Original

Effect of 5-Fluorouracil on Growth of Human Pancreatic Cancer Cells and Expression of Bcl-2 Family Genes

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Abstract : Pancreatic cancer is one of the most devastating malignant tumors and the development of an effective novel modality of treatment is awaited.

We studied the mechanism of growth-inhibition of 5-fluorouracil (5-FU) on human pancreatic cancer cells focusing on p53 status and expression of bcl-2 family genes. COLO-357 cells with wild-type p53 gene and T3M4, Panc-1 and AsPC-1 cells with mutant p53 gene were used. Growth of COLO-357 and T3M4 cells was inhibited by the treatment with relatively low concentrations of 5-FU, and growths of Panc-1 cells was suppressed by treatment with relatively high concentrations of 5-FU under serum-supplemented conditions. In contrast, 5-FU had no effect on growth of AsPC-1 cells. While DNA fragmentation in COLO-357 cells was induced by a low concentration of 5-FU and then declined with higher concentrations of 5-FU, in T3M4 cells DNA fragmentation was gradually induced with increasing concentrations of 5-FU. Expression of bax mRNA and protein was enhanced by 5-FU in COLO-357 cells. Although expression of bclXS mRNA was enhanced by 5-FU in both COLO-357 and T3M4 cells, BclXS protein was not detected on Western blotting. Increased expression of bax was associated with increased inhibition of cell growth by 5-FU in pancreatic cancer cells with a wild-type p53 gene and bclXS may play a role in the growth-inhibitory effect of 5-FU on pancreatic cancer cells both with wild-type and mutant p53 genes. Therefore, gene-therapy targeting BclX for pancreatic cancer may be effective. AsPC-1 cells provide a useful tool for the study of mechanisms of resistance to chemotherapy.

Key words : 5-FU, pancreatic cancer, p53, bcl-2 family, apoptosis

Introduction

Pancreatic cancer is one of the leading causes of cancer death in Western countries and in Japan^{1,2)}. Pancreatic cancer is rarely cured by surgical resection and is generally highly resistant to radiotherapy and chemotherapy¹⁾. Complete removal of macroscopically detectable tumor tissue does not prevent metastasis to the liver and local recurrence, which results in peritonitis carcinomatosa. To overcome these problems, the development of novel therapeutic strategies such as combination with chemotherapy and gene therapy is required for management of micrometastasis to the liver and peritonitis carcinomatosa³⁾.

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5-FU is widely used for treatment of many malignancies and 5-FU-based combination chemotherapy for the treatment of pancreatic cancer has been reported. Recently, biochemical modulation therapy with 5-FU and other anticancer agents for the treatment of pancreatic cancer and metastatic lesions was attempted, but to date the results have not been satisfactory⁴⁻⁶).

p53 is a tumor suppressor gene which is activated by many stimuli causing DNA damage, and induces G1 arrest or apoptosis through the activation of bcl-2 family genes such as bax⁷). p53 gene mutations are detected in more than a half of all cases of pancreatic cancer and give a growth advantage^{8,9}). A dual effect of mutant p53 protein in the chemosensitivity of human cancers was reported¹⁰). While an inhibitor of DNA synthesis such as 5-FU can induce apoptosis through a wild-type p53-dependent pathway, the cytotoxic effects of DNA-crosslinking chemotherapeutics such as cisplatin can be enhanced by mutated p53, which is no longer able to repair drug-induced DNA damage. Bcl-2 family genes such as bcl-2, bax, bclXL and bclXS form homodimers or heterodimers with each other and can thereby have either apoptotic or anti-apoptotic effects¹¹).

In this study, we investigated the mechanism of growth inhibition by 5-FU in human pancreatic cancer cells focusing on p53 status and the expression of bcl-2 family genes.

Materials and Methods

Cell culture

Human pancreatic cancer cells originating from ductal cells, AsPC-1, COLO-357, Panc-1 and T3M4 cells, were a gift from Dr. M. Korc of the University of California, Irvine. AsPC-1 and T3M4 cells were grown in RPMI 1640 medium, and COLO-357 and Panc-1 cells were grown in Dulbecco's modified Eagle medium (DMEM). Both media were supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 µg/ml). FBS was purchased from JRH Biosciences (Lenexa, KS, USA) and RPMI, DMEM, penicillin and streptomycin were purchased from GIBCO BRL (Grand Island, NY, USA).

Growth assay

To assess the effect of 5-FU on growth of cells, AsPC-1, COLO-357, Panc-1 and T3M4 cells were plated at a density of 15,000 cells/well in 96-well plates and grown overnight in RPMI 1640 or DMEM medium supplemented with 10% FBS and antibiotics. After washing with phosphate-buffered saline (PBS), the cells were incubated with 5-FU ranging from 0 to 25 µg/ml in medium with 10% FBS for 48 hours. Cell growth was assessed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. In brief, 12.5 µl of MTT (5 mg/ml PBS) was added to the medium and incubated for 4 hours. Then medium was aspirated and MTT dye crystals were dissolved with acidified isopropanol (0.04 N HCl). Optical density was measured at 570 nm with an enzyme-linked immunosorbent assay plate reader (Immuno Reader NJ2001 : Inter Med Japan, Tokyo, Japan) and an absorbance of 650 nm was subtracted to correct for absorbance of cellular debris. Effect of 5-FU on cell growth was assessed by comparison of OD values of 5-FU treated cells with that of non-treated cells as a control. 5-FU was obtained from Wako Pure Chemical Industries (Osaka, Japan). Other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

Detection of DNA fragmentation and caspase 3 activity

To identify apoptotic cells, a cellular DNA fragmentation ELISA kit (Boehringer-Mannheim, Mannheim, Germany) and caspase 3 calorimetric assay kit (R&D Systems, Minneapolis, MN, USA) were employed. For the detection of DNA fragmentation, COLO-357 and T3M4 cells were plated onto 96-well plates at a density of 15,000 cells/well and grown in media with 10% FBS containing 10 μ M of 5'-bromo-2-deoxy-uridine (BrdU). On the following day, BrdU-containing medium was aspirated and after washing with PBS, cells were treated with 5-FU up to 25 μ g/ml for 48 hours in the conditioned medium the same as in the growth assay. Then both culture media and cell lysates were transferred to 96-well plates precoated with anti-DNA antibody. An immunosorbent assay was done according to the manufacturer's instructions, and DNA fragmentation was detected by anti-BrdU peroxidase conjugate. Values at OD 450 nm of non-treated samples were used as a control.

For the measurement of caspase 3 activity, COLO-357 and T3M4 cells treated with 5-FU up to 25 μ g/ml for 48 hours were lysed with cell lysis buffer after washing with PBS and lysates were incubated with p-nitroanilide-conjugated caspase-specific substrate (DEVD-pNA). Caspase 3 activity was spectrophotometrically measured at OD 405 nm and was expressed as relative intensity of OD value to protein. Protein of cell lysates was measured by the Bradford method (Bio-Rad Lab, Hercules, CA, USA).

Expression of bcl-2 family genes

COLO-357 and T3M4 cells were plated in 6 cm-plates and grown in medium with 10% FBS to 70–80% confluence. After washing with PBS, medium containing 10% FBS with or without 5-FU up to 25 μ g/ml were added. Total RNA was extracted by the acid guanidinium thiocyanate phenol chloroform method 48 hours after the addition of 5-FU. One μ g of total RNA was reverse transcribed to cDNA (Takara Shuzo Co., Ltd., Shiga, Japan) and the resulting cDNA was amplified by polymerase chain reaction (PCR) using following primer sets specific to bcl-2, bax and bclXL and bclXS.

bcl-2 : sense 5'CGACGACTTCTCCCGCCGCTACCGC3'
antisense 5'CCGCATGCTGGGGCCGTACAGTTCC3'

bax : sense 5'CGAGTGGCAGCTGACATGTT3'
antisense 5'ATGGTTCTGATCAGTTCCGG3'

bclXL and bclXS: sense 5'CGGGCATTGAGTGACCTGAC3'
antisense 5'TCAGGAACCAGCGTTGAAG3'

PCR conditions were as follows: denaturation at 95°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s, 40 cycles for bcl-2¹²⁾, denaturation at 94°C for 60 s, annealing at 46°C for 120 s, and extension at 72°C for 120 s, 35 cycles for bax and denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, 30 cycles for bclXL and bclXS¹³⁾. Amplification products were electrophoresed through a 2 % agarose gel and stained with ethidium bromide.

Western blotting of Bcl-2 family proteins

COLO-357 and T3M4 cells were plated and treated with 5-FU as described for the extraction of total RNA. Treated cells were lysed with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 1 % tritonX100 and protease inhibitors (aprotinin, leupeptin and

4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride). After centrifugation at 15,000 g for 15 minutes, supernatant was retained for Western blotting. Each sample containing 20 μ g of total protein was loaded onto a 12.5% polyacrylamide slab gel. After electrophoresis under reducing condition, fractionated proteins were transferred to nitrocellulose sheets (Millipore Corp., Bedford, MA, USA). Immunoreactive proteins were detected with a chemiluminescence kit (Amersham ECL reagent kit, Amersham International, Buckinghamshire, UK) and exposed to X-ray film. Antibody for Bcl-2 was purchased from DAKO (Glostrup, Denmark) and antibodies for Bax (I-19) and BclXL and BclXS (L-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse IgG horse radish conjugate for Bcl-2 and protein A for Bax, BclXL and BclXS were used as secondary antibodies.

Statistical analysis

All experiments were repeated at least three times with separate cell preparations. Data were expressed as mean+SE. Analysis of variance and post hoc testing by Scheffe's F-test were used for statistical analysis. A P value less than 0.05 was considered statistically significant.

Results

Effect of 5-FU on growth of pancreatic cancer cells

Regardless of presence or absence of a p53 gene mutation, growth of human pancreatic cancer cells except for AsPC-1 cells was inhibited by 5-FU treatment. A significant growth-inhibitory effect of 5-FU on COLO-357 and T3M4 cells was observed at relatively low concentrations (0.25 μ g/ml) and compared with growth of non-treated cells, growth of cells was suppressed to 62.0% in COLO-357 cells and 60.9% in T3M4 cells, respectively. In Panc-1 cells, 0.25 to 0.5 μ g/ml 5-FU had no effect on growth, but growth was inhibited at higher concentrations with a maximal effect at 25 μ g/ml. AsPC-1 cells did not show any response to 5-FU treatment (Fig. 1).

Determination of DNA fragmentation and caspase 3 activity

In COLO-357 cells, DNA fragmentation was induced by 5-FU treatment and the peak level, which was a 3.2-fold increase in OD value compared to non-treated cells, was observed at 1 μ g/ml of 5-FU, after which the OD value declined with further increases in concentration. DNA fragmentation in T3M4 cells gradually increased in a dose-dependent manner up to 25 μ g/ml of 5-FU and the maximal OD value was a 2.2-fold increase over that of non-treated-cells (Fig. 2). While caspase 3 activity in COLO-357 cells gradually increased with increasing concentration of 5-FU up to 25 μ g/ml, in T3M4 cells caspase 3 activity was increased at 1 μ g/ml 5-FU and remained high up to 25 μ g/ml of 5-FU (Fig. 3).

bcl-2 family gene mRNA expression

In COLO-357 cells, bax mRNA expression was enhanced by treatment with 5-FU at more than 5 μ g/ml for 48 hours in medium with 10% FBS. However, the changes in bax mRNA expression with 5-FU treatment in T3M4 cells were not consistent in repeat experiments. The expression of bclXL mRNA was not changed by 5-FU treatment, but bclXS mRNA expression was increased by 5-FU in both COLO-357 and T3M4 cells (Fig.

Fig. 1. Effect of 5-FU on growth of human pancreatic cancer cells
Pancreatic cancer cells were treated with 5-FU at 0, 0.25, 1.0, 5.0, 10.0, 25.0 $\mu\text{g}/\text{ml}$ for 48 hours. * $p < 0.01$ (compared to non-treated cells)

Fig. 2. DNA fragmentation in COLO-357 and T3M4 cells
Pancreatic cancer cells were treated with 5-FU at 0, 1.0, 5.0, 10.0, 25.0 $\mu\text{g}/\text{ml}$ for 48 hours.

Fig. 3. Caspase 3 activity in COLO-357 and T3M4 cells
Pancreatic cancer cells were treated with 5-FU at 0, 1.0, 5.0, 10.0, 25.0 µg/ml for 48 hours.

Fig. 4. Expression of bax and bclX mRNA in COLO-357 and T3M4 cells
Pancreatic cancer cells were treated with 5-FU at 0, 5.0, 10.0, 25.0, 50.0 µg/ml for 48 hours.

4). Several non-specific bands were detected for bcl-2 mRNA and the results were not consistent between repeat experiments (data not shown).

Bcl-2 family protein expression

In COLO-357 cells, expression of Bax protein was enhanced by 5-FU treatment, consistent with the increased expression of bax mRNA. Bcl-2 and BclXL protein expression was not changed with 5-FU treatment. Expression of BclXS protein was very weak, and clear bands could not be detected on conventional Western blotting (Fig. 5). In T3M4 cells, there was no correlation between expression level of Bcl-2 family proteins and 5-FU treatment (data not shown).

Discussion

Pancreatic cancer is a devastating disease. When pancreatic cancer is clinically detected, it is usually already inoperable or rarely cured by surgical resection. Pancreatic cancer is thought to be highly resistant to radiotherapy and chemotherapy¹⁾ and metastasis to the

Fig. 5. Expression of Bcl-2 family proteins in COLO-357
COLO-357 cells were treated with 5-FU at 0, 5.0, 10.0, 25.0, 50.0 $\mu\text{g}/\text{ml}$ for 48 hours.

liver and local recurrence are not completely prevented even when macroscopically detectable cancer tissue is removed. To improve the poor prognosis of pancreatic cancer, it is important to develop novel therapeutic modalities for the management of micrometastasis to the liver and peritonitis carcinomatosa.

Genomic and transcriptional alterations have been reported in human pancreatic cancer and p53 gene mutation is detected in more than half of pancreatic cancers and gives a growth advantage^{8,9)}. While the wild-type p53 gene is activated by noxious stimuli causing DNA damage and induces G1 arrest or apoptosis through induction of bcl-2 family proteins such as bax⁷⁾, mutant p53 gene loses all function. Mueller and Eppenberger reported the dual effect of mutant p53 protein in chemosensitivity of human cancers¹⁰⁾. While the cytotoxic effects of DNA-crosslinking chemotherapeutics such as cisplatin could be enhanced by mutated p53, which is no longer able to repair drug-induced DNA damage, an inhibitor of DNA synthesis such as 5-FU can induce apoptosis through p53-dependent pathway. Therefore mechanisms of 5-FU-mediated growth inhibition should differ from p53 gene status. In this study, we investigated the mechanism of growth inhibition by 5-FU in human pancreatic cancer cells focusing on p53 status and the expression of bcl-2 family.

In a preliminary study, deprivation of serum from culture medium induced apoptosis in pancreatic cancer cells. However, since in the clinical setting 5-FU may be delivered through vessels, the growth assay was performed in serum-supplemented conditions. Regardless of p53 gene mutation, growth of COLO-357, Panc-1 and T3M4 human pancreatic cancer cells was inhibited by 5-FU. However, growth of AsPC-1 cells was not influenced by 5-FU up to 25 $\mu\text{g}/\text{ml}$. AsPC-1 cells were reported to be resistant to adriamycin, cisplatin, and vinblastin and Umezawa suggested that multidrug resistance of AsPC-1 cells was not due to P glycoprotein and was partially dependent on highly activated tyrosine kinase of insulin-like growth factor I receptor^{14,15)}. Therefore, AsPC-1 cells are a good model to study the mechanism of resistance to chemotherapy.

We then examined the difference in mechanism of 5-FU-induced cell death of COLO-357 cells with wild-type p53 gene and T3M4 cells with variant-type p53 gene by measuring DNA fragmentation and caspase 3 activity, the final step of the caspase cascade¹⁶⁾. DNA fragmentation in COLO-357 cells was induced by low concentrations of 5-FU and the peak

level was observed at $1\mu\text{g/ml}$, then declined suggesting necrosis. On the other hand, DNA fragmentation in T3M4 cells gradually increased up to $25\mu\text{g/ml}$ 5-FU. In contrast to the result of DNA fragmentation, caspase 3 activity in COLO-357 cells gradually increased up to $25\mu\text{g/ml}$ of 5-FU and that in T3M4 cells was increased already at $1\mu\text{g/ml}$ of 5-FU and was maintained at a high level up to $25\mu\text{g/ml}$ of 5-FU. This discrepancy in the results of these two assays would be partially due to the samples employed. For the detection of DNA fragmentation we used both cells attached to the culture plate and culture medium, which would contain released cells and cell fragments during 5-FU treatment, but only attached cells were used in the detection of caspase 3 activity, because it was difficult to obtain enough protein for the determination of caspase 3 activity in the same condition as growth assay and DNA fragmentation assay. It was reported that apoptotic cells in cell culture easily detached and escaped phagocytosis, resulted in secondary necrosis in culture medium¹⁷⁾. Thus apoptosis was induced by low concentrations of 5-FU ($1\text{--}5\mu\text{g/ml}$) in both COLO-357 and T3M4 cells, and at higher 5-FU concentrations primary and secondary necrosis was probably induced in COLO-357 cells, but further study is needed to confirm this.

As to the relation between the growth-inhibitory effect of 5-FU and p53 and bcl-2 family genes, expression of bax mRNA and protein was enhanced by 5-FU in COLO-357 cells with wild-type p53 gene, but was not influenced by 5-FU in T3M4 cells with a mutant p53 gene. It is well known that activated wild-type p53 enhances the expression of bax gene and protein product^{7,18)} and our results were consistent with this. Although we could not show BclXS protein on Western blotting, the expression of bclXS mRNA was enhanced by 5-FU in COLO-357 and T3M4 cells irrespective of their p53 status. BclX, a member of the bcl-2 family, can function as a bcl-2-independent regulator of apoptosis and alternative splicing results in two distinct bclX mRNAs. While the protein product of the larger mRNA, BclXL, inhibits apoptosis, the smaller one encodes BclXS which induces apoptosis¹⁹⁾. Overexpression of BclXS sensitized MCF-7 human breast cancer cells to VP-16- or taxol-induced apoptosis²⁰⁾ and BclXL antisense sensitized human colon cancer cells to 5-FU²¹⁾. Immunohistochemical analysis revealed that BclX was expressed in 90% of patients with invasive ductal adenocarcinoma, while expression of Bcl-2 and Bax was 23% and 53%, respectively (22). Although the detection of mutation of genes including p53 was tried in pancreatic juice obtained by endoscopic retrograde pancreatography and biopsied pancreatic specimen, it would be difficult to perform these procedures and to obtain definitive results in all patients with pancreatic cancer. Our study suggested that BclX would not be influenced by p53 status and that bclX is a promising target for gene therapy for pancreatic cancer.

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