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ORIGINAL ARTICLE

Alteration of the Intrinsic Apoptosis Pathway Is Involved in Notch-induced Chemoresistance to Gemcitabine in Pancreatic Cancer

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Background and Aims. Chemoresistance is a major challenge in pancreatic cancer (PC) treatment. Limited data have shown that members of the Notch signaling pathway are involved in resistance to gemcitabine (GEM) in PC. However, further evidence is needed and the underlying mechanisms remain to be elucidated. The current study aims to investigate the role of alterations of the intrinsic apoptosis pathway in Notch-induced GEM resistance of PC.

Methods. The Notch signaling pathway was inhibited or activated in three PC cell lines (AsPC-1, BxPC-3, and MIA PaCa-2) by γ-secretase inhibition and Notch intracellular domain (NICD) overexpression, respectively. Subsequent analyses included inhibition rates of cell proliferation by GEM, cell apoptosis, and expression of proteins involved in the intrinsic apoptosis pathway.

Results. Hes-1 expression was significantly elevated after GEM treatment, indicating Notch activation. Inhibition of the Notch signaling pathway by DAPT, a γ-secretase inhibitor, resulted in a significant increase of the inhibition rates by GEM in all PC cell lines. In addition, there was more frequent apoptosis, higher caspase-3 activity, up-regulation of Bax, and down-regulation of Bcl-2 and Bcl-xL. Conversely, transient transfection of NICD, which enhances the activity of the Notch signaling, caused a remarkable decrease of the chemosensitivity to GEM.

Conclusions. An alteration of the intrinsic apoptosis pathway is involved in Notch-induced chemoresistance to GEM in PC cells.

Key Words: Pancreatic cancer, Notch, Intrinsic apoptosis pathway, Chemoresistance, Gemcitabine.

Introduction

Pancreatic cancer (PC) has been well known as a lethal malignant neoplasm, ranking as the fourth leading cause of cancer-associated death (1). Currently, chemotherapy is widely used to treat the majority of patients with unresectable PC (2) because the resection rate remains low (3,4). Among agents for chemotherapy, gemcitabine (GEM) has become a first-line agent (4). However, it is unfortunate that inherent/acquired chemoresistance to GEM is common and negatively influences the outcome of patients with PC (5). Therefore, the relative mechanisms of GEM resistance are of particular interest.

Many signaling pathways such as NF-κB, PI3K/Akt, Notch and Hedgehog, are involved in drug resistance in PC (6). Among them, the Notch signaling pathway has been demonstrated to play crucial roles in tumor initiation, growth, invasion and apoptosis inhibition in cell lines and animal models (7–15). Moreover, this pathway influences chemosensitivity to some chemotherapeutic agents such as GEM and taxane (16–18). On the other hand, the γ-secretase inhibitor (GSI) MRK-003, which downregulates Notch signaling, has a synergistic effect with GEM on growth

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inhibition and apoptosis induction of PC (19,20). These findings support the hypothesis that the Notch signaling pathway may be a key mediator of chemoresistance in PC. However, the underlying mechanisms of Notch-induced GEM resistance in PC have not been extensively studied.

The present study aimed to explore the role of alterations of the intrinsic apoptosis pathway in Notch-induced chemoresistance to GEM in PC cells.

Materials and Methods

Cell Culture

Human PC cell lines AsPC-1, BxPC-3 and MIA PaCa-2, which originate from metastatic or primary sites and express different baseline levels of Notch1 (Figure S1), were kind gifts from Prof. Helmut Freiss, Heidelberg University, Germany. The cells were maintained in Dulbecco's modified Eagle's medium or RPMI 1640 (Hyclone, Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Hyclone) at an atmosphere with 5% CO_2 at 37°C.

Drug Treatments

To evaluate the effects of DAPT, a GSI, cells were seeded in six-well plates at 2×10^5 cells per well or 96-well plates at 4×10^3 cells per well. After treatment with GEM (10^3 nmol/L, Gemzar, Lilly, France), GEM (10^3 nmol/L) combined with DAPT (100 μmol/L, Sigma Chemical Co., St. Louis, MO), or culture medium alone (control) for 72 h, the cells were harvested for further analyses. Similarly, cells were seeded in 96-well plates at 1×10^4 cells per well and transfected with a plasmid carrying the sequence encoding the Notch intracellular domain (NICD) for 48 h. GEM was then added to the cells at 1 mmol/L, 1 μmol/L and 1 nmol/L followed by a further 48 h of incubation. The cells were then subjected to further analyses.

Plasmid Construction and Transfection

Notch intracellular domain (NICD) pIRES2-EGFP plasmid (Figure S2) was constructed and identified by endonuclease digestion and DNA sequencing (data not shown). Cells were seeded in six-well plates at 5×10^5 cells per well. At 80% confluence, the cells were transiently transfected with 4 μg of NICD or mock (control) plasmids for 48 h using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Western Blotting

Total protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking with 5% skim milk, the membranes were incubated with primary antibodies (Hes-1, Bax, Bcl-2, Bcl-xL and β-actin, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature. Protein bands were detected by an ECL kit (Millipore). Beta-actin served as the internal control.

Cell Proliferation Inhibition Assay

Cell proliferation was first measured using a cell count kit (CCK-8). After incubation in culture medium containing CCK-8 reagent for 3 h, the absorbance (450 nm) was detected using a microplate reader (Wellscan MK3, Thermo/Labsystems, Finland). Inhibition rate was then calculated according to the following formula:

$$\text{Inhibition rate} = 1 - (A \text{ of treatment group} - A \text{ of blank control group})/A \text{ of cell control group} - A \text{ of blank control group.}$$

where A represents the absorption value.

Annexin V/Propidium Iodide (PI) Staining for Measurement of Apoptosis

Apoptotic cells were detected by Annexin V-FITC/PI double staining. Treated cells were washed, trypsinized, centrifuged and then resuspended at 1×10^6 cells/mL. Cells were then incubated in binding buffer containing annexin V-FITC (5 μL) and PI (10 μL) for 15 min in the dark. The cells were then analyzed by an Accuri C6 Flow Cytometer (Accuri Cytometers, Ann Arbor, MI).

Detection of Caspase-3 Activity

Treated cells were collected and caspase-3 activity was analyzed by a CaspACE Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

Statistical Analysis

The values of continuous variables derived from experiments performed in triplicate were expressed as mean ± standard deviation. The independent sample t-test was used for comparisons. The statistical software package SPSS 13.0 (SPSS Inc., Chicago, IL) was employed for all analyses; p value <0.05 was defined as statistically significant.

Results

Inhibition of the Notch Signaling Pathway Enhances Chemosensitivity to GEM

The expression of Hes-1, a target protein of Notch, was obviously upregulated after GEM treatment (Figure 1A),