Involvement of Phosphatidylinositol 3-Kinase/Akt Pathway in Gemcitabine-Induced Apoptosis-Like Cell Death in Insulinoma Cell Line INS-1

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This study demonstrated gemcitabine-induced cytotoxicity in the insulinoma cell line INS-1. Gemcitabine inhibited INS-1 cell proliferation and maintained consistent cell number for 24 h, and then caused apoptosis within 48 h of incubation. Since gemcitabine activates the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway, which is involved in the resistance of pancreatic exocrine cancer to gemcitabine, we investigated the participation of this pathway in gemcitabine-induced cytotoxicity in INS-1 cells. LY294002 and wortmannin, two PI3-K inhibitors, significantly prevented gemcitabine-induced cytotoxicity in INS-1 cells, indicating that the PI3-K/Akt pathway is involved in gemcitabine-induced cytotoxicity. Gemcitabine-induced Akt phosphorylation in INS-1 cells was prevented by LY294002. Although gemcitabine induced cell cycle arrest at the G1 and early S phases, LY294002 did not inhibit the cell cycle. These data suggest that PI3-K activation does not influence gemcitabine-induced cell cycle arrest. In gemcitabine-treated cells, nuclear fragmentation and DNA ladder formation were observed. These findings suggest that gemcitabine induced apoptotic cell death in INS-1 cells through the activation of the PI3-K/Akt pathway.

Key words gemcitabine; phosphatidylinositol 3-kinase pathway; apoptosis; pancreatic β cell

Living bodies maintain homeostasis between proliferation and apoptosis, and abnormal cell proliferation leads to cancers such as ovarian, lung, breast, and pancreatic carcinomas. Gemcitabine (2',2'-difluorodeoxycytidine; Gemzar) is an anticancer drug used to treat pancreatic cancer because it inhibits DNA chain extension, terminates cell duplication, and induces apoptosis. Although it is the first-line drug for the treatment of pancreatic ductal adenocarcinoma, its use is restricted because of serious side effects and the development of drug resistance.

There are two main cell proliferation signaling pathways. One is the mitogen-activated protein kinase (MAPK) pathway consisting of the following three families: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. The other is the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway. PI3-K is a heterodimeric enzyme consisting of a regulatory subunit, p85, and a catalytic subunit, p110. On activation by environmental factors, such as growth factors and cytokines, PI3-K converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, resulting in Akt activation. At a later stage, active Akt translocates to the nucleus, where many of its substrates are located, and enhances the transcription of oncogenes, resulting in cell growth, transformation, differentiation, motility, and survival. Therefore, abnormal PI3-K activation can induce abnormal cell proliferation, thus, including cancer development. Numerous studies have demonstrated that the Akt gene is mutated to a constitutively active form in various human cancers, such as human breast, ovarian, and pancreatic carcinomas. In some cases, the resistance of cancer cells to anticancer drugs is due to the PI3-K/Akt pathway. Akt overexpression is involved in cisplatin chemoresistance in human uterine cancer cells. Furthermore, pancreatic ductal adenocarcinoma with PI3-K overexpression was more resistant to gemcitabine under certain conditions. However, some studies have observed an inverse relationship between the activity of the PI3-K/Akt pathway and the effect of anticancer drugs. PI3-K inhibitors may improve the efficacy of classical chemotherapeutic treatments such as gemcitabine administration.

Gemcitabine has been reported to have adverse effects; 27% of patients were found to have increased urine glucose concentrations with gemcitabine administration. From this finding, we speculate that gemcitabine influences pancreatic endocrine cells located near the exocrine cells. However, gemcitabine-induced cytotoxicity in pancreatic endocrine cells has not been examined in detail. In addition to regulation of cell proliferation, the PI3-K/Akt pathway plays an important role in insulin secretion from islet β-cells and its cell lines. Furthermore, the knockout of p110α, one of the catalytic subunits of PI3-K, resulted in a blunted glucose-stimulated insulin secretion and insulin secretion was prevented by PI3-K inhibitors. These findings suggested that PI3-K activity plays a positive role in insulin secretion from pancreatic β-cells. In addition, unlike other cell types, significant phosphatidylinositol 3,4,5-trisphosphate levels were detected in β-cells under non-stimulatory conditions. These findings suggest the possibility that gemcitabine-induced cytotoxicity in pancreatic β-cells is influenced by the regulation of PI3-K activation, which is associated with some events involved in insulin secretion.

INS-1 cell line was established by an X-ray-induced rat transplantable insulinoma, which has the capacity to secrete insulin in response to glucose and is similar to native β-cells. Here we investigated whether gemcitabine induces apoptosis in INS-1 cells, and if so, whether the activity of PI3-K/Akt pathway influences apoptosis. Furthermore, we
demonstrated that the PI3-K/Akt pathway may be involved in gemcitabine-induced apoptosis in INS-1 cells.

MATERIALS AND METHODS

Chemicals  Gemcitabine was obtained from LKT Laboratories, Inc. (St. Paul, MN, U.S.A.). LY294002, MEK1/2 inhibitor \( \text{E}-\alpha-(\text{amino}-(4-(\text{aminophenyl})thio)methylene)-2-(\text{trifluoromethyl})\text{benzenacetonitrile}, \) nocardazole, rapamycin, U0126, and wortmannin were purchased from Calbiochem (San Diego, CA, U.S.A.). Propidium iodide (PI), RPMI 1640 medium, sodium pyruvate, 2-mercaptoethanol, and pifithrin-\( \alpha \) (PFT-\( \alpha \)) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was obtained from MP Biochemicals, Inc. (Aurora, OH, U.S.A.). Penicillin, streptomycin, and 0.25% trypsin-ethylenediaminetetraacetic acid were obtained from Gibco (Grand Island, NY, U.S.A.). Z-VAD-FMK was obtained from R&D Systems (Minneapolis, MN, U.S.A.). The Cell Proliferation Assay kit and Ac-DEVD-CHO were obtained from Promega Co. (Madison, WI, U.S.A.). Hoechst 33258 was purchased from Dojindo Co., Inc. (Kumamoto, Japan) and MitoTracker Red from Molecular Probes (Eugene, OR, U.S.A.). The Apoptosis Ladder Detection Kit, loading buffer, and 4% paraformaldehyde phosphate buffer solution were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ECL Plus Western detection reagents and high-performance chemiluminescence film were purchased from GE Healthcare (Buckinghamshire, U.K.). The Akt antibody and phospho-Akt (Ser473) antibody were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Goat anti-rabbit immunoglobulin G (IgG)-conjugated horseradish peroxidase (HRP) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Dimethyl sulfoxide (DMSO) was obtained from Nakarai Tesque Inc. (Kyoto, Japan). Phosphate-buffered saline (PBS) tablets were obtained from TaKara Bio Inc. (Otsu, Japan). The BCA protein assay reagent kit and 1× radio immunoprecipitation assay (RIPA) buffer were purchased from Pierce (Rockford, IL, U.S.A.). All other chemicals used in this study were of the highest grade available from commercial suppliers.

Culture and Treatment of Cells  Rat insulinoma INS-1 cell line was a kind gift from Dr. C. B. Wollheim. The cells were cultured in RPMI 1640 medium, containing heat-inactivated 10% FBS, 10mM \( \text{N}-(2\text{-hydroxyethyl})\text{piperazine-\text{N}^{-2}\text{-ethanesulfonic acid (HEPES)}}, 2\text{mM l-glutamine}, 100\text{U/mL penicillin}, 100\text{mg/mL streptomycin}, 1\text{mM sodium pyruvate}, \) and 50\( \mu \text{M} \) 2-mercaptoethanol, and incubated at 37°C in an atmosphere of 5% \( \text{CO}_2\)/95% air as described previously.\(^{25,26}\) After removing the medium, the cells were harvested by trypsinization. These cells were resuspended in the culture medium and washed twice with PBS by centrifugation at 300×g for 5 min. The cell pellets were resuspended in PBS, and the cell number was determined using a hemocytometer (Erma, Tokyo, Japan).

The cells were seeded in 96-well plates at a density of 10\(^4\) cells/100\( \mu \)L and 12-well plates at a density of 10\(^5\) cells/mL of culture medium. Gemcitabine (final concentration: 250, 500\( \mu \)m) was added to the medium after an overnight preincubation, and the cells were incubated for the indicated times.

Cytotoxicity Assay  Cytotoxicity was measured as the index capability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT) using a minor modification of a previously described protocol.\(^{26,27}\) Briefly, after incubation with gemcitabine, the medium was removed. To assay the ability of MTT reduction, fresh culture medium (50\( \mu \)L) and a dye solution of MTT (7.5\( \mu \)L) were added to each of the cell-containing wells and incubated for 2h at 37°C. A solubilization/stop solution (50\( \mu \)L) was then added for an additional overnight incubation. The absorbance at 540 nm (formation of formazan) and 690 nm (reference) was recorded using a microplate reader (Bio-Rad, Tokyo, Japan). Proliferation was determined by comparing the cell number to that at 0 h. Cell death was determined by PI staining. After the cells were detached from the plate by trypsinization, they were resuspended in 500\( \mu \)L of PBS containing 10\( \mu \)g/mL PI and incubated at 37°C for 30 min. The cells were washed with PBS by centrifugation at 600×g for 5 min and then filtered through a nylon mesh (35\( \mu \)m). To determine cell death, PI fluorescence was measured by a flow cytometer (Coulter ELITE cytometer; Beckman Coulter, Inc., CA, U.S.A.) using the FL3 channel (EM: 620 nm).

To determine DNA content, cells were fixed in 25% ethanol containing 15\( \mu \)g/mL MgCl\(_2\) and 0.1 mg/mL RNase A for 1 h at 37°C. The cells were washed with PBS by centrifugation at 600×g for 5 min and stained with 50\( \mu \)g/mL of PI for 30 min at 4°C. PI fluorescence was measured using a flow cytometer. To judge cell cycle progression, the cells were treated with nocardazole (30ng/mL) for 12h before measurement.\(^{28}\)

Morphological alterations in cells were observed by phase-contrast microscopy (EVOS; Nikon Eclipse E400, Tokyo, Japan). Briefly, the cells were seeded in 12-well plates at a density of 10\(^6\) cells/mL and incubated as described in the figure legends.

To determine apoptosis, the cells were seeded in 12-well plates at a density of 10\(^6\) cells/mL of culture medium. Gemcitabine was added to the medium after an overnight preincubation, and cells were incubated for the indicated times. DNA ladder formation (DNA fragmentation pattern) in INS-1 cells was examined using by 2% agarose gel electrophoresis with a minor modification of a previously described protocol.\(^ {27}\) Electrophoretic patterns were visualized by staining with 0.5\( \mu \)g/mL ethidium bromide for 15 min and photographed by an Epi-Light UV FA1100 epifluorescence detector (Aisin Cosmos, Toyama, Japan). Nuclear fragmentation in cells treated with gemcitabine for 24–48 h was observed using an FV10i confocal laser microscope (Olympus, Tokyo, Japan). Briefly, after incubation with 25\( \mu \)M MitoTracker Red for 30 min, the cells were harvested by trypsinization and washed with PBS by centrifugation at 600×g for 5 min. The cells were then fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and washed with PBS by centrifugation at 600×g for 5 min. After the addition of Hoechst 33258 (10 mg/mL) to the suspension, cells were incubated for 10 min and then washed. The prepared cells were imaged using an excitation wave of 405 nm and emission wave of 420–460 nm for nuclei detection (Hoechst 33258) and an excitation wave of 559 nm and emission wave of 570–620 nm for cytosol detection (MitoTracker Red) under a confocal laser microscope.

Western Blotting  After an 18-h preincubation, INS-1 cells were treated with gemcitabine for 4 h. Adherent cells were removed by scraping in PBS and washed twice with PBS by centrifugation at 600×g for 5 min. The pellets were either stored at −80°C or immediately used for the experiments.
For protein extraction, cells were resuspended in 1× RIPA buffer and allowed to stand for 30min at room temperature. Protein content was measured using the BCA protein assay kit with bovine serum albumin as the standard. A solution of the sample protein was diluted with an equal volume of 0.1 m Tris–HCl buffer at pH 6.8 containing 4% sodium deoxycholate (SDS), 12% 2-mercaptoethanol, 20% glycerol, and 0.01% bromophenol blue, and the mixture was boiled for 5min. Proteins (50 mg/well) from each sample were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, U.K.). The membrane was blocked with 5% skim milk and incubated overnight with primary antibodies at 4°C. Probing of the membranes was performed with a polyclonal Akt antibody (1:1000 dilution) and a polyclonal phospho-Akt antibody (1:1000 dilution). Following a 30-min wash, the membranes were incubated with secondary antibodies, a donkey anti-rabbit IgG-conjugated HRP (1:20000 dilution) for 60 min at room temperature. The bound antibodies were detected with enhanced chemiluminescence (ECL) plus Western blotting system detection reagents, and immunocomplexes were visualized using Epi-Light UV FA1100. For quantification of band density as a measurement of the phosphorylation state, films were analyzed using Luminous Imager software (Aisin Cosmos, Toyama, Japan).

Statistical Analysis  Data are represented as the mean± S.D. and were statistically analyzed by Student’s t-test or Welch's t-test following the F-test for paired data. We also used Dunnett’s multiple comparison test after one-way analysis of variance. *p<0.05 was considered statistically significant.

RESULTS

Gemcitabine-Induced Cell Death in INS-1 Cells  We investigated gemcitabine-induced apoptosis in insulinoma INS-1 cells. Figure 1A shows the morphological alterations in INS-1 cells treated with 250 nm gemcitabine. In the control cells, the cell number increased in a time-dependent manner for 48h (gemcitabine-un-treated cells). Although no change was observed after 24h in gemcitabine-treated cells, the cell number decreased and cell detachment from the bottom of the plates was observed at 48h (Fig. 1A). We examined cytotoxicity in gemcitabine-treated INS-1 cells by the MTT assay and PI staining (Figs. 1B, C). In control cells, MTT reduction increased in a time-dependent manner, and the value at 48h was approximately 1.7 times higher than that at 0h. Gemcitabine at concentrations of 250 and 500 nm did not decrease MTT reduction at 24h, but it did at 36 and 48h. MTT reductions at 48h after the addition of 100, 250 and 500 nm of gemcitabine were 0.71, 0.57 and 0.58 times, respectively, compared with control cells at 0h, but little effects was observed by 1 and 10 nm of gemcitabine. Next, to determine whether gemcitabine induces death, we performed PI staining. In the control cells, an increase in the number of PI-positive cells was not observed until 48h. However, in gemcitabine-treated cells the number of PI-positive cells increased slightly at 24h (6.6±0.7%) and considerably at 48h (51.0±6.0%) as compared to that in control cells (0h incubation). These results suggest that gemcitabine induced the arrest of cell proliferation until 24h and caused cell death after 24h.

Gemcitabine is known to induce cell cycle arrest by inhibiting DNA synthesis in some cell lines. 29 To determine whether it induces cell cycle arrest in INS-1 cells, we analyzed the cell cycle at 24h after the addition of gemcitabine. In comparison to control cells, the cell number in gemcitabine-treated cells increased during the G1 and/or early S phase and decreased during the G2/M phases. (Fig. 1D, top). On addition of nocodazole, an inhibitor of microtubule polymerization that arrests cells at M phase of the cell cycle, 30 the cell number at the G2/M phases increased and that at the G1 phase decreased (Fig. 1D, left bottom). The addition of nocodazole to gemcitabine-treated cells did not affect the cell cycle distribution pattern (Fig. 1D, right bottom). These results suggest that gemcitabine induces cell cycle arrest at the G1 and/or early S phase within 24h in INS-1 cells.

PI3-K Inhibitors Protect INS-1 Cells from Gemcitabine-Induced Cell Death  The activation of the PI3-K pathway has been demonstrated to be involved in the resistance to the anticancer effects of gemcitabine in pancreatic exocrine cancer cells. 31 To determine whether the inhibition of the PI3-K pathway enhances gemcitabine-induced apoptosis in INS-1 cells, we investigated the effects of two PI3-K inhibitors, LY294002 and wortmannin, on gemcitabine-induced cell death. As shown in Fig. 2A, pretreatment with LY294002 significantly prevented the decrease in viability induced by gemcitabine in a concentration-dependent manner (without LY294002: 34±8%, 10 µM LY294002: 75±3%). Treatment with LY294002 alone at a concentration of 10 µM did not influence MTT reduction in control cells. Furthermore, it did not completely prevent the decrease in viability induced by gemcitabine. However, we could not perform the experiment using a higher concentration of LY294002 because it induced the cytotoxicity in control cells (data not shown). LY294002 inhibited gemcitabine-induced cell death when it was added to the cells 4h after, but not 24h after, the addition of gemcitabine (Fig. 2B). As shown in Figs. 2C and D, wortmannin significantly inhibited gemcitabine-induced cell death when it was added to the cells 1h before and 4h after the addition of gemcitabine (1h before: 65±4%, 4h after 53±4%). However, the inhibitor did not inhibit the cell death when it was added 24h after the addition of gemcitabine. These results suggest that gemcitabine may induce the activation of the PI3-K pathway within 24h after its addition, and that PI3-K activation may be involved in gemcitabine-induced apoptosis.

A PI3-K Inhibitor Prevented Gemcitabine-Induced Apoptosis in INS-1 Cells  It has been reported that gemcitabine induces apoptosis in some cells. 32 To determine whether it has a similar effect on INS-1 cells, we observed two features of apoptosis: nuclear fragmentation and DNA laddering. Figure 3A shows images of INS-1 cells that were subjected to double staining by Hoechst 33258 for nuclei and MitoTracker Red for cytosol. In gemcitabine-treated cells, although no change was observed at 24h, nuclear fragmentation was clearly observed at 48h. In the presence of LY294002, nuclear fragmentation was scarcely observed at 36 and 48h, respectively, and was similar to control cells. Treatment with gemcitabine for 48h also induced nucleosomal DNA ladder formation in INS-1 cells (Fig. 3B). Pretreatment with LY294002 completely prevented DNA laddering. These results suggest that PI3-K activation is involved in gemcitabine-induced apoptosis.

Gemcitabine Induces the Activation of the PI3-K/Akt Pathway  To determine whether gemcitabine induces the
activation of the PI3-K pathway under the present conditions, we investigated the phosphorylation of Akt (Ser473), one of the downstream targets of PI3-K, by Western blotting. As shown in Fig. 4, treatment with gemcitabine for 4h significantly increased Akt phosphorylation compared to control cells (phospho-Akt/total Akt was 2.6±0.2). However, pretreatment with LY294002 significantly decreased gemcitabine-induced Akt phosphorylation (phospho-Akt/total Akt was 1.2±0.5).

To determine whether the activation of mammalian target of rapamycin (mTOR), one of the downstream targets of the PI3-K/Akt pathway, is involved in gemcitabine-induced apoptosis, we investigated the effects of rapamycin, an inhibitor of mTOR, on gemcitabine-induced apoptosis. As shown in Fig. 5, pretreatment with rapamycin (0.5, 2µM) significantly prevented gemcitabine-induced apoptosis in a concentration-dependent manner (without rapamycin: 42±8%, 2µM rapamycin: 65±7%). Treatment with rapamycin alone at a concentration of 2µM did not influence MTT reduction by control cells. These results suggest that gemcitabine induces the activation of the PI3-K/Akt/mTOR pathway.

Figure 6 shows the effect of several inhibitors of intracellular signaling pathways. MG132, an inhibitor of NF-κB, and PFT-α, an inhibitor of p53, did not prevent gemcitabine-induced cytotoxicity. U0126, an inhibitor of MEK1/2 in the classical MAPK pathway, prevented gemcitabine-induced cytotoxicity, but another MEK1/2 inhibitor did not. These results suggest that NF-κB, p53, and MEK1/2 activation was not involved in gemcitabine-induced apoptosis in INS-1 cells. Furthermore, Z-VAD-FMK, the pan-caspase inhibitor, and Ac-DEVD-CHO, the caspase-3/7 inhibitor, did not prevent gemcitabine-induced cytotoxicity.

**DISCUSSION**

In this study, we demonstrated that gemcitabine induces apoptosis of a pancreatic β-cell line, INS-1, and characterized the mechanism in which activation of the PI3K/Akt pathway is involved. Gemcitabine may induce an increase in urine glucose concentration as an adverse effect. However, the cause and molecular mechanism of this gemcitabine-induced cytotoxicity have not been examined in detail. The concentration of gemcitabine (250nM) used here was less than one-third of that circulating in gemcitabine-treated cancer patients.

Our experimental model using INS-1 cells should be valid to elucidate the cytotoxic effects of gemcitabine in β-cells. Here...
we indicate the possibility that the induction of β-cell death could be the cause of increase in urine glucose concentration observed in gemcitabine-treated patients.

The present study demonstrated that gemcitabine induces apoptosis, which was indicated by cell cycle arrest in the early S phase, nuclear fragmentation and DNA ladder formation in INS-1 cells. Apoptosis was observed 48 h after treatment with gemcitabine, but scarcely at 24 h. As far as we know, we first showed that gemcitabine induced apoptosis in pancreatic β-cell line. The apoptosis with Akt phosphorylation induced by gemcitabine was prevented by PI3-K inhibitors, suggesting that the activation of the PI3-K/Akt pathway is involved in gemcitabine-induced apoptosis in INS-1 cells. In embryonic fibroblast-adipose like cell line 3T3L1 and leukemic monocyte lymphoma cell line U937, however, LY294002 and wortmannin did not prevent, but led to enhance cell death induced by various concentrations of gemcitabine (data not shown). In pancreatic adenocarcinoma cell lines PK1 and PK8, PI3K inhibitors enhanced apoptosis induced by gemcitabine.19) The combination of gemcitabine and either LY294002 or wortmannin significantly increased DNA fragmentation in metastatic colon cancer cell line KM20.34) In non-Hodgkin lymphoma cell lines Pfeiffer, mTOR inhibitor enhanced cell death induced by gemcitabine.35) These findings suggest that the activity of PI3K/Akt/mTOR pathway may play a significant role in mediating gemcitabine resistance in numerous cells. Although PI3-K/Akt pathway is well known for its role in promoting cell proliferation and suppressing apoptosis, this pathway can, under certain conditions, be involved in apoptosis. Byun et al. proposed the possibility that mammalian cells possess mechanisms that induce caspase-independent apoptosis in response to oncogenic Ras signals through the activation of both PI3-K and Rac1 to avoid the development of malignancy.36) Hofheinz et al. demonstrated that bile salt-induced hepatocyte apoptosis was partly mediated by a PI3-K-dependent signaling pathway, potentially involving JNK.37) Aki et al. reported the possibility that PI3-K is associated with apoptosis in myoblast cells under hypoxia.38) Shack et al. proposed that PI3-K accelerated death in uterine cancer cells treated with arsenious acid.39) Lee indicated that PI3-K accelerated death in monotopic leukemia cells under conditions of depleted blood.40) Recently, Lim et al. proposed that PI3-K activation by mild heat shock causes a switch from necrosis to apoptosis through an increase in p53 and Hsp27 activity.41) From these findings, we speculate that PI3-K activation by gemcitabine is involved in initiation of apoptosis in pancreatic β-cell line INS-1.

The addition of LY294002 1 h before and 4 h after the addition of gemcitabine, but not after 24 h, inhibited the cell death. These results suggest that PI3-K activation was immediately induced after the addition of gemcitabine in INS-1 cells, and that the initial step of apoptosis appears to be a later event occurring after PI3-K activation. This study demonstrated...
that gemcitabine-induced apoptosis was prevented by PI3-K inhibitors. In addition, rapamycin, an inhibitor of mTOR, significantly prevented gemcitabine-induced apoptosis. mTOR is a downstream target of the PI3-K/Akt pathway, and is involved in cell proliferation and growth through the regulation of activation of translational or cell cycle regulator proteins.42) These results suggest that the activation of the PI3-K/Akt/mTOR pathway is involved in gemcitabine-induced death in INS-1 cells. An inhibitor of MEK1/2, U0126, also prevented apoptosis,43) but another MEK1/2 inhibitor that has higher specificity did not do the same. Although the precise...
mechanism is unknown, U0126 can prevent the activation of mTOR/p70S6K, which is downstream of the PI3-K/Akt pathway. From these findings, we hypothesize that the MEK/ERK pathway may not be involved in gemcitabine-induced apoptosis. In addition, gemcitabine-induced cell death was not prevented by the caspase inhibitors Ac-DEVD-CHO and Z-VAD-FMK. We confirmed that caspase-3 activation was not detected in INS-1 cells treated with gemcitabine by the immunofluorescence assay using a flow cytometer (data not shown). Although we previously reported that cytotoxic compounds such as chloroacetatealdehyde and ferric-nitritolactate induced apoptosis through the activation of caspase signaling, the conflicting data from the present study and some literatures suggest that some compounds induce caspase-independent apoptosis. Apoptosis is now recognized not to represent a signal entity and some cells yield to developmental cell death in a caspase-independent fashion. Chipuk and Green proposed apoptosis-like cell death probably includes most forms of caspase-independent cell death. Recent evidences indicate that apoptosis-inducing factor (AIF) release from mitochondria and translocation to nucleus play a role in caspase-independent cell death such as apoptosis, necrosis and autophagy. AIF is likely to recruit other protein with nuclease properties that are involved in DNA fragmentation, although the identity of endonuclease is not known. Mukubou et al. have shown that autophagy can be activated in the pancreatic cancer cell line treated with gemcitabine and/or ionizing radiation. From the present results and these findings, We interpret the death as apoptosis-like cell death in INS-1 cells treated with gemcitabine.

It is known that cell cycle arrest is involved in the induction of apoptosis. Arrest of proliferation and cell cycle at the early S phase was observed within 24 h in gemcitabine-treated INS-1 cells, but was inhibited by LY294002. This result suggests that cell cycle arrest may not be directly associated with PI3K activity. Gemcitabine can upregulate the transcription factor E2F-1, which plays a central role in cell cycle regulation when deoxynucleotide triphosphates (dNTPs) are depleted. E2F-1 activation leads to progression to the S-phase and should increase the incorporation of gemcitabine into DNA, resulting in cell cycle arrest and DNA damage. Further studies are required to clarify the relationship between cell cycle arrest and the activation of the PI3-K/Akt pathway during apoptosis-like cell death in gemcitabine-treated INS-1 cells.

Resistance to gemcitabine is thought to be a major cause of treatment failure in pancreatic ductal adenocarcinoma patients. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway is an attractive target for cancer therapy because TRAIL induces cell death through its interaction with either TRAIL-receptor 1 or -receptor 2. In contrast, TRAIL binding to TRAIL-receptor 4 confers resistance to cell death. Laloue et al. demonstrated that the activation of TRAIL-receptor 4 triggered the initiation of signal pathway leading to cell survival and proliferation in HeLa cells through the phosphorylation of Akt. The combination treatment with death receptor 5 agonist and gemcitabine provided to be more efficacious. In addition, inhibitors of PI3K improved the anticancer efficacy of gemcitabine treatment. Our data and these findings suggest that the efficacy of gemcitabine can be influenced by the activation of some pathways containing the phosphorylation of PI3K/Akt, the activation of caspase cascade or the stimulation of TRAIL receptors. Further studies are required to clarify the process that activation of some signaling pathways will improve the curative effect of gemcitabine.

To explain the mechanism of gemcitabine-induced increase in urine glucose concentration, an adverse effect of gemcitabine treatment, we investigated the mechanism of gemcitabine-induced cytotoxicity in INS-1 cells. To the best of our knowledge, this is the first study to reveal that the activation of the PI3-K/Akt/mTOR pathway is involved in the induction of apoptosis-like cell death. The role of the PI3-K/Akt pathway is interesting in pancreatic β-cells because this pathway is involved in the release of insulin from β-cells. It has been demonstrated that activated PI3-K plays a positive role in glucose-stimulated insulin secretion in insulinoma INS-1 cells and human β-cells. Alternatively, alloxan, a diabetogenic compound, selectively induced the cytotoxicity of β-cells in vivo and in vitro. We previously demonstrated that alloxan induces apoptosis in INS-1 cells. Alloxan-induced apoptosis is also prevented by LY294002 and wortmannin (unpublished data). These findings suggest the possibility that abnormal activation of the PI3-K/Akt pathway can lead to apoptosis during insulin secretion in pancreatic β-cells. Further investigation on the mechanism of selective apoptosis in pancreatic β-cells is required.

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