

Mitogen-Activated Protein Kinases and Chemoresistance in Pancreatic Cancer Cells

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Background. Chemoresistance is an important clinical problem in pancreatic cancer. As the mitogen-activated protein kinases (MAPKs) have been found to be involved in the development of chemoresistance in a variety of cancer cell lines, the aim of the current study was to assess the role and mechanism of MAPK signaling in mediating chemoresistance in pancreatic cancer cells.

Materials and methods. The effects of pharmacological inhibition of MAPKs on resistance of pancreatic cancer cells to apoptosis induced by treatment with chemotherapeutic drugs were analyzed.

Results. Compared with parental cells, the activity of extracellular signal-regulated kinase (ERK) was elevated in all of the three chemoresistant sublines at basal conditions. Inhibition of the ERK pathway by PD98059 sensitized cells to 5-fluorouracil (5-FU), whereas cells became more resistant to Adriamycin (ADM; Meiji Seika, Tokyo, Japan) and gemcitabine (GEM). 5-FU induced apoptosis primarily via a caspase-8-dependent pathway, and ADM and GEM via caspase-9. PD98059 enhanced the activity of caspase-8 and inhibited the activation of caspase-9. In addition, PD98059 regulated the level of phospho-Bcl-2.

Conclusions. These data suggest that although constitutive activation of the ERK pathway might be a marker of chemoresistance, the effects of this pathway on chemoresistance of pancreatic cancer cells are drug dependent. This study also provides evidence for

a possible link between the ERK pathway and activation of the caspases and Bcl-2. © 2006 Elsevier Inc. All rights reserved.

Key Words: MAPK; pancreatic cancer; chemotherapy; drug resistance; apoptosis.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in Western industrialized countries [1]. In 2006, it is estimated that more than 33,700 new cases of pancreatic cancer will be diagnosed in the United States, with virtually the same number of deaths (32,300) from this disease [2]. Chemotherapy has proven to be effective in the cure and palliation of many human malignancies, such as testicular cancer and leukemia. However, drug resistance remains a major obstacle in the treatment of other cancers, especially pancreatic adenocarcinoma [3, 4].

The mitogen-activated protein kinase (MAPK) pathway is one of the most important membrane-to-nucleus signaling mechanisms, functioning as a mediator of cellular responses to a variety of cellular stimuli [5, 6]. In mammalian cells, there are three well-characterized subfamilies of MAPKs: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK, also known as the stress-activated protein kinases), and the p38 MAPK kinases. Each MAPK is activated through a specific phosphorylation cascade. The ERK pathway is thought to be activated in response to growth factors, conferring a survival advantage to cells [5, 6]. In contrast, JNK and p38 MAPK are activated in response to a variety of environmental stressors and inflammatory cytokines, which are fre-

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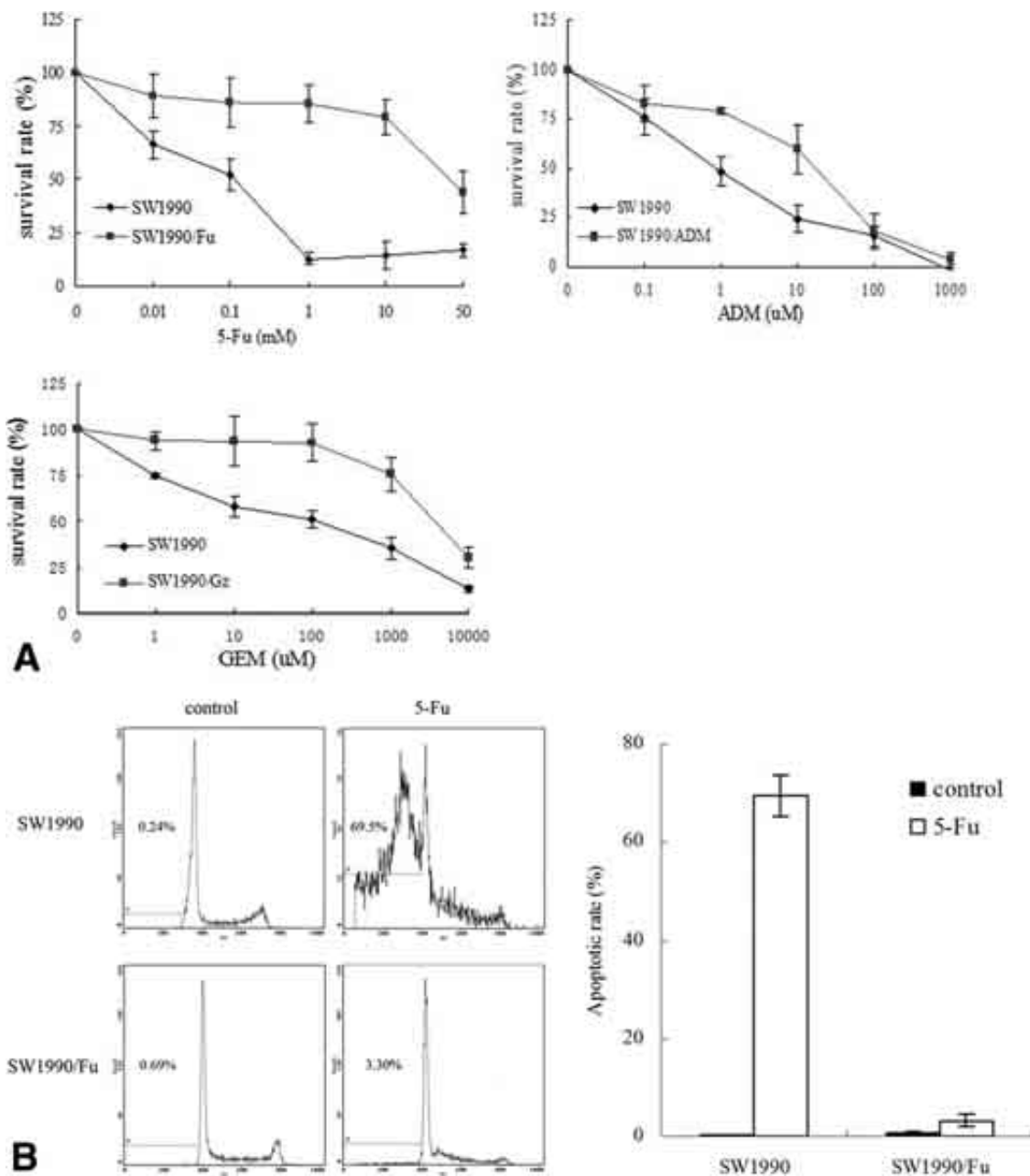


FIG. 1. Increased resistance of chemoresistant sublines to chemotherapeutic drugs compared with the parental cell line SW1990. **A**, Concentration-survival curves of the SW1990 cell line and the acquired chemoresistant sublines. Cells were seeded in 96-well plates and cultured for 24 h. Cells were then treated with the indicated concentrations of chemotherapeutic drugs for 48 h, followed by a WST-8 assay according to the manufacturer's instructions. There were five duplicate wells for each concentration, and experiments were repeated three times. **B**, Increased resistance of SW1990/FU to apoptosis induced by 5-FU. Exponentially growing SW1990 cells and resistant subline SW1990/FU cells were cultivated in 6-well plates for 24 h with drug-free medium and were then treated with the same concentration of 5-FU (5 mM) for 72 h. Both adherent and non-adherent cells were collected and stained with propidium iodide, and the number of apoptotic cells was counted with a Coulter Elite flow cytometer. Results represent the mean \pm SD of three independent experiments.

quently associated with the induction of apoptosis [5, 6]. When activated, MAPK can phosphorylate specific serines and threonines of target protein substrates and regulate diverse biological functions. Recent advances in cancer research have shown that the MAPK pathway may regulate tumor cell proliferation, invasion, survival, and apoptosis [7, 8].

The ERK, JNK, and p38 MAPK pathways have been molecular targets for drug development, and phase I/II clinical trials with pathway inhibitors have been completed in patients with locally advanced and metastatic cancer [9–13]. Some studies have suggested that activation of the MAPK pathway may be involved in chemoresistance in certain cancers. However, little is

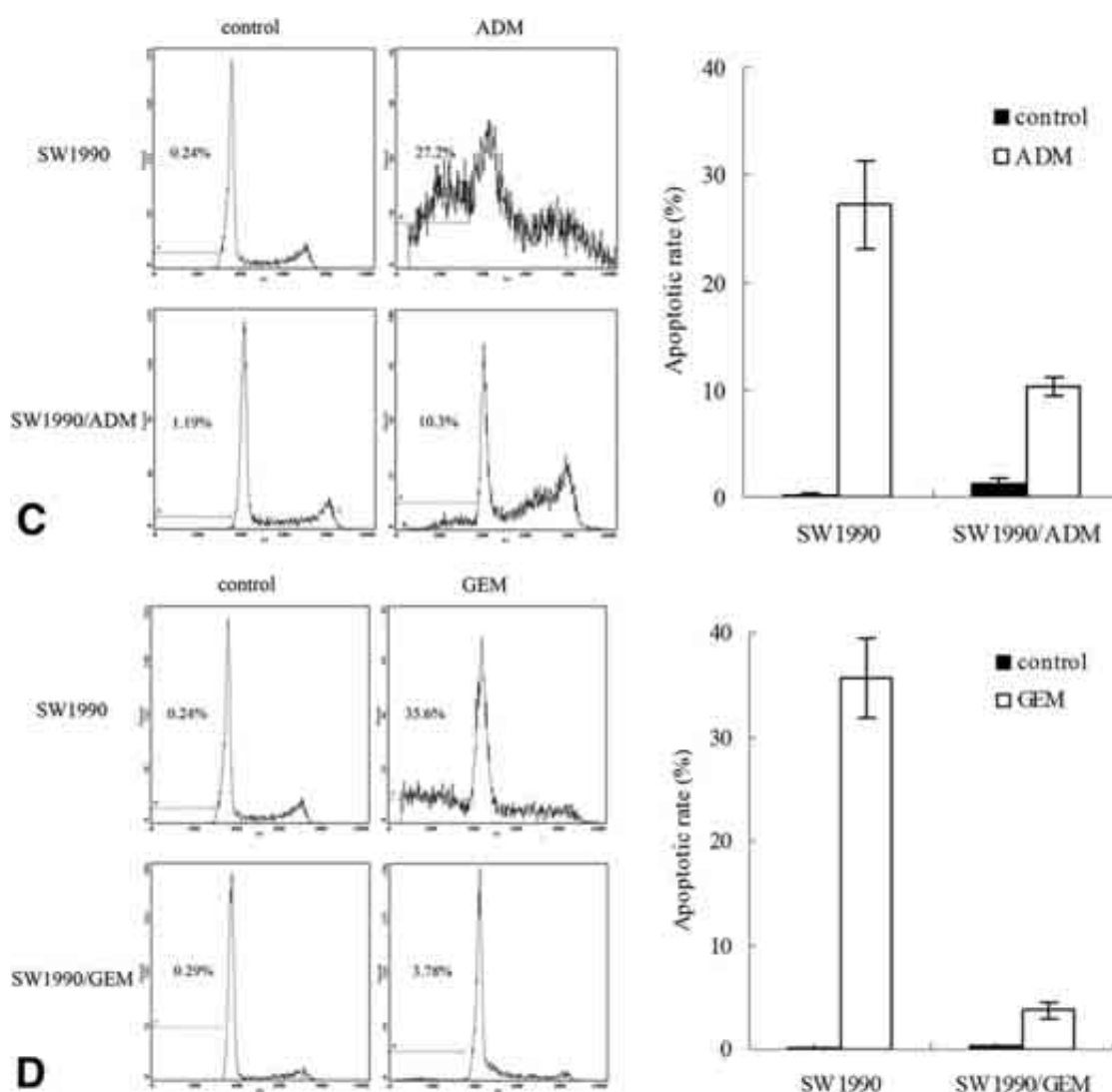


FIG. 1—Continued. C, Increased resistance of SW1990/ADM to apoptosis induced by 50 μM ADM for 72 h. D, Increased resistance of SW1990/GEM to apoptosis induced by 1 mM GEM for 72 h.

known regarding the role of the MAPK pathway in chemoresistance in pancreatic cancer. The phosphatidylinositol-3 kinase (PI3K)/AKT pathway is another important pathway in the regulation of cell survival which may mediate resistance to the pro-apoptotic effects of chemotherapeutic drugs in a variety of cancer types [14–17].

Although some studies have found that MAPK and PI3K/Akt pathways may regulate apoptosis of cancer cells induced by chemotherapeutic drugs, the mechanisms at the molecular level remain unclear. It is widely accepted that chemotherapy acts primarily by inducing apoptosis, and that alterations in the apoptotic pathway can make cancer cells resistant to therapy [16, 18, 19].

Apoptosis can be initiated either through the death receptor or the mitochondrial pathway. Caspases are activated by cleavage, which can be activated in both

pathways [16, 20]. The Bcl-2 family is a central regulator of programmed cell death, and members that inhibit apoptosis, such as Bcl-2 and Bcl-XL, are overexpressed in many cancers and contribute to tumor resistance to chemotherapy [20, 21]. However, whether MAPK and PI3K pathways mediate chemoresistance by modulating activity of caspases and members of the Bcl-2 family has, until now, not been analyzed in pancreatic cancer.

In the present study, we investigated the role of the MAPK (including ERK, JNK, and p38 MAPK) and PI3K/Akt signaling pathways in mediating chemoresistance of pancreatic cancer cells.

MATERIALS AND METHODS

Cell Lines

The human pancreatic cancer cell line SW1990 was purchased from American Type Culture Collection (ATCC, Manassas, VA). The

chemoresistant subline SW1990/FU was established by exposing SW1990 cells to 5-fluorouracil (5-FU, Sigma, St. Louis, MO) in step-wise increments of concentration from 1 $\mu\text{g}/\text{mL}$ to 2000 $\mu\text{g}/\text{mL}$ over 12 months [22]. The chemoresistant sublines SW1990/ADM and SW1990/GEM were selected over 10 months of pulse drug treatments with Adriamycin (ADM; Meiji Seika, Tokyo, Japan) and gemcitabine (GEM; Eli Lilly, Indianapolis, IN), respectively [23, 24]. In each pulse treatment, SW1990 cells growing exponentially were exposed to ADM for 24 h or GEM for 48 h, and then cultured in drug-free growth medium. The parental cell line and the established chemoresistant sublines were grown in RPMI 1640 culture media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$) in a humidified 5% CO_2 incubator at 37°C. Experiments were performed using cells in the exponential phase of growth. The chemoresistant sublines were used after they had been grown in drug-free medium for at least 2 months.

Reagents

The following antibodies used in Western blot analysis were purchased from Cell Signaling Technology (Beverly, MA): phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, p44/42 MAP kinase antibody, phospho-p38 MAP kinase (Thr180/Tyr182) antibody, p38 MAP kinase antibody, phospho-SAPK/JNK (Thr183/Tyr185) antibody, SAPK/JNK antibody, phospho-Akt (Ser473) antibody, Akt antibody, PARP antibody, cleaved caspase-3 (Asp175) antibody, cleaved caspase-9 (Asp315) antibody (human specific), caspase-8 (1C12) mouse mAb, phospho-Bcl-2 (Ser70) antibody (human specific), Bcl-xL antibody, and goat anti-rabbit IgG HRP-linked antibody.

Drug Treatments

The concentrations of chemotherapeutic drugs were chosen based on the results of preliminary studies that explored their effects on cell cycle inhibition and induction of apoptosis. Equal numbers of cells were treated with the vehicle (<1% DMSO), PD98059 (Cell Signaling Technology, Beverly, MA), and chemotherapeutic drugs as single agents or in combination with PD98059 for 72 h. When PD98059 and chemotherapeutic drugs were combined, PD98059 was given 1 h before the chemotherapeutic drugs.

Chemosensitivity Testing

The relative cytotoxicity of 5-FU, ADM, and GEM in SW1990 cells and chemoresistant sublines was assessed with a WST-8 assay using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan) [25]. This assay is based on the reduction of a tetrazolium compound to a soluble derivative by the dehydrogenase enzymes of metabolically active cells. The absorbance (450 nm) is directly proportional to the number of living cells in culture. Briefly, cells were added to 96-well tissue culture plates (1×10^5 cells/well) and cultured for 24 h with a drug-free medium supplemented with 10% FBS. Then cells were exposed to increasing concentrations of chemotherapeutic drugs for 48 h, after which the number of remaining living cells was determined according to the manufacturer's instructions. There were five duplicate wells for each concentration and each experiment was repeated at least three times. Chemosensitivity was defined as the drug concentration that inhibited cell proliferation by 50% (IC50) and was determined from concentration-survival curves. The resistance index (RI) was calculated by comparing the IC50 values between the chemoresistant subline and the parental cell line.

Western Blot Analysis

The cells were lysed with ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1.0 mM EDTA, 1 mM sodium orthovanadate, 2.5 mM sodium PPi, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 25 $\mu\text{g}/\text{mL}$ leupeptin and 25 $\mu\text{g}/\text{mL}$ aprotinin). After 30 min incubation on ice,

samples were separated by centrifugation at 12,000 rpm for 15 min and the supernatants were stored at -80°C . Protein content in the lysed extracts was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (60 $\mu\text{g}/\text{lane}$) were separated by 10% to 12% SDS-PAGE (Bio-Rad, Hercules, CA) and transferred to Hybond-P nitrocellulose membranes (Amersham Biosciences, Little Chalfont, United Kingdom). Blots were probed with appropriately diluted primary antibody and peroxidase-conjugated secondary antibody. The bound antibody was detected by enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Santa Cruz, CA).

Detection of Apoptosis

Flow Cytometry

After treatment, trypsinized and floating cells were pooled, centrifuged for 5 min at 1,000 rpm, washed with PBS (PBS), and fixed in 70% (v/v) ethanol at 4°C overnight. Before analysis, cells were centrifuged, washed twice in PBS, and subsequently suspended in a DNA staining solution (50 μg propidium iodide and 100 μg RNase A per 1 mL of PBS), and incubated for 30 min at room temperature in the dark. Samples were then analyzed for DNA content by the Coulter EPICS XL flow cytometer (Beckman-Coulter, Miami, FL). The data from 10,000 events were collected and analyzed using XL Analysis Software (version A27153 3.0). The percentage of cells in the sub- G_0 phase was quantitated as an estimate of cells undergoing apoptosis.

Fluorescence Microscopy

Cells were seeded on sterile cover glasses placed in six-well plates and cultured overnight. After treatment with drugs as indicated, cells were fixed, washed twice with PBS, and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, Haimen, Jiangsu, China). Image capture and slide evaluations were performed using a Nikon 80i fluorescence microscope equipped with ACT-2U Imaging Software (Nikon, Tokyo, Japan). Apoptotic cells were defined by the condensation of nuclear chromatin, fragmentation, or margination to the nuclear membrane.

Statistical Analysis

All experiments were performed at least three times, and the results were expressed as mean \pm SD. Statistical analysis and *P* value determinations were done by the two-tailed paired *t*-test. *P* < 0.05 was considered to be significant.

RESULTS

Drug Sensitivity Assay Between Chemoresistant Sublines and the Parental Cell Line SW1990

Compared with the parental cell line SW1990, the established chemoresistant sublines, SW1990/Fu, SW1990/ADM, and SW1990/GEM, were more resistant to 5-FU, ADM, and GEM, with RIs of 339.7, 11.9, and 56.6, respectively (Fig. 1A). All three chemotherapeutic agents induced apoptosis in pancreatic cancer cells in a time-dependent and dose-dependent manner (data not shown). Apoptosis levels were compared between the resistant and parental cells treated with chemotherapeutic drugs (5 mM 5-FU, 50 μM ADM, or 1 mM GEM) for 72 h. As shown in Fig. 1B–D, the increased resistance of the chemoresistant sublines to each of the

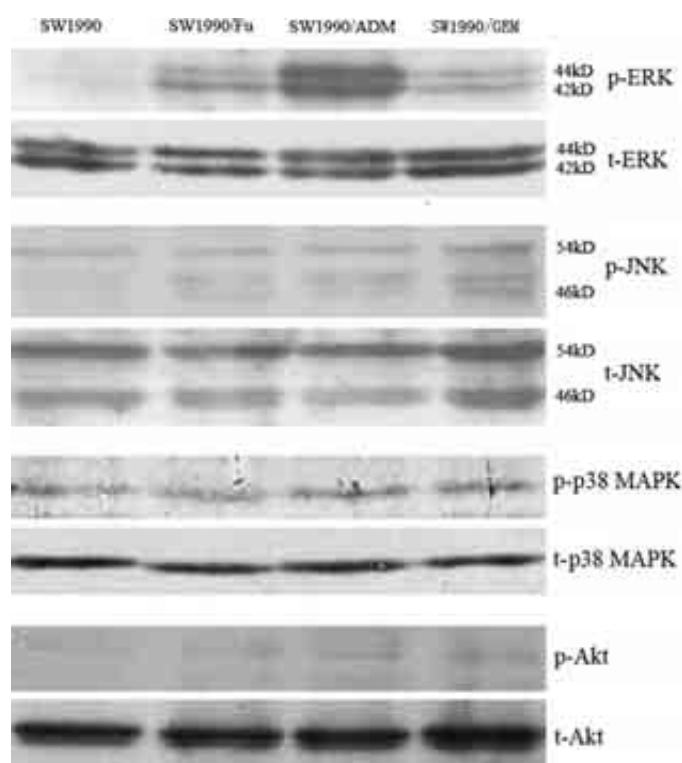


FIG. 2. Phosphorylation levels of ERK1/2, JNK, p38 MAPK and Akt kinases detected by Western blot analysis in SW1990 cells and SW1990 chemoresistant sublines: Equal amounts of total cellular protein from exponentially growing cells were separated by electrophoresis and subjected to Western blot analysis with phospho-specific and total protein antibodies to ERK1/2, JNK, p38 MAPK, and Akt. The non-phosphorylated forms also serve as the loading controls. Compared with the parental cell line SW1990, the phosphorylation levels of ERK1/2 were elevated in all three chemoresistant sublines. Representative blots of at least three independent experiments are shown.

corresponding drugs was associated with reduced induction of apoptosis as determined by flow cytometry.

Phosphorylation Levels of ERK1/2, JNK, p38 MAPK, and Akt in Chemoresistant Sublines and the Parental Cell Line SW1990

To determine the potential involvement of various protein kinase pathways in chemoresistance of pancreatic cancer cell lines, the phosphorylation status (indicative of activation) of the four major protein kinases, ERK1/2, JNK, p38 MAPK, and Akt, was measured by Western blotting (Fig. 2). Compared with the parental cell line SW1990, the phosphorylation levels of ERK1/2 were elevated in all three chemoresistant sublines: SW1990/FU, SW1990/ADM, and SW1990/GEM. Enhanced activation of ERK1/2 was not accompanied by changes in total ERK1/2 protein levels. In contrast, there were no significant differences in the phosphorylation and total protein levels of JNK, p38 MAPK, and Akt between the parental cell line SW1990 and the three chemoresistant sublines.

Effect of ERK1/2 Pathway Inhibition on Chemoresistance of the Chemoresistant Sublines SW1990/Fu, SW1990/ADM, and SW1990/GEM

To further determine the role of the ERK1/2 signaling pathway in the chemoresistance of pancreatic cancer cells, we used a specific ERK signaling pathway inhibitor, PD98059, in combination with 5-FU, ADM, or GEM. PD98059 exerts its effects by binding to the inactive form of MEK1/2 and preventing MEK1/2 activation by Raf-1, thus inhibiting the activation of ERK1/2 [26].

After SW1990/Fu, SW1990/ADM, and SW1990/GEM cells were treated for 72 h with 20 mM 5-FU, 200 μ M ADM, and 10 mM GEM, respectively, the phosphorylation level of ERK1/2 was further increased. However, treatment with 50 μ M PD98059 markedly decreased the activation of ERK1/2 (Fig. 3A).

As shown in Fig. 3B, flow cytometry analysis revealed that PD98059 alone failed to change the number of apoptotic cells, whereas apoptosis of SW1990/FU cells induced by 20 mM 5-FU was significantly increased following administration of PD98059. Furthermore, the inhibition effect of ERK by PD98059 was dose dependent (Fig. 3B). The results of fluorescence microscopy after Hoechst 33258 staining showed that treatment with 50 μ M PD98059 alone did not induce apoptosis, but when combined with 5-FU, more cells underwent apoptotic morphological changes than with 5-FU treatment alone (Fig. 4A).

For SW1990/ADM cells, flow cytometry analysis showed that PD98059 alone did not induce apoptosis, but apoptosis of SW1990/ADM cells induced by ADM was markedly decreased by the addition of PD98059. This effect was in striking contrast to the effect of PD98059 on SW1990/FU cells (Fig. 3C). The resistant effect of PD98059 in SW1990/ADM was dose dependent (Fig. 3C). The observed decrease of apoptosis by the combination treatment of PD98059 and ADM was confirmed by fluorescence microscopy (Fig. 4B).

The effect of ERK1/2 pathway inhibition on resistance of SW1990/GEM cells to GEM was the same as that of SW1990/ADM cells. Thus, PD98059 increased the resistance of SW1990/GEM cells in a dose-dependent manner (Fig. 3D and Fig. 4C).

The Role of the ERK1/2 Signaling Pathway in Regulating PARP (Poly[ADP-Ribose] Polymerase), Caspases, and Bcl-2 Activation

To investigate the possible mechanism of the ERK1/2 pathway in mediating chemoresistance in pancreatic cancer cells, Western blotting was used to analyze activation of apoptosis-related pathways after cells were treated by chemotherapeutic drugs in combination with or without PD98059.

After pancreatic cancer cells were treated with 20 mM 5-FU, 200 μ M ADM, or 10 mM GEM for 72 h, activation of PARP and caspase-3 was readily apparent (Fig. 5).

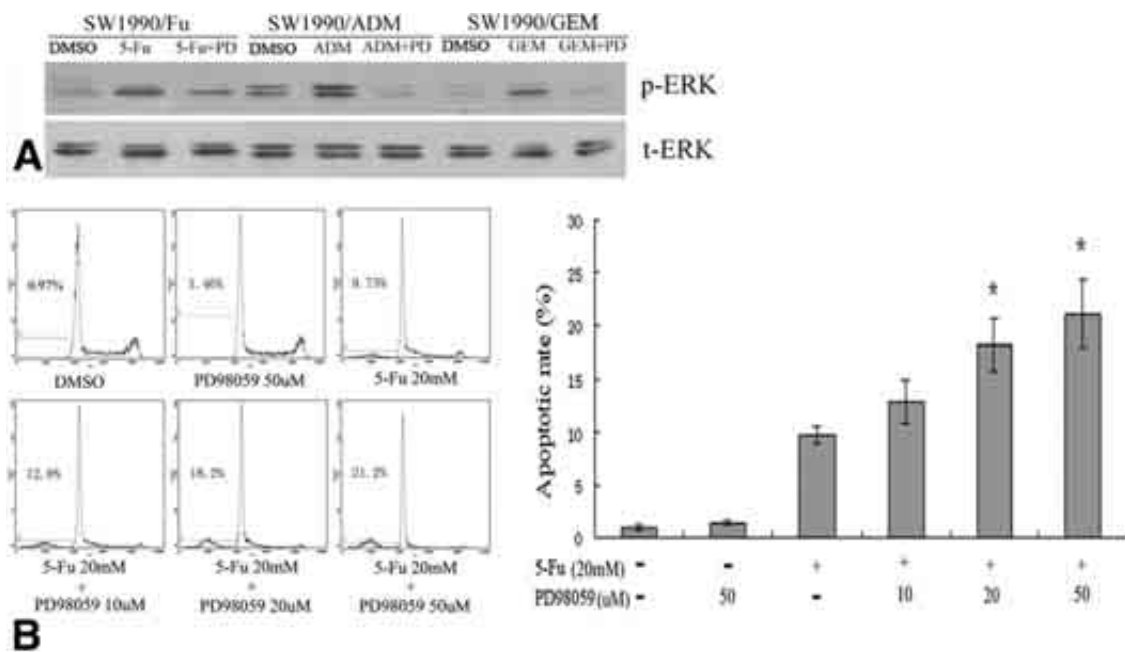


FIG. 3. Effect of ERK1/2 pathway inhibition by PD98059 in pancreatic cancer cells. A, Inactivation of the ERK1/2 pathway by PD98059. After treatment by DMSO (control) or chemotherapeutic drugs (20 mM 5-FU, 200 μ M ADM, and 10 mM GEM) with or without 50 μ M PD98059 for 72 h, cells were lysed and equal amounts of total protein (60 μ g/lane) were subjected to Western blot analysis using phospho-specific and non-phospho-specific antibodies of ERK1/2. The non-phosphorylated forms also serve as the loading controls. B, Sensitization effects of PD98059 on SW1990/Fu cells in combination with 5-FU. Exponentially growing cells were cultivated in 6-well plates for 24 h with a drug-free medium and then treated with DMSO (control) or 5-FU (20 mM) alone or combined with different doses of PD98059 for 72 h. Both adherent and non-adherent cells were collected and stained with propidium iodide, and the number of apoptotic cells was counted with a flow cytometer. Results represent the mean \pm SD of three independent experiments.

ERK1/2 pathway inhibition by 50 μ M PD98059 did not change the level of cleaved PARP and caspase-3 induced by 5-FU, ADM, or GEM (Fig. 5).

Although PARP and caspase-3 were activated by 5-FU, ADM, or GEM, these drugs might induce apoptosis of pancreatic cancer cells through different pathways. After cells were treated by 5-FU, caspase-8 cleavage was pronounced; however, ADM and GEM treatment primarily resulted in caspase-9 cleavage (Fig. 5). Intriguingly, the caspase-8 cleavage was enhanced, while caspase-9 cleavage was blocked by the addition of PD98059 (Fig. 5), which paralleled the resistance to apoptosis after ERK1/2 pathway inhibition.

The expression levels of phospho-Bcl-2 and Bcl-xL were down-regulated by GEM treatment, in contrast to the up-regulation by ADM (Fig. 5). 5-FU treatment resulted in up-regulation of phospho-Bcl-2 expression, but did not change the expression level of Bcl-xL (Fig. 5). Inhibition of the ERK1/2 pathway by PD98059 decreased the expression of phospho-Bcl-2 induced by 5-FU treatment, whereas it had no effect on the expression of phospho-Bcl-2 and Bcl-xL induced by ADM or GEM treatment (Fig. 5).

DISCUSSION

A major obstacle in tumor chemotherapy is the phenomenon of chemoresistance, whereby tumors cells be-

come refractory to drug treatment. In pancreatic cancer, acquired chemoresistance is a problem, notably in therapy with 5-FU, ADM, and GEM, which are widely used in the treatment of this disease. The mechanisms of acquired chemoresistance in pancreatic cancer are largely unknown. In the current study, we compared the resistance of the pancreatic cancer cell line SW1990 with that of several sublines that acquired chemoresistance after long-term exposure to chemotherapeutic agents. Because 5-FU, ADM, and GEM act by inducing apoptosis in pancreatic cancer cells, the resistance to apoptosis induced by these chemotherapeutic drugs was compared. As expected, we found that the chemoresistant sublines had higher resistance to apoptosis, which correlated with the resistance index. This finding is consistent with other reports that have showed that resistance to apoptosis is a main contributor to chemoresistance [16].

The importance of the MAPK signaling pathway in regulating apoptosis under conditions of stress has been widely investigated. The various pathways are illustrated in Fig. 6. A number of studies have supported the view that activation of the ERK pathway delivers a survival signal that counteracts pro-apoptotic effects associated with JNK and p38 MAPK activation [5, 6]. Consistent with such a pro-survival function of ERK, some investigators have reported that

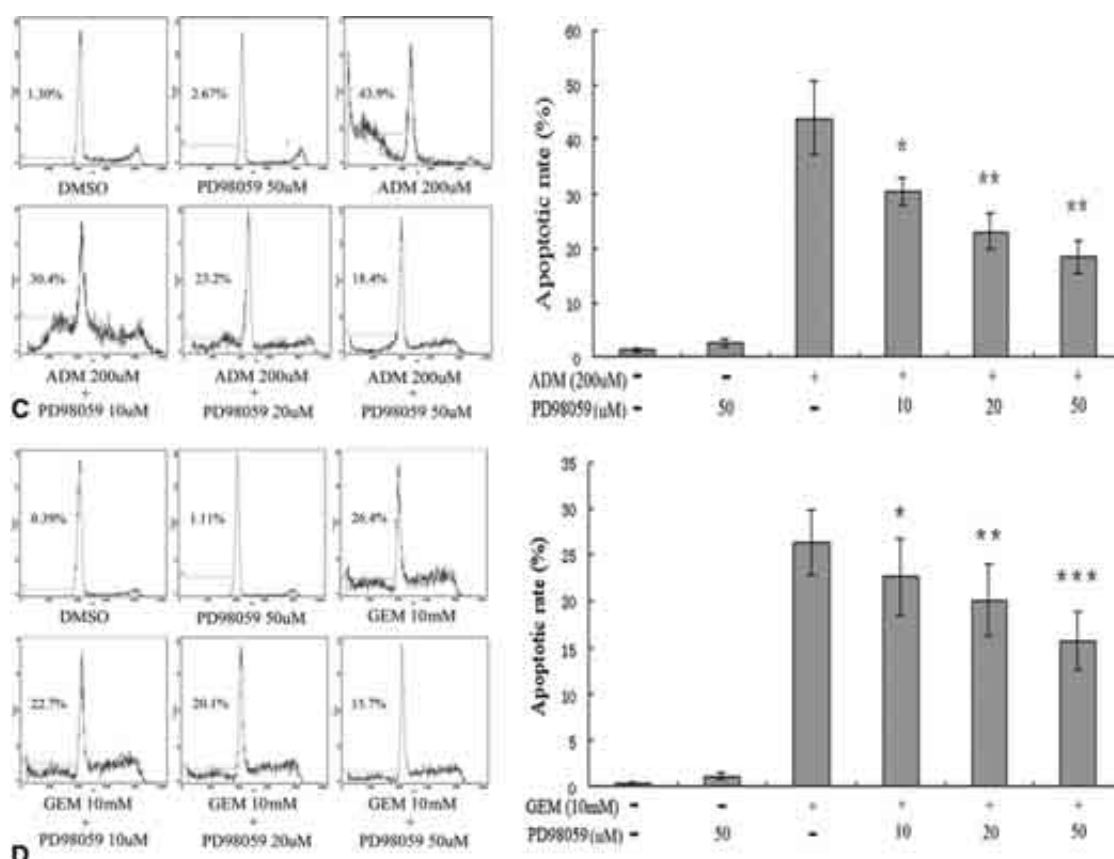


FIG. 3—Continued. C, Effect of PD98059 on apoptosis of SW1990/ADM induced by ADM (200 μ M, 72 h). D, Effect of PD98059 on apoptosis of SW1990/GEM induced by GEM (10 mM, 72 h) (* P < 0.05; ** P < 0.01; *** P < 0.001, compared with cells treated with chemotherapeutic drugs alone.).

activation of the ERK pathway is associated with resistance to apoptosis induced by chemotherapeutic drugs, and inhibition of the ERK signaling pathway leads to increased sensitivity of cancer cells to chemotherapy [27–30]. However, some studies have demonstrated that activation of the ERK pathway plays a promoting role in the apoptosis induced by chemotherapeutic drugs [31–33]. Furthermore, a few studies have found that activation of the ERK pathway can induce cell death independent of drug exposure [34, 35].

We have previously reported that the MAPK pathway might regulate tumorigenicity and proliferation of pancreatic cancer cells [36]. In the present study, however, we have provided evidence that the role of the ERK pathway in regulating chemosensitivity of pancreatic cancer cells varies according to the type of chemotherapeutic drug administered. At basal conditions, the ERK pathway was not activated in parental SW1990 cells; however, the pathway was highly activated in all three chemoresistant sublines. This observation is consistent with a recent report demonstrating that drug-resistant cells exhibited higher levels of ERK phosphorylation than parental cells [37]. These findings suggested that activation of the ERK pathway was related to the drug

resistance of pancreatic cancer cells. PD98059 treatments alone did not induce apoptosis of pancreatic cancer cells. However, inhibition of ERK activation by PD98059 resulted in different effects on apoptosis induced by different drugs. The sensitivity of pancreatic cancer cells to 5-FU was markedly increased by ERK inhibition, whereas the resistance to ADM and GEM was enhanced by PD98059. Thus, the ERK pathway exerted an anti-apoptotic effect on 5-FU-induced apoptosis, while it played a pro-apoptotic role in ADM- and GEM-induced apoptosis. To date, there have been few studies on the role of the MAPK pathway in chemoresistance of pancreatic cancer cells [38, 39]. Yokoi *et al.* observed that the resistance of the pancreatic cancer cell line L3.6pl to GEM under hypoxic conditions was partially because of activation of the ERK pathway [38]. However, Habiro and co-authors found that phosphorylation of p38 MAPK, rather than ERK or JNK, might play a key role in GEM-induced apoptosis in two pancreatic cancer cell lines (PK-1 and PCI-43) [39]. In addition, inhibition of the p38 MAPK pathway significantly inhibited GEM-induced apoptosis. Thus, although a number of studies have been carried out on the role of the ERK pathway in apoptosis induced by chemo-

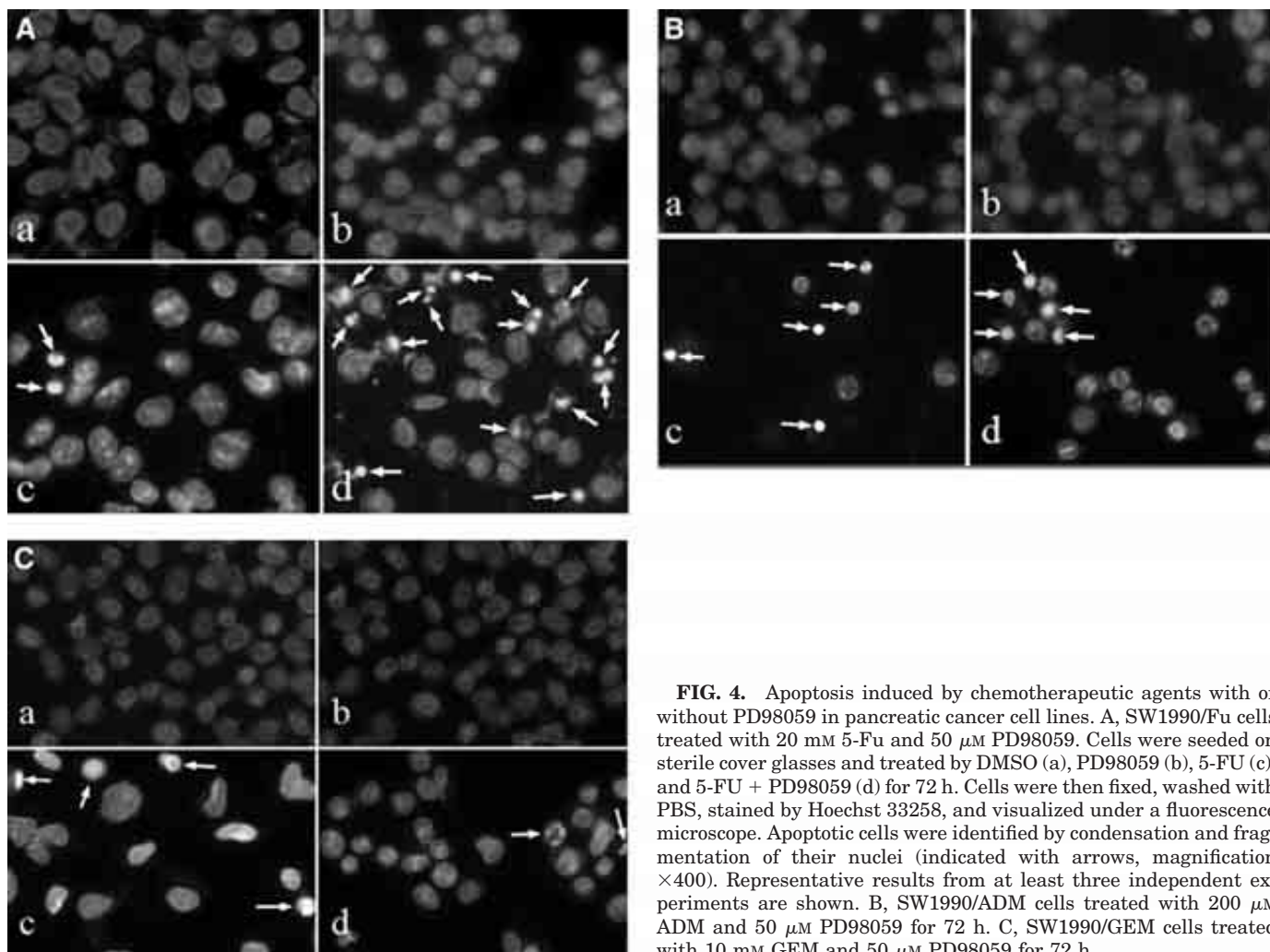


FIG. 4. Apoptosis induced by chemotherapeutic agents with or without PD98059 in pancreatic cancer cell lines. A, SW1990/Fu cells treated with 20 mM 5-Fu and 50 μ M PD98059. Cells were seeded on sterile cover glasses and treated by DMSO (a), PD98059 (b), 5-FU (c), and 5-FU + PD98059 (d) for 72 h. Cells were then fixed, washed with PBS, stained by Hoechst 33258, and visualized under a fluorescence microscope. Apoptotic cells were identified by condensation and fragmentation of their nuclei (indicated with arrows, magnification $\times 400$). Representative results from at least three independent experiments are shown. B, SW1990/ADM cells treated with 200 μ M ADM and 50 μ M PD98059 for 72 h. C, SW1990/GEM cells treated with 10 mM GEM and 50 μ M PD98059 for 72 h.

therapeutic drugs, the role for this signaling pathway in determining survival is far from clear. The differential effects observed suggest that the role of the ERK pathway in chemoresistance is dependent on cell type and conditions.

Many anti-tumor agents cause cancer cell apoptosis, a process that is mediated by caspases, a ubiquitous family of cysteine proteases which are activated by proteolytic cleavage [20, 40–42]. Caspase-9 cleavage is triggered by an intrinsic apoptotic pathway, which is mediated by mitochondria [43, 44]. However, caspase-8 is activated by the extrinsic apoptotic pathway, which proceeds via the formation of a death-inducing signaling complex (DISC) at the cell surface [45]. Moreover, caspase-3 is one of the effector caspases, which are activated in both intrinsic and extrinsic pathways. This caspase cascade ultimately leads to proteolytic cleavage of a variety of cellular proteins, such as PARP, and induces the morphological and biological changes that are characteristic of apoptosis.

5-FU, ADM, and GEM represent three different kinds of chemotherapeutic drugs. In the current

study, we found that the pathways through which these agents trigger apoptosis in pancreatic cancer cells varied. 5-FU primarily activated the caspase-8 pathway, while ADM and GEM mainly activated the caspase-9 pathway. Both caspase-3 and PARP were markedly activated in cells treated with these drugs. These observations are in agreement with those of several other reports [46–50]. Importantly, our study provides evidence that inhibition of the ERK pathway by PD98059 significantly enhances the cleavage of caspase-8, whereas it decreases the activation of caspase-9. Surprisingly, the cleavage of caspase-3 and PARP was not affected by ERK pathway inhibition. We speculate that the ERK pathway might regulate the response of pancreatic cancer cells to anti-cancer drugs at least partially by modulating apoptotic pathways. The cross-talk between the ERK pathway and apoptotic pathways might be at the level of caspase-9 and caspase-8, rather than caspase-3 and PARP, although other interfering points cannot be excluded. Indeed, some studies have shown that the ERK pathway could

regulate apoptosis by regulating cytochrome *c* release and caspase activation [31, 51, 52].

The Bcl-2 family plays a crucial role in the function of mitochondria during the apoptotic process [20, 21, 53]. The Bcl-2 family includes anti-apoptotic members, such as Bcl-2 and Bcl-XL, which block the mitochondrial release of pro-apoptotic factors, and pro-apoptotic members, such as Bax and Bid, which promote this release [20, 21]. Cellular Bcl-2 content directly correlates with the cytotoxicity of gemcitabine in pancreatic cancer [54], and Bcl-XL antisense oligonucleotide treatment increases the sensitivity of pancreatic cancer cells to GEM [55]. Phosphorylation of Bcl-2 at the evolutionarily conserved Ser-70 site was first considered to inactivate Bcl-2 and result in cell death [56], but recent evidence supports the view that phosphorylation may be required for its full antiapoptotic function [57, 58]. Our results demonstrate that GEM decreases the level of phospho-Bcl-2 and Bcl-xL, which might enhance its pro-apoptotic effect. In contrast, the effects of ADM and 5-FU on phospho-Bcl-2 and Bcl-xL were different and require further analysis. The ability of PD98059 to reduce the high expression level of phospho-Bcl-2 induced by 5-FU implies that there might be some cross-talk between the ERK pathway and the Bcl-2 family [28, 52, 58, 59].

In summary, the current study delineates the role of the ERK pathway in chemoresistance of pancreatic cancer cells. Although much evidence has accumulated indicating that the ERK pathway is important in promoting cell survival [5, 6, 29, 30], and although ERK pathway inhibitors represent a promising approach for treatment of human malignancies

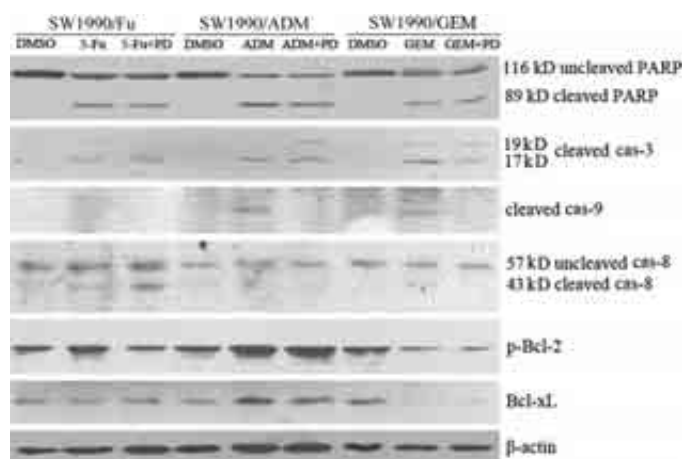


FIG. 5. Effects of ERK pathway inhibition and chemotherapeutic drugs on PARP, caspases, and Bcl-2 activation. After treatment by DMSO (control) or chemotherapeutic drugs (20 mM 5-FU, 200 μ M ADM, and 10 mM GEM) with or without 50 μ M PD98059 for 72 h, protein lysates (60 μ g/lane) were subjected to Western blot analysis. Cleaved PARP and cleaved caspases represented their activated forms and were detected by corresponding antibodies. β -actin served as a loading and transfer control. The results are representative of at least two independent experiments.

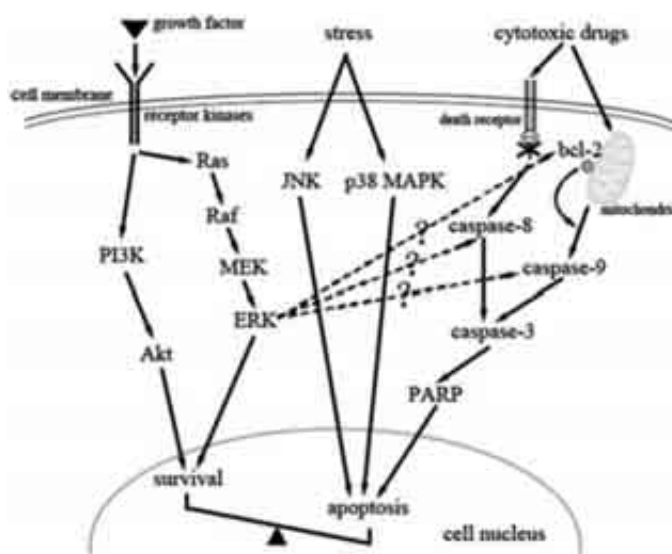


FIG. 6. A brief diagram of the mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways and the apoptotic pathways. The MAPKs include extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and the p38 MAPK kinases. Generally, it is considered that ERK and PI3K/Akt is activated in response to growth factors, conferring a survival advantage to cells. In contrast, JNK and p38 MAPK is highly activated in response to a variety of environmental stress, which is most frequently associated with induction of apoptosis. Cytotoxic drugs can induce apoptosis either through the death-receptor or the mitochondrial pathway. Caspases are activated by cleavage, which can be activated in both pathways. Bcl-2 family is a central regulator of mitochondria-mediated apoptosis. There might be some cross-talks between the MAPK pathways and the apoptotic pathways. The balances of the survival and the apoptosis signals determine the future of cells.

[9–13], our findings indicate a need for caution with respect to the generality of this approach. Our study also provides preliminary evidence that the ERK pathway might function upstream of caspase-8, caspase-9, and Bcl-2 in regulating apoptosis induced by chemotherapeutic drugs. In addition, our results suggest that measuring ERK activity in pancreatic tumors could have predictive value in terms of potential response to different chemotherapeutic agents. Further elucidation of the mechanism by which the ERK pathway influences apoptotic pathways will provide insights into the underlying causes of intrinsic and acquired drug resistance and facilitate the development of novel anticancer therapies.

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