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Biochemical and Biophysical Research Communications 316 (2004) 71–77
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Involvement of p38 mitogen-activated protein kinase in gemcitabine-induced apoptosis in human pancreatic cancer cells☆

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Received 16 December 2003

Abstract

In this study, we investigated the involvement of Akt and members of the mitogen-activated protein kinase (MAPK) superfamily, including ERK, JNK, and p38 MAPK, in gemcitabine-induced cytotoxicity in human pancreatic cancer cells. We found that gemcitabine induces apoptosis in PK-1 and PCI-43 human pancreatic cancer cell lines. Gemcitabine specifically activated p38 MAPK in a dose- and time-dependent manner. A selective p38 MAPK inhibitor, SB203580, significantly inhibited gemcitabine-induced apoptosis in both cell lines, suggesting that phosphorylation of p38 MAPK may play a key role in gemcitabine-induced apoptosis in pancreatic cancer cells. A selective JNK inhibitor, SP600125, failed to inhibit gemcitabine-induced apoptosis in both cell lines. MKK3/6, an upstream activator of p38 MAPK, was phosphorylated by gemcitabine, indicating that the MKK3/6-p38 MAPK signaling pathway is indeed involved in gemcitabine-induced apoptosis. Furthermore, gemcitabine-induced cleavage of the caspase substrate poly(ADP–ribose) polymerase was inhibited by pretreatment with SB203580, suggesting that activation of p38 MAPK by gemcitabine induces apoptosis through caspase signaling. These results together suggest that gemcitabine-induced apoptosis in human pancreatic cancer cells is mediated by the MKK3/6-p38 MAPK-caspase signaling pathway. Further, these results lead us to suggest that p38 MAPK should be investigated as a novel molecular target for human pancreatic cancer therapies.

Keywords: p38 MAPK; AKT; ERK; Gemcitabine; Pancreatic cancer

Pancreatic cancer is resistant to almost all chemotherapeutic agents [1]. Gemcitabine (2′,2′-difluorodeoxycytidine) is a relatively new chemotherapeutic agent that appears to be the only drug that is clinically active against pancreatic cancer [2]. However, the precise molecular mechanism by which gemcitabine exerts its effects against pancreatic cancer is not known. Understanding this molecular mechanism would allow us to improve therapeutic strategies for pancreatic cancer.

Previous theories about how chemotherapy works are largely based on the rationale that administration of chemotherapeutic drugs results in the death of tumor cells via apoptosis. Mitogen-activated protein kinases (MAPKs) are essential components of the intracellular signal transduction pathways that regulate cell proliferation and apoptosis. Mammalian systems have three subgroups of MAPKs: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal protein kinases (JNKs), and p38 MAPKs. Extensive research has documented the pivotal roles of the ERK subgroup in proliferative responses and of the stress-activated protein kinases (SAPKs) in the JNK and p38 subgroups in stress responses and in programmed cell death [3–5]. However, it is not known whether MAPKs are involved in the gemcitabine-induced cytotoxicity of human pancreatic cancer cells. The present study was performed to test the hypothesis that MAPK signaling pathways may be involved in this process.

☆ This work was supported in part by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, Culture, Science, Sports and Technology, Japan.

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doi:10.1016/j.bbrc.2004.02.017

Materials and methods

Cell lines. The human pancreatic adenocarcinoma cell lines PK-1 and PCI-43 were used in this study. The PK-1 cell line was obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan) [6] and the PCI-43 cell line was provided by Dr. H. Ishikura at Hokkaido University (Sapporo, Japan) [7]. Cells were grown in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified 5% CO_2 incubator at 37 °C. Experiments were performed using cells in the exponential phase of growth.

Chemicals and antibodies. Gemcitabine was provided by Eli Lilly (Indianapolis, IN). Stocks of the selective p38 MAPK inhibitor SB203580 (Calbiochem, La Jolla, CA) were made in DMSO (Sigma, St. Louis, MO). The following antibodies were used for Western blotting: phospho-specific p38 MAPK (Thr180/Tyr182) and p38 (Cell Signaling Technology, Beverly, MA); phospho-specific AKT (Ser473) and AKT (Cell Signaling Technology), phospho-specific ERK1/2 (Thr202/Tyr204) and ERK1/2 (Cell Signaling Technology); phospho-specific JNK (Thr183/Tyr185) and JNK (Cell Signaling Technology); phospho-specific MKK3/6 (Ser189/207) (Cell Signaling Technology); PARP and cleaved PARP (Cell Signaling Technology); and MKK3/6 (Upstate Biotechnology, Lake Placid, NY).

Drug treatments. Gemcitabine concentrations were chosen based on the results of preliminary studies that explored its effects on cell cycle inhibition and induction of apoptosis. Cells were treated with the vehicle (<1% DMSO), gemcitabine, SB203580, or SP600125 as single agents or in combination for 3 days. All experiments were performed in triplicate.

Chemosensitivity testing. The relative cytotoxicity of gemcitabine in PK-1 and PCI-43 cells was assessed with a WST-1 assay using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). 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. Cells were added to 96-well tissue culture plates (3×10^3 cells/well) and exposed to increasing concentrations (10^{-1}–10^3 μM) of gemcitabine, after which the number of remaining living cells was determined according to the manufacturer's instructions. Chemosensitivity was expressed as the drug concentration that inhibited cell proliferation by 50% (IC_{50} values) and was determined from concentration–effect relationships.

Cell extracts and Western blot analysis. Cells were harvested and extracted with lysis buffer [50 mM Tris–HCl (pH 7.5), 137 mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μg/ml aprotinin], and cellular extracts (50 μg) were resolved in SDS–polyacrylamide (10%) gels. Insoluble material was removed by centrifugation at 4 °C for 10 min at 18,400*g*. Protein concentration was determined with a Protein Assay Kit (BioRad, Hercules, CA). Samples were then electrophoretically transferred onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). The indicated primary antibodies were incubated, washed, and visualized by incubation with horseradish peroxidase-conjugated secondary antibodies (Calbiochem) and Renaissance Chemiluminescence Reagent Plus (NEN Life Science, Boston, MA). Kinase activation levels were assessed using antibodies specific to their phosphorylated forms [8,9]. Signals were quantitated by scanning the film and the intensity of the bands was analyzed using the NIH image 1.61. Although equal amounts of total cellular protein were loaded per lane, all filters were incubated with an antibody to total protein to compare the protein levels of the different substrates. The intensity of the signals was equilibrated to the intensity of the total protein expression signals.

Detection of apoptosis by flow cytometry. After drug treatment, adherent cells were released by trypsinogen, combined with nonadherent cells, collected by centrifugation, washed with PBS, and fixed in 70% (v/v) ethanol at −20 °C for up to 1 week. After removing the cell tablet, cells were pelleted, washed once with PBS, and resuspended in propidium iodide solution [50 μg propidium iodide and 0.5 mg/ml RNase in PBS (pH 7.4)] for 30 min in the dark. FACS analysis was performed 3 days after the treatments. Flow cytometric analysis was performed on a FACScan Flow Cytometer (Beckton–Dickinson, San Jose, CA). The data from 10,000 cells were collected and analyzed using Cell Quest Cell Cycle Analysis Software. The sub-G_1 population was calculated to estimate the apoptotic cell population. All experiments were repeated at least three times; representative results are presented.

Results

Gemcitabine induces apoptosis in human pancreatic cancer cells

We determined that physiologically relevant doses of gemcitabine can induce apoptosis in PK-1 and PCI-43 human pancreatic cancer cells. After treatment with gemcitabine, cell viability was assessed using the WST-1 assay. Control cells were treated with 0.1% DMSO alone. As shown in Fig. 1A, gemcitabine induced apoptosis in a dose-dependent manner in these cells. IC_{50} values were 2.0 μM in PK-1 cells and 0.5 μM in PCI-43 cells. Based on these data and the report that micromolar concentrations of gemcitabine can be achieved clinically [10], we used 10 μM gemcitabine for all subsequent experiments. Flow cytometric analysis showed that 10 μM gemcitabine significantly increased the sub-G_1 population of apoptotic cells in both PK-1 and PCI-43 cells (Fig. 1B). These results demonstrate that gemcitabine evokes apoptosis in human pancreatic cancer cells.

Gemcitabine increases phosphorylation of p38 MAPK, but not Akt, ERK1/2, or JNK

To determine the potential involvement of various protein kinase pathways in gemcitabine-induced apoptosis, we surveyed the phosphorylation status (indicative of activation) of the four major protein kinases, Akt, ERK1/2, JNK, and p38 MAPK, after exposure of pancreatic cancer cells to gemcitabine for 24 h (Fig. 2). Expression of phosphorylated and total protein was assessed by Western blotting. Signal intensities of the bands were determined using a densitometer. Gemcitabine had little effect on the phosphorylation status of Akt, ERK1/2, and JNK, but dramatically increased phosphorylation of p38 MAPK when compared to treatment with DMSO alone. The total protein expression levels of these four kinases were not affected by gemcitabine. These observations suggest that gemcitabine specifically activates the p38 MAPK signaling pathway in pancreatic cancer cells.

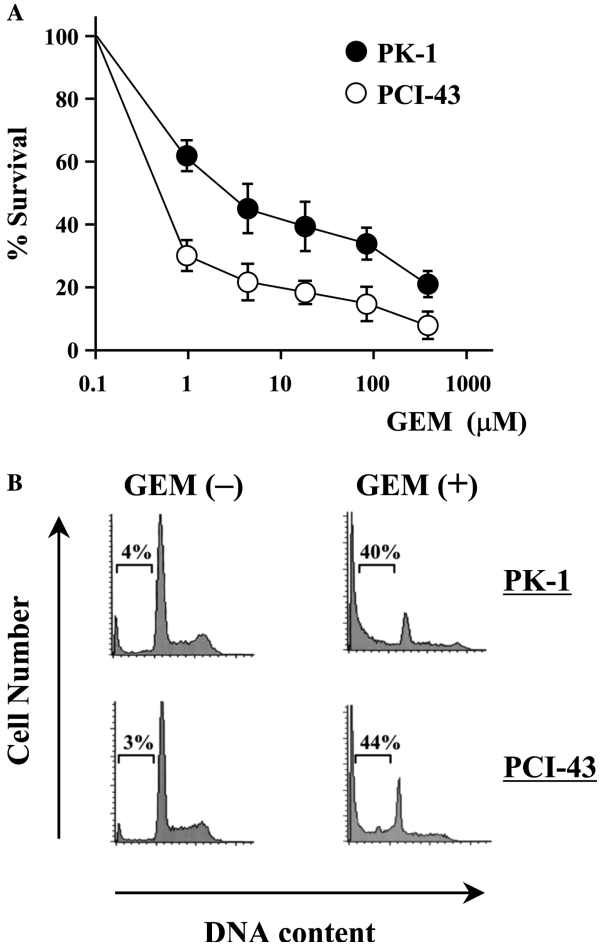

Fig. 1. Effect of gemcitabine on human pancreatic cell lines PK-1 and PCI-43. (A) The relative cytotoxicity of gemcitabine against cells was assessed using a WST-1 assay. Cells were added to a 96-well tissue culture plate and exposed to increasing concentrations (10^{-1}–10^{3} μM) of gemcitabine, after which the number of remaining living cells was determined according to the manufacturer's instructions. Bars, SD. (B) After removal of the tablet cells, flow cytometric analysis was performed on the cells in the presence and absence of 10 μM gemcitabine for 72 h.

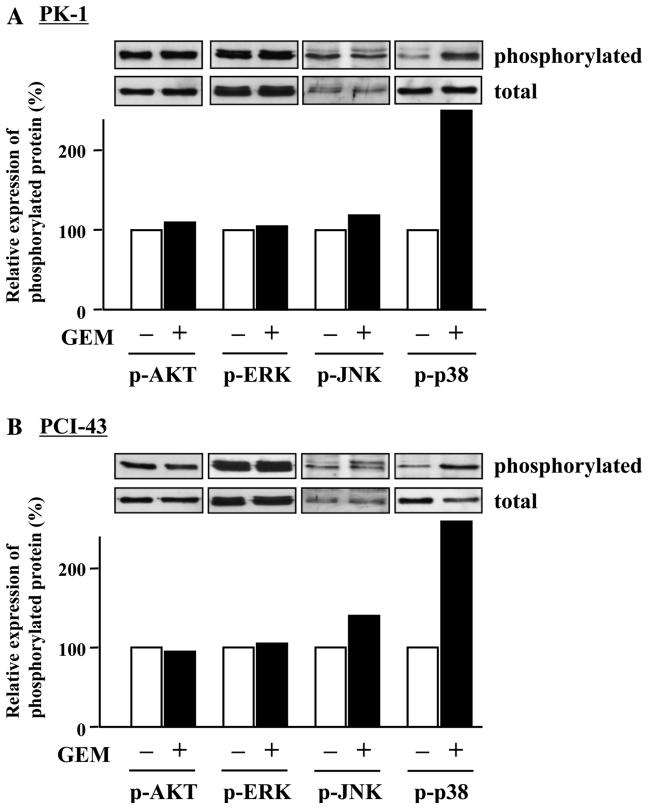

Fig. 2. In gemcitabine-treated PK-1 and PCI-43 cells, the phosphorylation levels of four major kinases were assessed by determining the amount of phosphorylated proteins using antibodies against their phosphorylated forms. Equal amounts of total cellular protein from exponentially growing cells were separated by electrophoresis and subjected to Western blot analysis with phospho-specific and total protein antibodies to Akt, ERK, JNK, and p38 MAPK, respectively. (A) PK-1 cells; (B) PCI-43 cells.

As shown in Fig. 3A, p38 MAPK was phosphorylated in response to gemcitabine treatment in a concentration-dependent manner. Phosphorylation of p38 MAPK was detected as early as 1 h after continuous exposure of 10 μM gemcitabine. Maximal phosphorylation occurred at 6 h and was sustained over the next 42 h (Fig. 3B).

Gemcitabine-induced apoptosis is inhibited by the p38 MAPK inhibitor SB203580 but not JNK inhibitor SP600125

To further demonstrate that gemcitabine-induced apoptosis is mediated through the p38 MAPK signaling pathway, we used a specific p38 MAPK inhibitor, SB203580, in combination with gemcitabine. As shown in Fig. 4, flow cytometric analyses revealed that SB203580 alone failed to change the number of apoptotic cells, whereas gemcitabine-induced apoptosis was markedly inhibited by addition of SB203580 (20 μM). The degree of inhibition of gemcitabine-induced apoptosis by SB203580 was higher in PCI-43 cells than in PK-1 cells. To further examine the involvement of JNK pathway, we pretreated SP600125 (20 μM), a specific JNK1/2 inhibitor. The flow cytometric analyses revealed that SP600125 alone did not change the number of apoptotic cells, and gemcitabine-induced apoptosis was not affected by SP600125 pretreatment (Fig. 5). These results support that gemcitabine-induced apoptosis is mediated through the p38 MAPK signaling pathway, but not JNK signaling pathway in PK-1 and PCI-43 cell lines.

Activation of MKK3/6 in gemcitabine-treated cells

Two MAPK kinases, MKK3 and MKK6, have been identified as immediate upstream activators of p38 MAPK [11]. To confirm the involvement of the entire p38 MAPK cascade in gemcitabine-induced apoptosis, we analyzed whether gemcitabine activates MKK3/6.

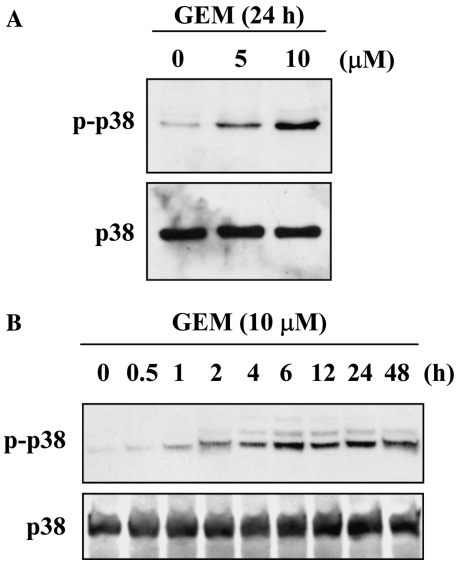

Fig. 3. Dose- and time-dependent induction of p38 MAPK activation in gemcitabine-treated PCI-43 cells. (A) p38 MAPK was phosphorylated in the presence of the indicated concentrations of gemcitabine. (B) Phosphorylation of p38 MAPK was detected 1 h after continuous exposure to 10 μM gemcitabine. Maximal phosphorylation occurred at 6 h and was sustained over the next 42 h.

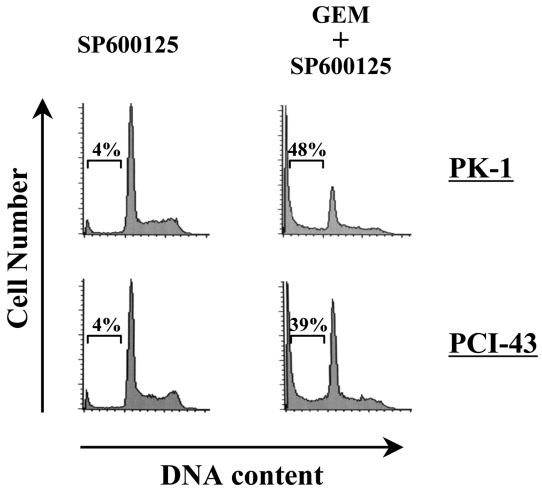

Fig. 5. Effects of the JNK inhibitor, SP600125, on gemcitabine-induced apoptosis. Both cell lines were pretreated with SP600125 (20 μM) in combination with or without gemcitabine. After 72 h, adherent and nonadherent cells were collected, and the sub-G_1 population, which is indicative of apoptosis, was determined by flow cytometry.

Using antibodies that recognize the phosphorylated form of MKK3/6, we found that MKK3/6 is activated in response to gemcitabine in both PK-1 cells and PCI-43 cells (Fig. 6). These results indicate that gemcitabine activates the MKK3/6-p38 MAPK signaling pathway to induce apoptosis.

Gemcitabine-induced PARP cleavage is inhibited by the p38 MAPK inhibitor SB203580

The proteolytic cleavage of poly(ADP–ribose) polymerase (PARP), which synthesizes poly(ADP–ribose) from β-nicotinamide adenine dinucleotide in response to DNA strand breaks, is an early biochemical event during apoptosis [12]. Since PARP cleavage is a hallmark of caspase activation, we assayed PARP cleavage in pancreatic cancer cells following gemcitabine treatment using antibodies that detect intact (116 kDa) and cleaved PARP (85 kDa). As shown in Fig. 6, PARP was completely cleaved in PK-1 cells treated with 10 μM gemcitabine, demonstrating that the apoptosis machinery is activated upon gemcitabine challenge in these cells. Next, we examined whether gemcitabine-induced PARP cleavage is influenced by SB203580. Pretreatment with SB203580 resulted in potent inhibition of PARP cleavage (Fig. 7), further supporting the hypothesis that SB203580 reduces gemcitabine-induced apoptosis.

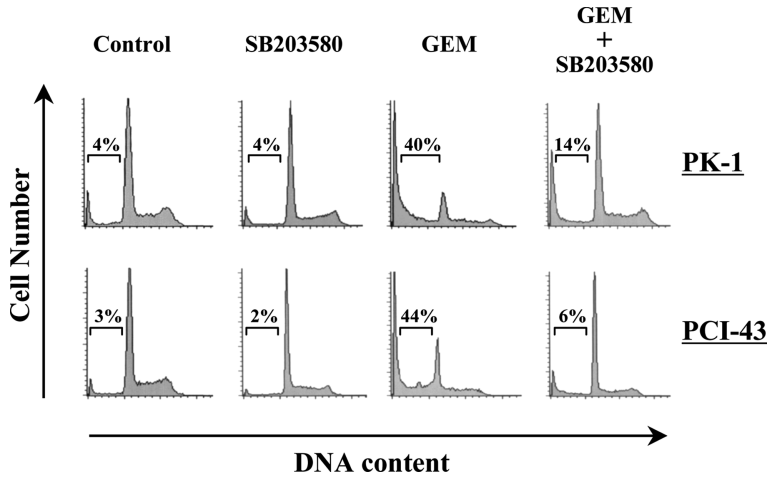

Fig. 4. Effect of the p38 MAPK inhibitor, SB203580, on gemcitabine-induced apoptosis. Both cell lines were pretreated with or without SB203580 (20 μM) in combination with gemcitabine. After 72 h, adherent and nonadherent cells were collected, and the sub-G_1 population, which is indicative of apoptosis, was subject to flow cytometric analysis.

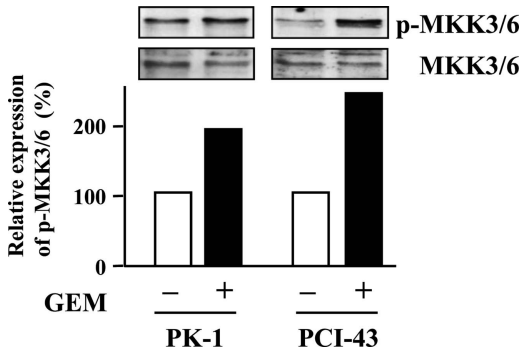

Fig. 6. Phosphorylation of MKK3/6, an upstream activator of p38 MAPK, in the presence and absence of 10 μM gemcitabine. Antibody binding was quantified by densitometric analysis. The intensity of the phosphorylated protein signals was equilibrated to the intensity of the total protein expression signals.

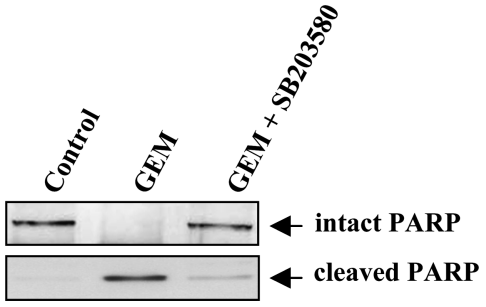

Fig. 7. PARP cleavage in gemcitabine-treated PCI-43 cells. Cells were pretreated with or without SB203580 for 24 h and then exposed to gemcitabine for 48 h. After 72 h, cells were harvested and lysed. Western blotting was performed using antibodies against intact and cleaved PARP.

These results also suggest that the caspase pathway is involved in gemcitabine-induced apoptosis, and that caspase signaling occurs downstream of p38 MAPK signaling.

Discussion

Recent reports have demonstrated that the activation status of the Akt and MAPK signaling pathways is often associated with sensitivity to chemotherapeutic agents. These protein kinase cascades have been investigated for their involvement in apoptosis induced by conventional chemotherapeutic agents. For example, cisplatin, a platinum-containing drug, has been reported to activate ERK1/2 and JNK but not p38 MAPK in human ovarian cancer cells [13], and inhibition of cisplatin-induced ERK1/2 activity causes enhanced cisplatin cytotoxicity. Topotecan, a topoisomerase I inhibitor, induces Akt dephosphorylation and promotes apoptosis in human lung cancer cells [14]. Inhibition of the Akt pathway has been shown to play an important role in exerting its cytotoxic effects. Additionally, paclitaxel (Taxol) has been shown to activate signaling cascades, including those involving ERK1/2 and JNK, in human lung and breast cancer cell lines [15]. Further, inhibition of ERK combined with paclitaxel treatment causes a significant enhancement of apoptosis. Thus, chemotherapeutic agents appear to trigger the activation of various MAPK signaling pathways, and the combination of chemotherapy with cellular response modifiers may be a promising new strategy for cancer chemotherapy.

Gemcitabine is a nucleotide analog that is converted to its triphosphate active form in cells and is subsequently incorporated into DNA to terminate strand elongation. As a novel anticancer drug, it has been approved for use in the treatment of pancreatic cancer and is also widely used for palliation of pancreatic cancer-associated symptoms [2]. However, there have been few published analyses on the cellular response of human pancreatic cancer cells to gemcitabine. We therefore attempted to demonstrate whether MAPK signaling pathways are involved in the gemcitabine-induced cytotoxicity of pancreatic cancer cells.

The major finding of this study is that gemcitabine specifically activates p38 MAPK in human pancreatic cancer cell lines. To test the hypothesis that p38 MAPK activation is involved in gemcitabine-induced apoptosis, we examined the effect of SB203580, a p38 MAPK inhibitor, on gemcitabine-induced apoptosis and clearly showed that p38 MAPK activation plays a vital role in the gemcitabine-evoked apoptosis in human pancreatic cancer cells. A lack of inhibition of the gemcitabine-induced apoptosis by SP600125 (20 μM), a specific JNK1/2 inhibitor, furthermore supports the conclusion that the p38 MAPK signalling pathway mediates the apoptotic change by gemcitabine in pancreatic cancer cells.

It has been demonstrated that p38 MAPK activation triggers an apoptotic response to cellular stress in tumor cells but not in normal cells [16–23], although there have also been some contrasting reports that p38 MAPK activation is central to the antiapoptotic and growth-promoting effects in leukemia and lymphoma cell lines [24,25]. Our data obtained from human pancreatic cancer cells further support the observation that p38 MAPK activation is involved in apoptotic signaling in cancer cells. We do not know the precise mechanism that determines whether p38 MAPK activation promotes or inhibits apoptosis in tumor cells, although the difference might simply depend upon cell type. Recent reports have demonstrated that oncogenic transformation can significantly affect p38 MAPK signaling, which may help explain the different degree of p38 MAPK signaling in tumor cells versus normal cells [22]. Comparison of stress signaling between non-transformed NIH3T3 cells and transformed NIH3T3 cells that overexpress epidermal growth factor receptor (EGFR), Her-2 kinase, or oncogenic Ras revealed that p38

MAPK activation is greatly augmented in transformed cells relative to non-transformed cells in response to various genotoxic agents [22]. Reactive oxygen species (ROS) production has also been demonstrated to be increased in oncogene-expressing NIH3T3 cells compared to parental NIH3T3 cells, and increased ROS generation in transformed cells has been shown to activate stress signaling via potentiation of p38 MAPK activation. Thus, oncogene-related molecule transformation might alter the properties of p38 MAPK. Although further studies are required to elucidate the precise mechanism, it is possible that a selective p38 MAPK activator may enhance the sensitivity of pancreatic cancer cells but not normal cells to gemcitabine and thereby increase selective tumor cell kill.

The present data demonstrate that MKK3/6, the upstream kinase of p38 MAPK, is activated by gemcitabine, which strongly supports the involvement of the p38 MAPK signaling pathway in gemcitabine-induced apoptosis. We also showed that SB203580, a specific p38 MAPK inhibitor, suppresses PARP cleavage in addition to blocking gemcitabine-induced activation of p38 MAPK in pancreatic cancer cells. Gemcitabine has recently been reported to induce apoptosis in multiple myeloma cells through activation of the caspase pathway, including caspase-3, -8, and -9, and addition of a caspase inhibitor to the cell culture prevents the gemcitabine-induced apoptotic process [26]. It has also been suggested that caspase activation is required for gemcitabine-induced apoptosis. Taken together with these observations, our data suggest that there is a close relationship between p38 MAPK activation and caspase pathways, and that gemcitabine may induce apoptosis through caspase signaling downstream of p38 MAPK in pancreatic cancer cells. Previous studies have demonstrated that induction of apoptosis by dopamine or nitric oxide depends on p38 MAPK activation followed by caspase activation [27,28]. These reports support the present evidence that p38 MAPK activation followed by caspase activation is involved in gemcitabine-induced apoptosis.

From a clinical point of view, it is important to clarify whether increased p38 MAPK activation correlates with resistance to gemcitabine in pancreatic cancer cells. We found in this study that the degree of p38 MAPK activation was consistently higher in PCI-43 cells than in PK-1 cells, and that the IC_{50} value was lower in PCI-43 cells than in PK-1 cells (Fig. 1). Furthermore, inhibition of gemcitabine-induced apoptosis by SB203580 was higher in PCI-43 cells than in PK-1 cells (Fig. 4). These data suggest that the degree of p38 MAPK activation may correlate with the sensitivity of pancreatic cancer cells to gemcitabine. In pancreatic cancer patients, gemcitabine therapy is frequently efficient in early stages of the disease, whereas advanced tumors are usually resistant to the same treatment [2]. Recently, it has been reported that p38 MAPK activity is reduced in hepatoma cells compared to normal liver tissue specimens, and is significantly lower in lesions larger than 20 mm in diameter [29]. It will be interesting to investigate whether p38 MAPK activity is reduced in pancreatic cancer compared to normal pancreatic tissues, and in metastatic tissue compared to tissues without distant metastasis.

In conclusion, our data strongly demonstrate a critical role of p38 MAPK activation in gemcitabine-induced apoptosis in pancreatic cancer cells. We suggest that the p38 MAPK signaling pathway be further investigated as a target for the development of novel pancreatic cancer therapies.

Acknowledgment

We thank Eli Lilly & Co. for the generous supply of gemcitabine used for this research.

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