Activation of p38 Mitogen-activated Protein Kinase is Necessary for Gemcitabine-induced Cytotoxicity in Human Pancreatic Cancer Cells

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Abstract. Background: Gemcitabine is a pyrimidine nucleoside analog that is clinically active against pancreatic cancer. We have recently demonstrated that p38 MAPK is specifically activated by gemcitabine and that pharmacological blockade of p38 MAPK signaling prevented gemcitabine-induced apoptosis in human pancreatic cancer cells. In this study, we further investigated the implication of p38 MAPK in the cytotoxic action of gemcitabine. Materials and Methods: Cells expressing a dominant-negative mutant of p38 MAPK were generated. Clonogenic assays were used to assess the long-term effect on cancer cell viability in the human pancreatic cancer cells, PK1 and PCI43. The p38 MAPK activation level was assessed using an antibody specific to the phosphorylated form. Results: Gemcitabine increased the activation level of p38 MAPK in a dose-dependent manner and induced apoptosis in the two tested human pancreatic cancer cell lines. The selective p38 MAPK inhibitors, SB203580 and SB202190, reduced gemcitabine-induced activation of p38 MAPK, prevented the gemcitabine-induced apoptosis and increased long-term clonogenic survival. Overexpression of a dominant-negative p38 mutant in cells resulted in the reduction of gemcitabine-induced p38 MAPK activation and apoptosis, and increases in clonogenic survival. Conclusion: These results strongly suggest that the activation of p38 MAPK signaling is necessary for gemcitabine-induced cell death in human pancreatic cancer cells. Based upon these results, we suggest that molecules of p38 MAPK signaling pathways should be listed as novel targets for gemcitabine-based therapy.

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cells. In the present study, to further clarify the importance of p38 MAPK in the gemcitabine-induced cytotoxicity, we made a dominant-negative p38 MAPK mutant and used clonogenic assays to assess the long-term effect on cancer cell viability in the human pancreatic cancer cells.

Materials and Methods

Cell culture. The human pancreatic adenocarcinoma cell lines PK1 and PCI43 were used in this study. The PK1 cell line was obtained from the Cell Resource Center for Biochemical Research (Tohoku University, Sendai, Japan) (14) and the PCI43 cell line was provided by Dr. H. Ishikura at Hokkaido University (Sapporo, Japan) (15). Cells were grown in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified 5% CO₂ incubator at 37°C. Experiments were performed using cells in the exponential phase of growth.

Drugs and treatments. Gemcitabine was provided by Eli Lilly (Indianapolis, IN, USA). The selective inhibitors of p38 MAPK, SB203580 and SB202190, were purchased from Calbiochem (San Diego, CA, USA). Cells were incubated with the vehicle (DMSO) or gemcitabine in combination with or without pretreatment with p38 inhibitors. All experiments were performed in triplicate.

Expression vector construction and transfection. The cDNA for human p38α was isolated and introduced into the expression vector, as previously reported (16, 17). The cDNA for full-length human p38α was obtained by RT-PCR using mRNA from PCI43 cells as a template. The product was cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Full activation of p38α requires phosphorylation of Thr180 and Tyr182 found within a Thr-Gly-Tyr tripeptide motif in the activation loop of the kinase (18). To generate a dominant-negative p38α mutant, site-directed mutagenesis was performed by PCR to replace Thr180 and Tyr182 with alanine and phenylalanine, respectively. An N-terminal hemagglutinin (HA) epitope-tag was introduced to distinguish between endogenous and transfected p38α. The p38α insert was sequenced on both strands to confirm the mutation.

PK1 and PCI43 cells were cultured in RPMI for 12 h before transfection. Both cell lines were defined as >20 cells. Briefly, the cDNA for full-length human p38α was obtained by RT-PCR using mRNA from PCI43 cells as a template. The product was cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Full activation of p38α requires phosphorylation of Thr180 and Tyr182 found within a Thr-Gly-Tyr tripeptide motif in the activation loop of the kinase (18). To generate a dominant-negative p38α mutant, site-directed mutagenesis was performed by PCR to replace Thr180 and Tyr182 with alanine and phenylalanine, respectively. An N-terminal hemagglutinin (HA) epitope-tag was introduced to distinguish between endogenous and transfected p38α. The p38α insert was sequenced on both strands to confirm the mutation.

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For transfections, the cells were plated in 100-mm dishes and transfected with an empty vector or the DN-p38α expression plasmid using GenePorter2 (Gene Therapy Systems), following the manufacturer’s instructions. In all transfections, a constant amount of DNA was transfected. Both cell lines were then transfected with empty vector or DN-p38α expression plasmids using GenePorter2 (Gene Therapy Systems), following the manufacturer’s instructions. The product was cloned into pcDNA3.1 vector, as previously reported (16, 17). Briefly, the cDNA for full-length human p38α was obtained by RT-PCR using mRNA from PCI43 cells as a template. The product was cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Full activation of p38α requires phosphorylation of Thr180 and Tyr182 found within a Thr-Gly-Tyr tripeptide motif in the activation loop of the kinase (18). To generate a dominant-negative p38α mutant, site-directed mutagenesis was performed by PCR to replace Thr180 and Tyr182 with alanine and phenylalanine, respectively. An N-terminal hemagglutinin (HA) epitope-tag was introduced to distinguish between endogenous and transfected p38α. The p38α insert was sequenced on both strands to confirm the mutation.

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Detection of apoptosis. To assess morphological changes in the chromatin structure of the cells undergoing apoptosis, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma). After transfection 24 h later, the cells were incubated with gemcitabine for 4 h, then trypsinized gently. Trypsin-released adherent cells and cells that were floating in the medium before trypsin treatment were mixed, pelleted by centrifugation, washed with PBS and fixed with 3.5% paraformaldehyde for 20 min. The cells were then stained with DAPI (1 μg/ml), mounted on glass slides and analyzed using a fluorescence microscope to assess chromatin condensation and nuclei fragmentation. Apoptotic cells were identified by the condensation and fragmentation of their nuclei. Cell staining experiments were performed in triplicate, and the percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. The incidence of apoptosis in each treatment was analyzed by counting 1,000 cells.

Clonogenic assay. Clonogenic assays were carried out on growing cells in 100-mm dishes. Before the induction of apoptosis, the cells were preincubated for 1 h with 20 μM SB203580 or SB202190. After treatment with 10 μM gemcitabine for 4 h, the cells were washed twice with PBS and incubated in normal growth medium. For transfections, the cells were plated in 100-mm dishes and transfected with an empty vector or the DN-p38α expression plasmid using GenePorter2 (Gene Therapy Systems), following the manufacturer’s instructions. At 24 h after transfection, the cells were incubated with 10 μM gemcitabine for 4 h. The cells were washed twice with PBS and incubated in growth medium in the presence of G418. Colonies formed within 2 weeks. These colonies were stained with 70% methanol and 1.5% crystal violet. Colonies were defined as >20 cells.

Results

Gemcitabine-induced activation of p38 MAPK is inhibited by the selective p38 inhibitors, SB203580 and SB202190. We first confirmed that gemcitabine activates p38 MAPK in two human pancreatic cancer cell lines. As shown in Figure 1A, the activation level of p38 MAPK was low, but detectable in
the absence of gemcitabine in both cell lines. Activation of p38 MAPK was markedly increased at a concentration of 10 μM gemcitabine at 24 h after the treatment. Next, we examined whether gemcitabine-induced activation of p38 MAPK is pharmacologically inhibited by selective p38 MAPK inhibitors. The pyridinylimidazole inhibitors SB203580 and SB202190 were used (16). Cells were preincubated with 20 μM SB203580 or SB202190 for 1 h, washed and then incubated with 10 μM gemcitabine for 4 h. The cells were lysed and the lysates were used for Western blotting. As shown in Figure 1B, both inhibitors strongly repressed activation of p38 MAPK by gemcitabine treatment, whereas the protein expression level of total p38 MAPK was unaffected.

**Pharmacological inhibition of p38 MAPK activation blocks gemcitabine-induced cell death and increases clonogenic survival.** To test the biological role of p38 MAPK in response to gemcitabine, a specific inhibitor of p38 MAPK, SB203580, was used. The cells were pretreated with 20 μM of SB203580 for 1 h, washed and then incubated with 10 μM gemcitabine for 4 h. The cells were washed and cultured for an additional 72 h. As shown in Figure 2, there was an increase in the viability of both cell lines pretreated with a p38 MAPK inhibitor. The results obtained by these methods essentially matched those observed by flow cytometry analysis in our previous report (13). Next, to evaluate the clinical significance of our finding, we decided to assess the role of p38 MAPK using clonogenic assays, because the most important parameter in the efficacy of a chemotherapeutic drug is its long-term effect on cancer cell viability. Cells were pretreated with 20 μM of SB203580 and washed with PBS, then incubated with 10 μM gemcitabine for 4 h. Subsequently, the cells were seeded into normal growth medium and the growth of cell colonies was scored 2 weeks later. Figure 3 demonstrates that gemcitabine significantly reduced colony formation. Pretreatment with SB203580 increased the number of colonies in both cell lines, suggesting that p38 MAPK signaling appears to mediate the gemcitabine-induced cytotoxicity in human pancreatic cancer cells.

**Overexpression of the DN-p38α mutant in cells inhibits gemcitabine-induced activation of p38 MAPK and increases clonogenic survival.** Although SB203580 and SB202190 are believed to be specific inhibitors of p38 MAPK, it is important to confirm experimental results obtained with these drugs through other strategies. Four different p38 MAPK members have been identified: p38α, β, γ and δ. These proteins may have both overlapping and specific functions (19-21). Among them, p38α is broadly expressed and is the most abundant p38 MAPK family member, being present in most cell types. To further our studies, we expressed a DN-p38α mutant with the G418 resistance gene to examine whether overexpression of DN-p38α in cells affects gemcitabine-induced apoptosis and clonogenic survival. First, the effect of a DN-p38α mutant on the activation level of p38 MAPK in response to gemcitabine was tested. As illustrated in Figure 4, overexpression of a DN-p38α mutant resulted in the reduction of p38 MAPK activation levels in both cell lines. Wild-type p38α showed no inhibitory effect on p38 MAPK activation. Next, we found that gemcitabine-induced apoptosis was inhibited in cells expressing DN-p38α (Figure 5). Since results may differ depending on whether long-term survival or short-term apoptosis assays are performed, we transfected cells with pcDNA3-DN-p38α.
and counted antibiotic-resistant colonies after 2 weeks in the presence of G418. As shown in Figure 6, relative to cells transfected with empty vector alone, less than 5% of the cells incubated with gemcitabine formed colonies. In contrast, incubation of DN-p38α-transfected cells with gemcitabine blocked the inhibitory effect of gemcitabine on cell proliferation.

The serine/threonine kinase Akt is a well-characterized kinase that is known to play a critical role in anti-apoptotic signaling pathways. To address whether inactivation of Akt is involved in gemcitabine-induced apoptosis, the effect of gemcitabine on Akt activation in the DN-p38 cells was examined (Figure 7). The membranes probed with anti-phospho-p38 MAPK antibody in Figures 1B and 5A were

![Figure 2](image1.png)  
*Figure 2. Gemcitabine-induced nuclear fragmentation was detected by fluorescent microscopy. The cells were pretreated with 20 μM SB203580 for 1 h and washed. After treatment with 10 μM gemcitabine for 4 h, the cells were washed, and further cultured for 72 h. Both attached and detached cells were collected, fixed with 3.5% paraformaldehyde and stained with DAPI. A total number of 1,000 cells was counted and the percentage of the cells showing nuclear morphological changes was calculated. (A) Representative photographs of the cells showing chromatin condensation and nuclei fragmentation analyzed with DAPI staining. (B) Results shown are the means of three experiments. Bars, SD.*

![Figure 3](image2.png)  
*Figure 3. Clonogenic assay performed on cells treated with 10 μM gemcitabine. After treatment with gemcitabine for 4 h with or without pretreatment of SB203580, the cells were washed and the medium was changed. The cells were cultured for 10 days and fixed with 70% ethanol. Next, the cells were stained with crystal violet. Colonies consisting of more than 20 cells were counted. The relative number of colonies was calculated. (A) Photographs were taken at 2 weeks after gemcitabine treatment. (B) Colonies were counted 2 weeks later and normalized to control. Bars, SD.*
reused for reprobing with anti-phospho-Akt (Ser473) antibody. No clear alteration of the Akt activation level was observed in gemcitabine-treated cells, indicating that Akt is not implicated in the gemcitabine-induced cytotoxicity mediated through p38 MAPK activation.

**Discussion**

Gemcitabine reportedly induces apoptosis in pancreatic cancer cells (22). However, little is known about the signaling pathway(s) leading to gemcitabine-induced apoptosis. We demonstrated, in a previous report, that the activation of the p38 MAPK signaling pathway is involved in the cellular response to gemcitabine in human pancreatic cancer cells (13). In the present study, we further investigated the role of p38 MAPK signaling in gemcitabine-induced cytotoxicity using a molecular approach in human pancreatic cancer cell lines. We demonstrated that gemcitabine-induced apoptosis is significantly blocked by p38 MAPK inhibitors and by DN-p38α expression, clearly suggesting the pro-apoptotic role of p38 MAPK activation in gemcitabine-induced cell death. Furthermore, our results indicate, for the first time, that this event is a key component of gemcitabine-induced cell death, and that the biological outcome of p38 MAPK activation in response to gemcitabine is modulated by the inhibition of p38 MAPK signaling in human pancreatic cancer cells.

The present study confirmed the requirement of p38 MAPK activation in gemcitabine-induced cell death by using clonogenic assays. The clonogenic assay is the most reliable method for assessing cell killing after treatment with genotoxic agents (23). Short-term assays may lead to incorrect assessments of overall cell kill rates, largely because they ignore kinetic differences in the manifestation of cell death. Cells do not die immediately after treatment; they can take hours to many days before dying, and this is highly dependent upon the cell type and the toxic agent being...
investigated. It has been reported that the sensitivity of cells to toxic agents, as assessed by their ability to form a colony, is greater than that of the short-term assay, which measures the proportion of cells undergoing apoptosis (24). This supports our present data, which show differences in the degree of apoptosis between the short-term assay (Figures 2 and 5) and the clonogenic assay (Figures 3 and 6).

The mechanisms underlying p38 MAPK-mediated apoptosis in response to gemcitabine remain to be clarified. With regard to this point, we examined whether Akt is implicated in the mechanism. Akt is a key oncogenic survival factor, and activation of Akt has been shown to induce resistance to apoptosis induced by chemotherapeutic drugs or irradiation (25-27). Some reports demonstrated an association between the activation status of p38 MAPK and the Akt survival pathway in tumor cells. Liao et al. found that p38 phosphorylation is down-regulated and Akt phosphorylation is up-regulated in multiple human tumor tissues, whereas the staining intensity of phospho-p38 protein was relatively strong while that of phospho-Akt was very weak in normal organs and parallel healthy tissues (12). Tanaka et al. reported that stimulation of protein kinase C by phorbol 12-myristate 13-acetate promotes apoptosis in prostate cancer cells through activation of p38 MAPK and inhibition of the Akt activity (28). These reports suggest that down-regulation of p38 MAPK activity is a common event in human cancer cells, particularly when the level of Akt activation is increased. On the other hand, p38 activation and Akt inactivation may contribute to increasing apoptosis induced by drugs. In the present study, it was demonstrated that gemcitabine induced p38 MAPK activation, but not Akt activation, in cells transfected with empty vector or DN-p38α. Thus, our data failed to provide evidence that gemcitabine has an effect on phosphorylation of Akt not only in untransfected, but in DN-p38α-transfected cells.

In conclusion, our data indicate that gemcitabine induces phosphorylation of p38 MAPK, and that this event is a key component of pancreatic cancer cell death in response to gemcitabine. The pro-apoptotic functions of p38 suggest

Figure 6. Clonogenic assay of PCI43 cells stably expressing DN-p38α treated with 10 μM gemcitabine. After treatment with gemcitabine for 4 h, the cells were washed and the medium was changed. The cells were cultured for 10 days in the presence of G418 (200 μg/ml) and fixed with 70% ethanol. The cells were then stained with crystal violet. Colonies consisting of more than 20 cells were counted. (A) Representative photographs of colonies taken at 2 weeks after gemcitabine treatment. (B) Relative number of colonies was calculated. Results shown are the means of three experiments. Bars, SD

Figure 7. Phosphorylation levels of p38 MAPK and Akt in gemcitabine-treated cells. The membranes in Figures 1B and 5A were simultaneously probed with antibodies to both phospho-Akt (S473) and phospho-p38 MAPK.
possible new approaches to targeted therapy and p38 MAPK modulators may have potential as chemotherapeutic drugs in gemcitabine-based therapy in human pancreatic cancer.

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