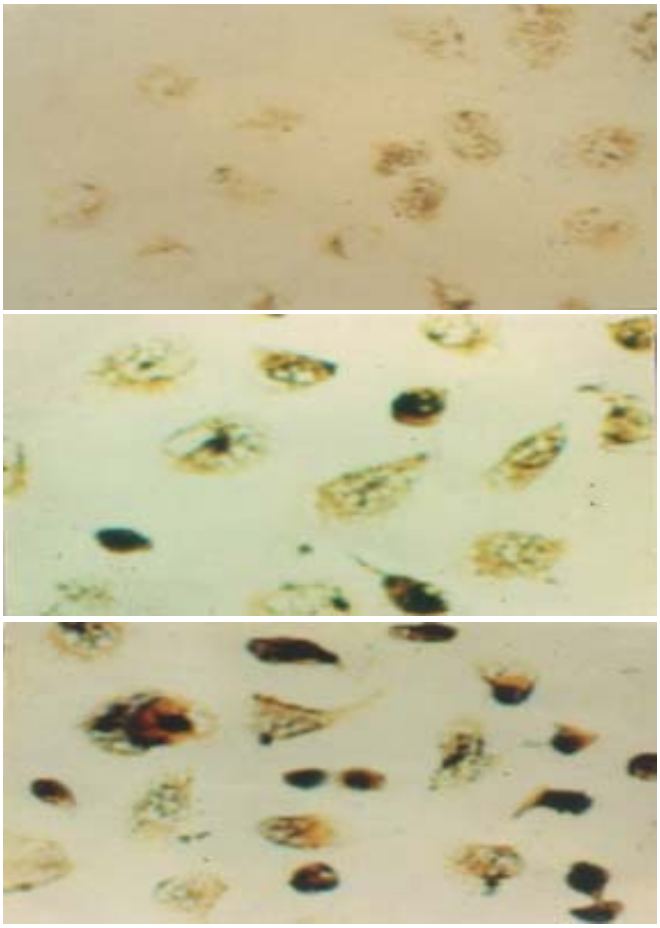

Figure 3 TUNEL assay demonstrating marked morphological changes in AGS cells after treatment with different concentrations of JTE-522. A: control; B: 0.1mmol/L JTE-522; C: 1mmol/L JTE-522

Effect of JTE-522 on caspases 8, 9 in AGS cells

In order to demonstrate the role of caspase 8 and caspase 9 in JTE-522-induced apoptosis, we examined the activities of caspase 8 and caspase 9 after treatment with JTE-522 for indicated time. The results showed that caspase 8 activity increased at 9h after JTE-522 treatment in AGS cells (Figure 4A), and the activity of caspase 9 also increased. To further confirm the activation of these two caspases, we conducted Western blot analysis (Figure 4B). The results showed that both caspases were activated, as judged by the decrease of the procaspases and the increase of their cleavage products. These experiments clearly showed that caspase 8 and caspase 9 played an important role during apoptosis induced by JTE-522. To investigate whether the activation of caspases 8 and 9 was required for the apoptosis induction, we examined the effect of caspase-specific inhibitors on apoptosis. The results showed extensive DNA fragmentation after treatment with JTE-522, while the treatment of Z-VAD.fmk, Ac-IETD-CHO or Ac-LEHD-CHO significantly blocked it (Figure 4C). These results suggested that both caspase 8 and caspase 9-like activity was required for JTE-522 apoptosis during the apoptotic process.

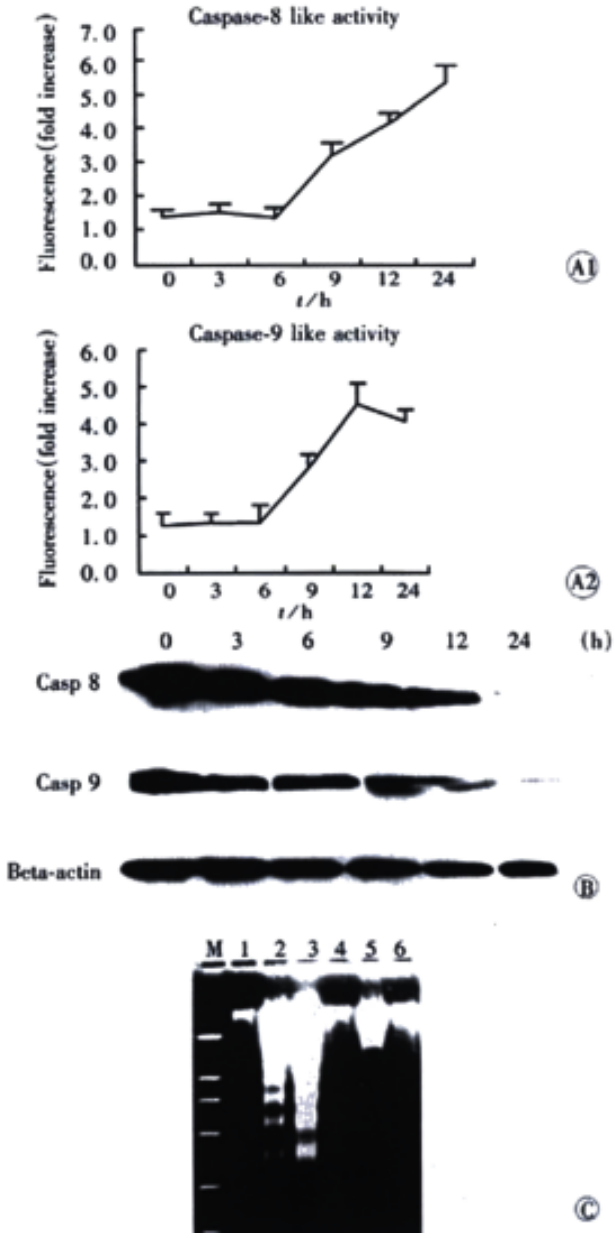

Figure 4 The role of procaspase 8 and procaspase 9 in JTE-522 induced apoptosis for indicated time. 4A: Effect of JTE-522 on caspase activities for indicated time. Data presented are the mean value of three separate experiments. 4B: Western blot shows the cleavage of procaspase 8 (Casp 8) and procaspase 9 (Casp 9). AGS cells were treated with JTE-522 for the indicated times. 4C: Effect of caspase inhibitor on JTE-522 induced DNA fragmentation. AGS cells were treated with: 1:control; 2: 1mmol/L JTE-522; 3: 0.5mmol/L JTE-522; 4: 50μmol/L Z-VAD.fmk+1mmol/L JTE-522; 5: 50μmol/L Ac-IETD-CHO +1mmol/L JTE-522; 6: 50μmol/L Ac-LEHD-CHO+1mmol/L JTE-522 for 24h.

Effect of JTE-522 on cytosolic release of cytochrome C and membrane translocation of bax

Cytochrome C, which is released from mitochondria, has recently been identified as a key factor that could activate caspase in a cell-free system[19]. In fact, it has been reported that levels of cytosolic cytochrome C were increased in response to various apoptotic stimuli such as staurosporine, *P*53, etoposide, and ultraviolet-B irradiation[20-22]. We therefore performed Western blotting to examine the distribution of cytochrome C in cytosol and membrane fractions before and after apoptosis induction. AGS cells were treated with JTE-522 for 24h, and the cells treated with 0.1μmol/L staurosporine for 24h were used as a positive control. After treatment, the cells were collected and suspended in the mitochondrial isolation buffer containing digitonin. Treatment with digitonin made small pores on cell membranes that allow the releases of cytosolic cytochrome C to the buffer fraction[19]. The supernatant and the pellet that represented cytosol and membrane fractions, respectively, were collected separately and subjected to Western blot analysis (Figure 5A). The result showed that cytochrome C in membrane fractions decreased significantly in both staurosporine and JTE-522 treated cells, while in cytosol fractions, high levels of cytochrome C were detected in both treated cells but not in control cells. On the contrary, bax protein increased in membrane fractions and decreased in cytosol fractions after treatment with staurosporine or JTE-522. Bcl-2 protein, as well as cytochrome C oxidase subunit IV (cyto oxi, as a control for mitochondrial protein loading), which were located in mitochondria and not released to cytosol. This result indicated that the cytosolic release of cytochrome C was specially triggered by apoptotic stimuli, but not due to the experimental manipulation.

There was a growing evidence for two pathways of cytochrome C release from mitochondria during apoptosis: rupture of the outer membrane following swelling, or through a specific pore in the outer membrane[23]. In liposomes and yeast Bax and Bak interacted with voltage-dependent anion channel (VDAC) to cause selective cytochrome C release[21]. Addition of recombinant Bax to isolated mitochondria induced cytochrome C loss without swelling. However, others had shown that Bax did induce the permeability transition in isolated mitochondria and within cells[24]. Therefore, we questioned whether the cytosolic release of cytochrome C and membrane translocation of Bax in JTE-522-induced apoptosis were caspase-dependent or not. The cells were pretreated with Z-VAD.fmk at 50μmol/L for 1h before treatment with staurosporine or JTE-522. Levels of cytochrome C and Bax in cytosol were examined by Western blotting (Figure 5C). The results showed that the increase of cytochrome C and the decrease of Bax in cytosol induced by staurosporine or JTE-522 were blocked by Z-VAD.fmk. The cleavage of procaspase 3 and 8 was also blocked. These results indicated that the Bax translocation and cytochrome C release were dependent on the activation of caspases.

Since it has been well documented that caspase-8 was the first caspase activated in Fas-, *P*53-, TRAIL- and TNF-induced apoptosis[20, 25, 26], we sought to determine whether caspase-8 activation occurs prior to, as in Fas-, *P*53- and TNF-induced apoptosis or after caspase-9, caspase 3 activation and cytochrome C release, and examined the effects of Ac-IETD-CHO on cytochrome C release and caspase 9 activation. The results showed that Ac-IETD-CHO effectively blocked cytochrome C release and the processing of caspase 8. The caspase 9-like activity was also blocked by both Z-VAD.fmk and Ac-IETD-CHO (Figure 5D). This result suggested that caspase 8-like caspase might work as an initiation caspase that triggered the release of cytochrome C and caspase 9 activation in this system.

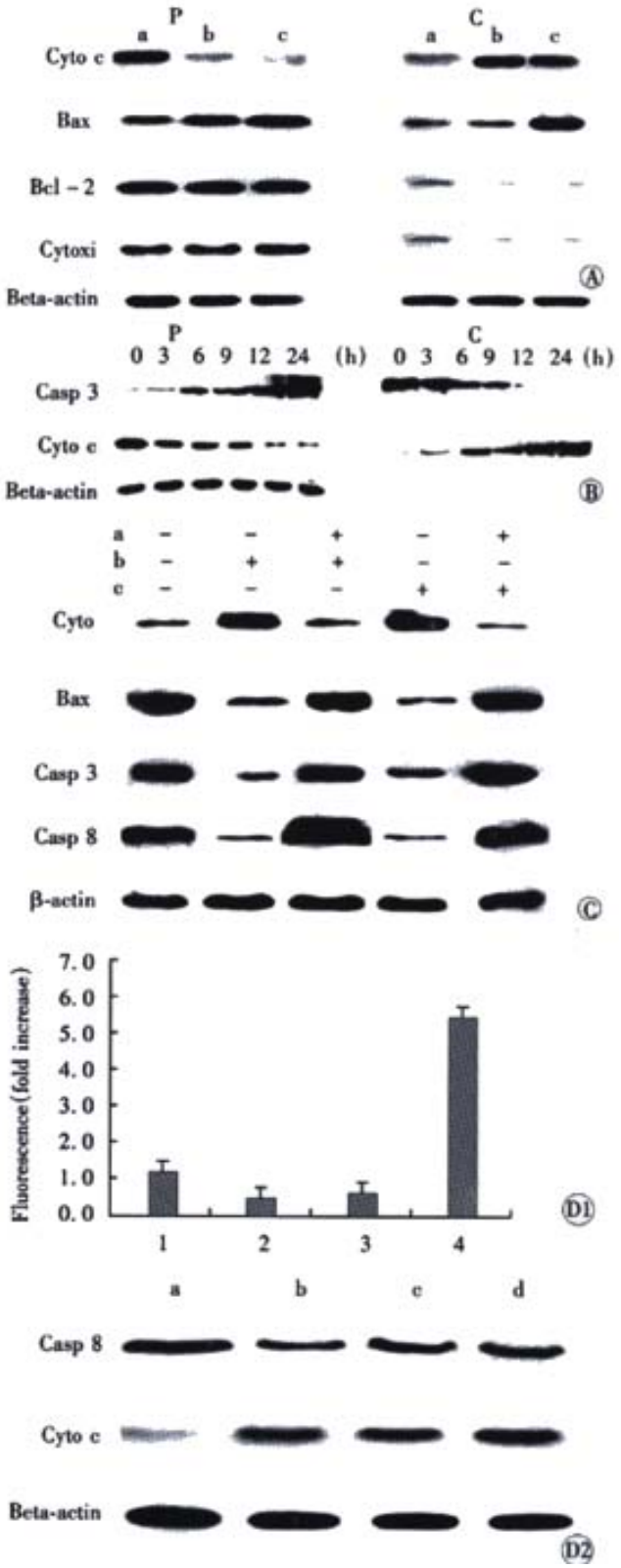

Figure 5 Effect of JTE522 on the activation of caspase, cytochrome C release and membrane translocation of Bax. Figure 5A: AGS cells were treated with: RPMI 1640 medium for control (a) or 0.1μmol/L staurosporine (b) or 1mmol/L JTE-522(c). After 24h, the cells were collected and suspended in mitochondria isolation buffer. Cytosol (C) and pellet (P) fractions were separated and subjected to Western blotting as described in Materials and Methods. Cytochrome C (cyto c), cytochrome C oxidase subunit IV (Cyt oxi); Figure 5B: Effect of JTE522 on the changes of cytosolic cytochrome C release and procaspase 3 (casp-3); Figure 5C: Effect of caspase inhibitors on cytochrome C release and translocation of Bax. AGS cells were treated with 50

μmol/L Z-VAD.fmk (a) for 1h or 0.1μmol/L staurosporine(b) or 1mmol/L JTE-522(c) or without any treatment. The cytosol was prepared as in (A) and analyzed by Western blotting with indicated antibodies, Casp 3 (procaspase 3), Casp 8 (procaspase 8); Figure 5D: AGS cells were cultured at RPMI 1640 medium or treated with caspase inhibitors: Figure 5D1: Effect of caspase inhibitor on the activity of caspase 9: 1: control; 2: Z-VAD.fmk+1mmol/L JTE-522; 3: Ac-IETD-CHO+1 mmol/L JTE-522; 4: 1mmol/L JTE-522 for 24h; Figure 5D2: Effect of caspase inhibitor on caspase 8 cleavage, cytochrome c release: 1: control(a); 2: Z-VAD.fmk+1mmol/L JTE-522 (b); 3: Ac-IETD-CHO+1mmol/L JTE-522(c); 4: Ac-LEHD-CHO +1 mmol/L JTE-522(d). Cells were collected and analyzed for caspase 8 cleavage, cytochrome C release, or caspase-like acitivity as described in Materials and Methods.

Effect of JTE-522 and Z-VAD.fmk on mitochondria membrane potential

It had been described that cytochrome C release could be induced by mitochordrial permeability transition (PT) characterized by loss of $\triangle\Psi m$[27]. As salicylates induced mitochondrial PT and loss of $\triangle\Psi m$ in isolated mitochondria and hepatocytes, we analyzed the effect of JTE-522 on $\triangle\Psi m$ in AGS cells using rhodamin 123 dye. As shown in Figure 6, JTE-522 had no significant effect on $\triangle\Psi m$ at 3h and decrease the uptake of rhodamin 123 at 6h, 9h and 12h, earlier than mitochondrial cytochrome C release and caspases 8 and 9 processing. In addition, Z-VAD.fmk blocked loss of $\triangle\Psi m$ induced by JTE-522 at 9h and had a marked inhibitory effect at 12h, indicating that loss of $\triangle\Psi m$ was caspase-dependent and this loss was in a time-dependent manner.

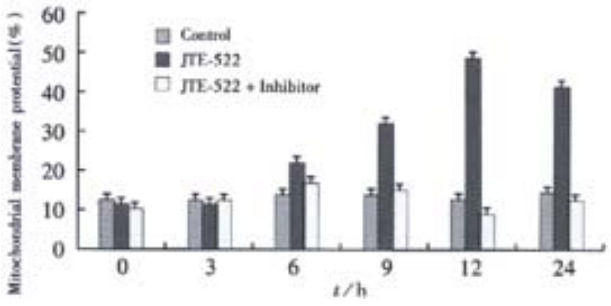

Figure 6 Time-lapsed changes of mitochondrial $\triangle\Psi m$ in AGS cells under the treatment of JTE-522. The data were the means of three experiment±SD

DISCUSSION

NSAIDs have another interesting effect which is seemly unrelated to their anti-inflammatory action. Administration of some NSAIDs to patients and animals suffering from colonic polyps and colon cancer causes regression of the aberrant growth, while epidemilolgical studies have shown that long-term consumption of NSAIDs, greatly reduces the risk of developing colon cancer and gastric adenocarcinoma[28-33]. However, the mechanism remains unclear. Many studies showed that apoptosis was likely to play an extremely important part in the pathogenesis of NSAIDs-induced ulcerogenesis, and also likely to be involved in regression of colon cancer and other neoplasms[34]. The mechanism underlying the inducting of apoptosis by JTE-522 and other NSAIDs is unknown and currently under research. So far, several pathways have been implicated inducing cyclooxygenase inhibition[32-35], arrest of cell cycle[36]and nuclear factor-kB inhibition[37,38]. Although several groups have demonstrated the involvement of caspases[5], the pathways leading to caspase activation remain unknown. In this study, we observed for the first time the caspase-dependent membrane translocation of Bax and cytosolic release of cytochrome C and the loss of $\triangle\Psi m$ in JTE-522-induced apoptosis. These results indicate that mitochondria play an important role in JTE-522-induced apoptosis.

A number of pro- and anti-apoptotic members of the Bcl-2 protein family regulate the release of cytochrome C and apoptosis inducing factor (AIF) from the mitochrondrial intermembrane space into the cytosol[39-43]. Cytochrome C then interacts with pro-caspase 9 and Apaf-1 to activate caspase 9 and thus switch on caspase 3, 6 and 7, leading to apoptosis. The cytosolic release of cytochrome C has been reported in apoptosis induced by various stimuli, including NSAIDs[43]. In this study, we observed the cytosolic release of cytochrome C, as well as the activation of caspase 9. In contrast to the caspase-independent release of cytochrome C in Raji cells induced by aspirin[44] the release of cytochrome C was almost completely blocked by caspase inhibitor in our system, suggesting that different mechanisms are involved in cytochrome C release in these two systems.

Disruption of mitochondrial function is a critical event in the apoptotic process leading to the elimination of cells treated with chemptherapeutic agents. Opening of the mitochondrial megachannel has been implicated as a key event in the disruption of mitochondrial membrane integrity during apoptosis. Many intrinsic factors can induce opening if the megachannel including members if the Bcl-2/Bax family, cellular redox status, and cytosolic Ca^{2+} levels[45]. Disruption of mitochondrial membrane integrity involves the loss of metabolic functions and release of proteins from the mitochondrial intermembrane space into the cytosol. Cytochrome C and AIF represent two such proteins, which are released into the cytosol, and promote caspase and/or nuclease activation. However, there are considerable uncertainties about how cytochrome C and AIF are released from mitochondria and about the events leading up to their appearance in the cytosol. One of them is that the pro-apoptotic proteins Bax and Bak that accelerate opening of the voltage-dependent anion channel (VDAC) in the outer membrane and thereby specifically release cytochrome C[46,47]. Bax is a proapoptotic member of Bcl-2 family proteins, which can form channels in synthetic membrane[48-50] and induce cytochrome C release from isolated mitochondria[51]. However, the mechanism by which Bax stimulates cytochrome C release is unclear. Bax contains the hydrophobic membrane-anchoring domain at its C-terminus, but the membrane-anchoring potential is repressed by its N-terminal domain, allowing Bax to distribute typically in cytosol[52]. Upon apoptotic stimulation, Bax inserts into mitochondria membrane, where it exerts its function[53,54]. The overexpression[55], enforced dimerization[56], and Bid-induced conformation change[57] trigger the redistribution of Bax. In this study, we observed JTE-522-induced membrane translocation of Bax and this translocation was not associated with the increase of Bax protein level, but dependent on caspase activity. Caspase 8 has been shown to cleave Bid and the cleaved Bid is reported to be more efficient for triggering the oligomerization and insertion of Bax into mitochondria membrane. In agreement with these observations, we detected caspase 8 activation by cleavage activity of a caspase 8-specific fluorogenic substrate, as well as cleavage of caspase 8 in our system. In addition, the caspase 8-preferential inhibitor effectively blocked cytochrome C release and caspase 9 activation, suggesting caspase 8 to be an upstream caspase.

The most informative measure of the changes associated with cytochrome C release is the mitochondrial membrane potential. However, such measurement in apoptotic cells has produced conflicting results, with reports of no decrease in potential until after cytochrome C release, a decrease in membrane potential associated with cytochrome C release[50], and an initial increase in potential followed by cytochrome C release without loss of membrane potential[58]. These measurements rely on the uptake of fluorescent dyes, which gives a qualitative indication of the membrane potential. In this study, our results indicate that JTE-522-induced decrease in $\triangle\Psi m$ precedes the release cytochrome C at an early phase of apoptosis. This result is different from the report that aspirin induced cytochrom c release precedes the decrease of $\triangle\Psi m$ in Raji cells. This

discrepancy might reflect that multiple pathways mediated NSAIDs-induced apoptosis depending on experimental systems.

In summary, The present data indicate a crucial association between activation of caspases 8 and 9, cytochrome C release, membrane translocation of Bax, loss of mitochondrial membrane potential and JTE-522-induced apoptosis in AGS cells.

ACKNOWLEDGEMENTS

We gratefully acknowledge Prof Geng-Tao Lui, Institute of Material Medica, Chinese Academy of Medical Sciences for the many helpful discussion and suggestions relating to this work; Special thanks to Chris Simmet and Pasricha Jerriment for proofreading the manuscript and for useful suggestions; Dr Cheng Wei He for technical advice and helpful discussion; Dr Gang Fei Peng for FCM; Dr Wang Tao for Western blot; Dr Guo Qing Xie and Mr Hai Nan Wang for photo processing.

REFERENCES

1 Qiao L, Shiff SJ, Rigas B. Sulindac sulfide alters the expression of cyclin proteins in HT-29 colon adenocarcinoma cells. *Int J Cancer* 1998;76:99-104
2 Shiff SJ, Koutsos MI, Qiao L. NSAIDs inhibits the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis. *Exp Cell Res* 1996; 222: 179-188
3 Robertson FM, Parrett ML, Joarder FS, Ross M, Harris RE. Ibuprofen-induced inhibition of cyclooxygenase isoform gene expression and regression of rat mammary carcinomas. *Cancer Lett* 1998; 122: 165-175
4 Duffy CP, Elliott RA, Heenan MM, Coyle S, Cleary IM, Clynes M. Enhancement of chemotherapeutic drug toxicity to human tumor cells *in vitro* by a subset of non-steroidal anti-inflammatory drugs. *Eur J Cance* 1998; 34: 1250-1259
5 Bellosillo B, Colomer D, Montserrat E, Pons G, Gil J. Aspirin and salicylate induce apoptosis and activation of caspases. *Blood* 1998; 92: 1406-1414
6 Zhang GS, Tu CQ, Zhang GY, Zhou GB, Zheng WL. Indomethacin induces apoptosis and inhibits proliferation in chronic myeloid leukemia cells. *Leukemia Res* 2000; 24: 385-392
7 Tomozawa S, Nagawa H, Tsuno N. Inhibition of haematogenous metastasics of colon cancer in mice by a selective COX-2 inhibitor, JTE-522. *Br J Cancer* 1999; 81: 1274-1279
8 Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997; 326: 1-6
9 Kumar S, Lavin MF. The ICE family of cysteine proteases as effectors of cell death. *Cell Death Differ* 1996; 3: 255-267
10 Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. *Cell* 1997; 91: 443-446
11 Luo X, Budihardjo L, Zou H, Slaughter C, Wang X. Bid, a Bcl-2 interacting protein, mediates cytochrome C release from mitochondria in response to activation of cell surface death reeptors. *Cell* 1998; 94: 481-490
12 Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome C and dATP-dependent formation of Apaf-1/caspasse-9 complex initiates an apoptotic protease cascade. *Cell* 1999; 91: 479-489
13 Shimizn S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome C by the mitochondrial channel VDAC. *Nature* 1999; 399: 483-485
14 Antonsson B, Montessuit S, Lauper S. Bax oligomerization is equired for channer-forming activity in liposomes and to trigger cytochrome C release from mitochondria. *Biochem J* 2000;345: 271-278
5 Goping IS, Gross A, Lavoie JN, Nguyen M, Shore GC. Regulated targeting of bax to mitochondria. *J Cell Biol* 1998; 143: 207-215
16 Hsu YT, Wolter KG, Youle RJ. Cytosol-to-membraane redistribution of Bax and Bcl-XL during apoptosis. *Proc Natl Acad Sci USA* 1997; 94: 3668-3672
17 Li HL, Zhang HW, Ren XD. Synergism between heparin and adriamycin on cell proliferation and apoptosis in human nasopharyngeal carcinoma CNE2 cells. *Acta Pharmacol sin* 2002;23:167-172
18 Li HL, Ye KH, Ren XD. Heparin induced apoptosis in human nasopharyngeal carcinoma CNE2 cells. *Cell Research* 2001;11:311-315
19 Eguchi Y, Srinivasan A, Tomaselli KJ, Shimizu S, Tsujimoto Y. ATP-dependent steps in apoptosis signal transduction. *Cancer Res* 1997; 59: 2174-2181
20 Gao CF, Ren S, Zhang LL, Ichinose S, Tsuchida N. Caspase-dependent cytosolic release of cytochrome C and membrane translocation of Bax in *P*53-induced apoptosis. *Exp Cell Res* 2001; 265: 145-151
21 Akihiro I, Uehara T, Tokumitsu A, Okuma Y, Nomura Y. Possible involvement of cytochrome C release and sequential activation of caspases in ceramide-induced apoptosis in SK-N-MC cells. *Biochim Biophy Acta* 1999; 1452: 263-274
22 Ferrer I, Lopez E. Staurosporine- and H-7-induced cell death in SH-SY5Y neuroblastoma cells is associated with caspase-2 and caspase-3 activation, but not with activation of the FAS/FAS-caspase-8 signaling pathway. *Mol Brain Res* 2000; 85: 61-67
23 Scarlett JL, Sheard PW, Hughes G, Ledgerwood EC, Ku HH, Murphy MP. Changes in mitochondrial membrane potential during staurosporine-induced apoptosis in Jurkat cells. *FEBS Lett* 2000; 475: 267-272
24 Zamzami N, Susin SA, Marchetti P. Mitochondrial control of nuclear apoptosis. *J Exp Med* 1996; 183: 1533-1544
25 Zhang JG, Cohen GM. Release of mitochondrial cytochrome C is upstream of caspase activation in chemical-induced apoptosis in human monocytic tumor cells. *Toxicol Lett* 1998; 102-103: 121-129
26 Murphy KM, Streips UN, Lock RB. Bax memebrane insertion during Fas (CD95)-innduced apoptosis precedes cytochrome C release and is inhibited by Bcl-2. *Oncogene* 1999; 18: 5991-5999
27 Petit PX, Susin SA, Zamzani N. Mitochondrial and programmed cell death: back to the future. *FEBS Lett* 1996; 396: 7-9
28 Thun MJ. Aspirin and gastrointestinal cancer. *Adv Exp Med Bio* 1997; 400A: 395-402
29 Waston AJM. Review article: manipulation of cell death-the development of novel strategies for the treatment of gastronintestinal disease. *Aliment Pharmacol* 1995; 9: 215-226
30 Smith ML, Havvcraft G, Hull MA. The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action. *Eur J Cancer* 2000; 36: 664-674
31 Zhu GH, Ching CK, Lai KC, Lam SK. Differential apoptosis by indomethacin in gastric epithelial cell through the constitutive expression of wild-type *P*53 and/or up-regulation of c-myc. *Biochem Pharmacol* 1999; 58: 193-200
32 Sheng M, Dinchuk JE, Kargman SL. Suppression of intestinal polyposis in Apc716 knockout mice by inhibition of COX-2. *Cell* 1996; 87: 803-809
33 Wu YL, Sun B, Zhang XJ, Wang SN, He HY, Qiao MM, Zhong J,Xu JY. Growth inhibition and apoptosis induction of Sulindac on human gastric cancer cells. *World J Gastroenterol* 2001;7:796-800
34 Huang S, Li JY, Wu J, Meng L, Shou CC. Mycoplasma infections and different human carcinomas. *World J Gastroenterol* 2001;7:266-269
35 Sheng GG, Shao J, Sheng H. A selective COX-2 inhibitor suppresses cells. *Gastroenterology* 1997; 113: 1883-1897
36 Antonsson B, Conti F, Ciavatta A, Montessuit SL, Sano H, Kawahito Y, Wilder R. Expression of COX-1 and COX-2 in human colorectal cancer. *Cancer Res* 1995; 55: 3785-3789
37 Qiao L, shiff SJ, Rigas B. Sulindac sulfide inhibits the proliferation of colon cancer cells: diminished expression of the proliferation markers PCNA and ki-67. *Cancer Lett* 1997; 115: 229-234
38 Kipp E, Ghosh S. Inhibition of NF-kB by sodium salicylate and aspirin. *Science* 1994; 265: 956-959
39 Giardina C, Boulares H, Inan MS. NSAIDs and butyrate sensitize a human colorectal cancer cell line to TNF and Fas ligation: the role of reactive oxygen species. *Biochim Biophys Acta* 1999; 1448: 425-438
40 Scarlett JL, Sheard PW, Hughes G, Murphy MP. Changes in mitochondrial membrane potential during staurosporine-induced apoptosis in Jarket cells. *FEBS lett* 2000; 475: 267-272
41 Xu CT, Huang LT, Pan BK. Current gene therapy for stomach carcinoma. *World J Gastroenterol* 2001;7:752-759
42 Kroemer G, Reed JC. Mitochondrial control of cell death. *Nature Med* 2000; 6: 513-519
43 Shen ZY, Shen J, Li QS, Chen CY, Chen JY, Zeng Y. Morphological and functional changes of mitochondria in apoptosis esophaged carcinoma cells induced by arsenic trioxide. *World J Gastroenterol* 2002;8:31-35
44 Du C, Fang M, Li Y, Wang X. Smac A. Mitochondrial protein that promotes cytochrome C dependent caspase activation by eliminating IAP inhibition. *Cell* 2000; 102: 43-53
45 Pique M, Pons G, Gil J. Aspirin induce apoptosis through mitochondrial cytochrome C release. *FEBS lett* 2000; 480: 193-196
46 Zhivotovsky B, Orrenius S, Brustugun OT.Injected cytochrome C release in aoptosis. *Nature* 1998; 391: 449-457
47 Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondriaal cytochrome C release in apoptosis occurs upstream of the DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998; 17: 37-45
48 Yang J, Liu X, BhallaK, Kim CN, Ibrado AM, Cai J, Peng T, Jones DP, Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome C from mitochondria blocked. *Nature* 1997; 275: 1129-1132
49 Adachi S, Cross AR, Babior BM, Gottieb RA. Bcl-2 and the outer mitochondrial membrane in the inactivation of cytochrome C during Fas-mediated apoptosis. *J Biol Chem* 1997; 272: 21878-21882
50 Ewis S, Martinou I, Bernasconi L, Bernard A, Mermod J, Maazzei G,

Maundrell K, Gambale F, Sadoul R, Martinou J. Inhibition of Bax channel-forming activity by Bcl-2. *Science* 1997; 272: 370-372
51 Jurgensmier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome C from isolated mitochondria. *Proc Natl Acad Sci USA* 1997; 95: 4997-5002
52 Goping I, Gross A, Lavoie JN, Nguyen M, Jemmeeerson R, Roth K, Korsmeyer SJ, Hore GC. Regulated targeting of BAX to mitochondria. *J Biol Chem* 1998; 143: 207-215
53 Wolter KG, Hsse YT, Smith CL, Nechushtan AX. Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997; 139: 1281-1292
54 Bruel A, Karenty E, Schmid M, Mcdonnell TJ, Lanottte M. Altered sensitivity to retinoid-induced apoptosis associated with changes in the subcellular distribution of Bcl-2. *Exp Cell Res* 1997; 223: 281-287
55 Rosse T, Oliver R, Monney L, Rager M, Conus S, Fellay I, Jansen B, Broner C. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome C. *Nature* 1998; 391: 496-499
56 Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation,mitochonddria dysfunction and apoptosis. *EMBO J* 1998; 17: 3878-3885
57 Desagher S, OsenSand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC. Bid-induced conformational cytochrome C release during aoptosis. *J Cell Biol* 1999; 144: 891-901
58 Eskes R, Desaggher S, Antonsson B, Marrtinou JC. Bid induced the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* 2000; 20: 929-935
59 Green DR, Reed JC. Mitochondria and aoptosis. *Science* 1998; 281: 1309-1312

Edited by Zhang JZ