Gemcitabine-Induced Programmed Cell Death (Apoptosis) of Human Pancreatic Carcinoma Is Determined by Bcl-2 Content

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**Background:** Gemcitabine is a new nucleoside analogue that produces a clinical response in 30% of patients with unresectable pancreatic carcinoma. The cytotoxic effects of many chemotherapeutic agents occur through induction of programmed cell death (apoptosis), which is controlled by the bcl-2 gene family. We determined whether induction of apoptosis by gemcitabine in pancreatic carcinoma is associated with cellular Bcl-2 content.

**Methods:** Four pancreatic carcinoma cell lines (MIA-PaCa-2, AsPC-1, Panc-1, and Panc-48) were screened by Western blotting for Bcl-2 protein expression. Dose-response relationships for the cytotoxic effects of gemcitabine were determined using methylthiotetrazole assays, and induction of apoptosis was confirmed by fluorescence-activated cell sorting analysis. MIA-PaCa-2 cells transfected with human bcl-2 were also analyzed for gemcitabine-induced apoptosis.

**Results:** Pancreatic cancer cell lines expressed varying amounts of Bcl-2, and the 50% lethal dose for gemcitabine-induced apoptosis was correlated with Bcl-2 content. Furthermore, Bcl-2 overexpression was associated with a significant increase in the 50% lethal dose for gemcitabine-induced apoptosis.

**Conclusions:** Cellular Bcl-2 content was directly correlated with the cytotoxicity of gemcitabine in pancreatic carcinoma. Therefore, routine immunohistochemical analyses may be useful in predicting gemcitabine efficacy, and patients who would likely not benefit could be spared gemcitabine administration. Furthermore, the effectiveness of gemcitabine and other chemotherapeutic agents may be increased by gene therapy-mediated alteration of bcl-2 gene family members.

**Key Words:** Gemcitabine—bcl-2—Pancreatic cancer—Apoptosis.

A major unsolved health problem, namely cancer of the exocrine pancreas, continues to cause approximately 27,000 deaths/year in the United States and 50,000 deaths/year in western Europe.¹² Pancreatic cancer is the fourth leading cause of adult deaths from cancer and is responsible for almost 5% of all cancer-related deaths.² Because of difficulties in diagnosis, the aggressiveness of pancreatic cancers, and the lack of effective systemic therapies, only 1-4% of patients with adenocarcinoma of the pancreas are alive 5 years after diagnosis.³⁴ The incidence rates are virtually identical with mortality rates.

Gemcitabine (2’-deoxy-2’-difluorodeoxycytidine, Gemzar; Eli Lilly Co., Indianapolis, IN) is a deoxycytidine analogue with structural and metabolic similarities to cytarabine. After a phase I study,⁵ gemcitabine was evaluated in a multicenter trial of 44 patients with advanced pancreatic cancer.⁶ Although objective responses were documented for only 12% of patients, the investigators noted frequent symptomatic benefit, often in the absence of an objective tumor response. Based on these observations, two subsequent trials of gemcitabine were performed with patients with advanced pancreatic cancer. In one randomized trial, patients treated with gemcitabine achieved modest but statistically significant improvements in response rate and median survival rate, compared with those treated with 5-fluorouracil (5-FU).⁷
In addition, more meaningful clinical effects on disease-related symptoms (pain control, performance status, and weight gain) were seen with gemcitabine than with 5-FU. Similar effects were documented for gemcitabine-treated patients whose illness progressed while they were receiving 5-FU. These results suggest that gemcitabine will become accepted as first-line therapy for patients with advanced pancreatic adenocarcinoma. Despite the recent encouraging results with gemcitabine, however, the median survival time for patients with metastatic pancreatic disease continues to be <6 months, with long-term disease stabilization remaining very rare.

The process of cell death produced by cytotoxic chemotherapy or ionizing radiation was once thought to begin with irreversible DNA damage, followed by mitotic failure. The belief now, however, is that nearly all chemotherapeutic agents, as well as ionizing radiation, use endogenous apoptotic mechanisms to induce programmed cell death. The Bcl-2 gene family seems to be centrally involved in regulating apoptosis triggered by a variety of stimuli, including chemotherapy. Although other members of the bcl-2 family stimulate programmed cell death, bcl-2 is the prototypic antiapoptotic gene. The levels of expression of these genes, and specifically that of bcl-2, have been shown to be correlated with the apoptotic responses of a variety of neoplastic cell lines after exposure to cytotoxic agents. Because resistance to chemotherapy and radiotherapy seems to be mediated by alterations in the pathways of apoptosis, elucidation of the mechanisms of apoptotic resistance to standard cancer therapy clearly will help eliminate continued administration of ineffective therapy and perhaps will identify alternative therapeutic modes.

The mechanism of resistance of pancreatic cancer to standard chemotherapeutic agents is not well understood. Furthermore, there is no reliable method by which responsiveness to gemcitabine can be determined a priori. The purpose of this study, therefore, was to determine whether levels of Bcl-2 are correlated with the cytotoxic effects of gemcitabine in cell lines in vitro.

MATERIALS AND METHODS

Cell Lines and Reagents

MIA-PaCa-2, AsPC-1, and Panc-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD), and the Panc-48 cell line was generously provided by Dr. Douglas Evans (Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, L-glutamine, vitamins, and antibiotics. Cells were maintained in a humidified incubator containing 10% CO₂, at 37°C.

All chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Gemcitabine was purchased from Eli Lilly Co., reconstituted in sterile phosphate-buffered saline (PBS), and stored in aliquots at −20°C. The monoclonal antibody to human Bcl-2 (clone 6C8) was generously provided by Dr. Timothy J. McDonnell (Department of Molecular Pathology, M. D. Anderson Cancer Center). A polyclonal antibody to actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The bcl-2 expression plasmid was a generous gift from Dr. John Reed (The Burnham Institute, La Jolla, CA). In brief, the human cDNA for the bcl-2 gene was cloned into the eukaryotic expression plasmid pCI-neo, containing the neomycin gene for selection. The bcl-2 gene is under control of the cytomegalovirus promoter, as previously reported.

Detection of Cellular Bcl-2 Protein Content

Cells were cultured in T25 flasks in appropriate medium and collected, at approximately 75% confluence, by trypsinization (0.25%, w/v, trypsin, 1 mmol/l EDTA). Cells were lysed for 1 hour at 4°C in lysis buffer containing 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, and 25 mmol/l Tris (pH 7.5). Debris was sedimented by centrifugation for 5 minutes at 12,000 × g, and the pellets and/or supernatants were solubilized for 5 minutes at 100°C in Laemmli’s sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing 100 mmol/l diethiothreitol. Protein concentrations of the lysates were determined with a Bio-Rad protein assay kit, and 50 μg of each sample were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis at 100 V for 1 hour. Separated polypeptides were then electrophoretically transferred to 0.2-μm nitrocellulose membranes (Schleicher & Schuell, Keene, NH) for 1 hour at 100 V. Membranes were blocked for 1 hour in Tris-buffered saline/Tween (25 mmol/l Tris, pH 8.0, 150 mmol/l NaCl, 0.05% Tween-20) containing 3% (w/v) nonfat dried milk. Blots were then incubated overnight with antibodies to Bcl-2 and developed using species-specific secondary and tertiary antisera. Immunoreactive material was detected by the enhanced chemiluminescence technique (Amershams, Arlington Heights, IL). Relative polypeptide expression was quantified by laser densitometry (Molecular Dynamics, Sunnyvale, CA).