The role of autophagy in the treatment of pancreatic cancer with gemcitabine and ionizing radiation

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Abstract. Autophagy has recently emerged as a significant mechanism in cancer treatment. Although gemcitabine and/or ionizing radiation are important modalities in the treatment of pancreatic cancer, the contribution of autophagy in such treatment has not been fully elucidated. This study investigated the role of autophagy in the treatment of pancreatic cancer with gemcitabine and ionizing radiation. To evaluate the effect of gemcitabine and/or ionizing radiation on autophagy, several human pancreatic cancer cell lines were used. The treatment of pancreatic cancer cell cultures in vitro and in vivo with gemcitabine and ionizing radiation resulted in synergistic cytotoxicity. After treatment with gemcitabine, the autophagy-related protein light chain 3-II (LC3-II) was upregulated. When gemcitabine was combined with ionizing radiation treatment, LC3-II upregulation was enhanced. In addition, electron microscopy of pancreatic cancer cells treated with gemcitabine and/or ionizing radiation detected the induction of autophagy. The blockage of autophagy by 3-methyladenine indicated that autophagy contributed to cell death after gemcitabine treatment and enhanced its cytotoxicity. The inhibitory effect and immune reactivity of the autophagy-related proteins LC3 and beclin-1 were the strongest after the combination treatment. In conclusion, these results suggest that autophagy can be activated by gemcitabine and/or ionizing radiation in the treatment of pancreatic cancer cells and that activated autophagy plays a role in cancer suppression. These findings may have important implications for future strategies involving GEM and/or IR.

Materials and methods

Pancreatic carcinoma cell lines and cell culture. Human PC cell lines PANC-1, AsPC1, and MIAPaCa-2 were obtained from the American Type Culture Collection (Rockville, MD, USA). PANC-1 and AsPC1 were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), and MIAPaCa-2 was maintained in Dulbecco’s modified Eagle’s medium (Gibco Invitrogen, Carlsbad, CA, USA). Both media were supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Specimens were kept at 37°C in a humidified 5% CO₂ incubator.

Introduction

Pancreatic cancer (PC) is an intractable tumor due to its aggressive local invasion and early metastasis. Despite the progress in diagnostic imaging, it is still difficult to detect PC at an early stage (1). Only 20-30% of PC cases can be treated by surgery, and the prognosis after a single surgical treatment is poor, even after a curative resection. Therefore, chemotherapy (gemcitabine; GEM) and radiotherapy (ionizing radiation; IR) are important modalities for the treatment of PC (2-6).

Autophagy, a type of non-apoptotic cell death, is characterized by sequestration of bulk cytoplasm and organelles in autophagic vesicles (also named autophagosomes) that are fused with lysosomes to generate autolysosomes and are degraded by the cells’ own lysosomal system (7). Autophagy plays essential roles in cell survival, growth and development, and tumorigenesis (8-10). Such cell death has profound effects on cancer progression or suppression.

In the present study, we evaluated the contribution of GEM and/or IR-induced autophagy in the treatment of PC by using cells from various human PC cell lines. Clarifying the role of autophagy in the treatment of PC may have important implications for future strategies involving GEM and/or IR.

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MIAPaCa-2 cells (1x10^5/well) were seeded in a 10-cm dish, at concentrations of 0 nM, IC50 nM, and double IC50 nM for 24 h and/or IR. To evaluate GEM as a potential radiosensitizer, forming assay was performed to assess the effect of GEM using a microplate reader (Bio-Rad, Tokyo, Japan). A colony-were also examined. Absorbance at 570 nm was measured by a DNA fragmentation assay using the APO-Direct™ Detection of apoptosis.

Western blot analysis. Equal numbers of control or experimental cells (PANC-1 and MIAPaCa-2) treated with IC50 nM of GEM for 24 h and/or 5 Gy of IR (2.5 Gy/min) were lysed using a lysis buffer consisting of Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 50 mM sodium fluoride, 1.0 g/ml leupeptin, 1.0 g/ml aprotinin, 1.0 g/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Equivalent amounts of total protein (20 μg) were separated by 16% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Portran; Schleicher and Schuell, Dassel, Germany). The membranes were blocked in 5% non-fat milk for 30 min at room temperature, washed 3 times, and then incubated with anti-LC3-I/II or β-actin antibodies overnight at 4°C. LC3-II is localized in autophagosome membranes during amino acid starvation-induced autophagy. Therefore, the amount of LC3-II present in the autophagosome membrane can be indicative of the extent of autophagosome formation (13). The primary antibodies were diluted 1:1000 and detected using the appropriate horseradish peroxidase-conjugated secondary antibodies. Immune complexes were detected by chemiluminescence and then by fluorescence using a LAS3000 mini lumino image-analyzer (Fujifilm, Tokyo, Japan). The density of each band was measured numerically using Multi Gauge Version 3.0 (Fujifilm), and the obtained findings were graphed in a bar chart (LC3-II/β-actin).

Detection of apoptosis. The induction of apoptosis was evaluated by a DNA fragmentation assay using the APO-Direct™ Assay Staining kit (BD Bioscience Pharmingen, San Diego, CA, USA). The APO-Direct assay is a single-step method for labeling DNA breaks with fluorescein isothiocyanate-deoxyuridine triphosphate (FITC-dUTP) followed by analysis using flow cytometry (FACS Calibur; Becton-Dickinson, Franklin Lakes, NJ, USA). This method is often used to detect fragmented DNA, and it uses a reaction catalyzed by exogenous TdT, often referred to as ‘end-labeling’ or ‘terminal deoxynucleotidyl transferase dUTP nicking and labeling’ (TUNEL). We used PANC-1 cells and MIAPaCa-2 cells treated with IC50 nM of GEM for 24 h and/or 5 Gy of IR (2.5 Gy/min) combined with or without 3-MA (1 mM for 1 h). Cultured cells were harvested by trypsinization and washed with phosphate buffered saline (PBS). Washed cells were fixed with 1% paraformaldehyde for 15 min and then with 70% ethanol overnight at -20°C. Fixed cells were combined with a DNA labeling solution containing conjugated FITC for 30 min. The stained cells were resuspended in PBS, and flow cytometry data were analyzed with CellQuest software (Becton-Dickinson). We measured the cells that were apoptotic as a percentage of the total number of cells using the CellQuest software package.

Electron microscopic analysis. The control and the GEM-and/or IR-treated cells were fixed with 2% glutaraldehyde, 4% paraformaldehyde, and 1% tannic acid in 0.1 mol/l cacodylate buffer (pH 7.4) for 24 h. After thio-carbohydrazide-osmium staining, the cells were dehydrated in a graded ethanol series and then immersed serially in 1:1 hexamethyldisilazane and absolute ethanol. Thin-cut sections and the gels were coated with 500-Å of gold in a JEOL Vacuum sputter coater; they were then viewed using a T300 electron microscope with a scanning attachment (JEOL, Tokyo, Japan). By using the images obtained by electron microscopy, we identified cells having 10 or more autophagic vacuoles in the cell cytoplasm as undergoing ‘cell-induced autophagy’. Ten slices at the same magnification were selected at random, and the number of cells in each group was graphed.

In vivo tumor growth assay. Male BALB/c nude mice aged 4-6 weeks (CLEA, Tokyo, Japan) were used. Equal numbers (2x10^6) of MIAPaCa-2 cells were injected subcutaneously into the hind limbs of the mice to form a tumor xenograft; GEM was then injected intraperitoneally. For tumor irradiation, the mice were immobilized in a customized harness that exposed the hind limbs of the mice to form a tumor xenograft; GEM was then injected intraperitoneally. For tumor irradiation, the mice were immobilized in a customized harness that exposed the hind leg while shielding the remainder of the body with 100 cm² of lead. Each mouse was then exposed to a single dose of 5 Gy (2.5 Gy/min). The mice were then randomized into 4 groups: sham, GEM alone (300 mg/kg/week), IR alone, and GEM combined with IR (GEM + IR). Each group consisted of 6 mice. The tumor volumes were measured, and the volume was calculated twice weekly using the formula L x W^2/2, where L is the longest diameter and W is the shortest diameter of the tumor, to assess the antitumor effect of GEM or IR. The 6 mice in each group were maintained in order to observe survival times, and Kaplan-Meier survival curves were constructed.

Immunohistochemical analysis. The excised tumors were fixed in 10% formalin and then embedded in paraffin. Sections (4 μm
thick) were stained with anti-LC3 or anti-beclin-1 rabbit polyclonal antiserum (1:50) and incubated with blocking buffer for 40 min at room temperature, followed by incubation with the primary antibodies overnight at 4°C. After incubation with the appropriate concentrations of secondary antibody, antibodies were detected in the sections using the ImmunoCruz System (Santa Cruz Biotechnology). The sections were then counterstained with hematoxylin. By using images obtained by light microscopy, we counted the number of cells stained for either LC3 or beclin-1 per 100 cells in 10 slices that had been selected at random. The number of cells for each group was then graphed.

Table I. *In vitro* cytotoxicity of gemcitabine (IC$_{50}$) against pancreatic cancer cell lines by MTT assay.

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>PANC-1 IC$_{50}$ (nM)</th>
<th>MIAPaCa-2 IC$_{50}$ (nM)</th>
<th>AsPC1 IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40±5.2</td>
<td>65±4.4</td>
<td>38±3.2</td>
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<td>3</td>
<td>31±4.3</td>
<td>58±4.1</td>
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<tr>
<td>5</td>
<td>20±5.5</td>
<td>35±4.1</td>
<td>27±2.9</td>
</tr>
<tr>
<td>7</td>
<td>13±3.3</td>
<td>28±4.4</td>
<td>24±5.2</td>
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</table>

IC$_{50}$ is shown as nM and the mean ± SEM of examined samples.

Figure 1. Synergistic effects of GEM and ionizing radiation (IR). (A) PANC-1: (●) IR alone; (○) IR + GEM 40 nM; (■) IR + GEM 80 nM. (B) MIAPaCa-2: (●) IR alone; (○) IR + GEM 65 nM; (■) IR + GEM 130 nM. IR dose was 0, 2, 4, 6, and 8 Gy. Error bars were calculated from 12 independent experiments. P<0.01 were considered to be statistically significant; GEM-free line vs. either the GEM (IC$_{50}$) line (*) or the GEM (double IC$_{50}$) line (*).

Figure 2. Detection of apoptosis by GEM, IR, and GEM + IR combined with or without 3-methyladenine (3-MA: 1 mM x 1 h) in PC cells. The IC$_{50}$ concentration of GEM was administered for 24 h, and 5 Gy of IR was administered at the rate of 2.5 Gy/min for 2 min. P<0.01 were considered to be statistically significant; control group vs. either the GEM group (*) or IR group (*), and the GEM group vs. the GEM + IR group (*).

Statistical analysis. Statistical analysis was performed using the GraphPad Prism software program (GraphPad Software, La Jolla, CA, USA) and the StatView Version 5.0 statistical software package (Abacus Concepts, Berkeley, CA, USA). Comparisons were made using two-tailed Student's t-test, and a significant difference was defined as P<0.05. Data are shown as the mean ± standard deviation (SD). The $\chi^2$ test for two-by-two tables was used to compare categorical data. The log-rank test was used to assess the statistical significance of the *in vitro* and *in vivo* analyses. The overall survival rates were calculated using the Kaplan-Meier methods. Statistical significance of differences in survival rates were examined using log-rank test for univariate analysis.

Results

*GEM-induced cytotoxicity and the synergistic effect of IR and cell growth curves.* We examined the cytotoxic effects of GEM at various incubation times (1, 3, 5 and 7 days) on the
PANC-1, AsPC1, and MIAPaCa-2 PC cell lines using an MTT assay. After a 24-h incubation period, the 3 PC cell lines showed similar levels of GEM-induced cytotoxicity (IC50 concentrations 38-65 nM). This trend continued as incubation periods were lengthened, with enhancement of GEM-induced cytotoxicity occurring in a time-dependent manner (Table I). We performed a colony-forming assay to determine the radio-sensitizing potential of GEM. The surviving fractions were described using a linear-quadratic equation. Decreasing graph lines proved that sensitization was demonstrated. In comparison to the GEM-free group, both the GEM (IC50) group line (P<0.01) and the GEM (double IC50) group line (P<0.01) decreased significantly in PANC-1 and MIAPaCa-2 cells. Therefore, synergistic effects were observed in the colony-forming assay when GEM was combined with IR (Fig. 1).

Induction of apoptosis by GEM and/or IR with or without 3-MA and determination of the GEM-induced apoptotic index. The induction of apoptosis was also assessed in the PANC-1 and MIAPaCa-2 PC cell lines (Fig. 2). As the graph shows, the inhibition of autophagy by 3-MA did not influence the proportion of apoptotic cells in these groups. A single treatment with GEM or IR increased the apoptotic cell population in PANC-1 (GEM, 3.2% and IR, 2.9%) and MIAPaCa-2 cells (GEM, 6.4% and IR, 1.3%). Combined GEM + IR treatment increased the proportion of apoptotic cells significantly more than GEM or IR treatment alone in both PANC-1 (18.3%, P<0.01) and MIAPaCa-2 cells (13.7%, P<0.01).
Detection of autophagy in the treatment with GEM and/or IR. The expression of LC3-I (18 kDa) and LC3-II (16 kDa) was examined in PANC-1 and MIAPaCa-2 cells treated with GEM and/or IR (Fig. 3). In comparison to the cells treated with GEM or IR alone, combined treatment in both PANC-1 and MIAPaCa-2 cells induced significantly higher levels of LC3-II (P<0.01; LC3-II/β-actin).

Inhibition of autophagy by the specific inhibitor 3-MA. As shown in Fig. 4, cell growth curves showed that PANC-1 and MIAPaCa-2 PC cell lines had reduced tumor growth following treatment with 3-MA at a concentration of 10 mM x 1 h compared to those treated with concentrations of 0 μM, 100 μM x 1 h, and 1 mM x 1 h. Therefore, in the MTT assay experiments, the 3-MA concentration used was 1 mM x 1 h since 3-MA treatment was not toxic at this concentration. The effect of 3-MA on GEM-induced cytotoxicity was examined in vitro (Fig. 5). In the PANC-1 and MIAPaCa-2 PC cell lines, 3-MA inhibited GEM-induced cytotoxicity following 1, 3, 5 and 7 days of incubation. The 3-MA-treated cells required significantly higher concentrations of GEM to suppress cell proliferation than did the untreated cells (P<0.05).

Detection of autophagy by electron microscopic analysis. Induction of autophagy was evaluated by electron microscopy. Mitochondrial disruption was observed in autophagic vacuoles in PANC-1 cells treated with the GEM, IR, and the combination of GEM + IR (Fig. 6A). Compared to the cells treated with a single treatment of GEM or IR, the number of autophagic vacuoles increased significantly in the cells treated with GEM + IR. The graph shows the cells counts of those with 10 or more autophagic vacuoles in the cytoplasm for each group. *P<0.01.

Figure 6. (A) Induction of autophagy evaluated by electron microscopy. Electron microscopy images show autophagic vacuoles in PANC-1 cells. (A) Control (a), GEM (b; IC50 nM for 24 h), IR (c; 5 Gy exposed at 2.5 Gy/min), and GEM + IR (d; GEM; IC50 nM for 24 h and IR 5 Gy exposed at 2.5 Gy/min). (B) Frequencies of cells with autophagy induced by GEM, IR, and GEM + IR in PANC-1 cells. The graph shows the cells counts of those with 10 or more autophagic vacuoles in the cytoplasm for each group. *P<0.01.

Figure 7. Enhancement of gemcitabine (GEM) antitumor effects by ionizing radiation (IR) on the growth of MIAPaCa-2 human PC xenografts in vivo. Male BALB/c nude mice aged 4-6 weeks were randomized into 4 groups: sham, GEM alone, IR alone, and GEM combined with IR (GEM + IR). Each group consisted of 6 mice. According to the protocol (A), the mice were exposed to a single dose of 5 Gy and 300 mg/kg/week; GEM was administered intraperitoneally. (B) The general appearance of representative mice bearing MIAPaCa-2 xenografts after 35 days. (C) The tumor volume curves on the growth of MIAPaCa-2 xenografts. Individual mice were monitored for tumor growth over a period of 35 days. The volume for individual tumors was calculated and each curve represents the mean tumor volume calculated for each group (n=6). Standard errors of the mean are represented by error bars. (D) The survival curve was plotted according to the methods of Kaplan-Meier (n=6). *P<0.05 were considered to be statistically significant for sham vs. either the GEM group or the IR group. **P<0.05 were considered to be statistically significant for GEM + IR vs. either GEM or IR alone.
autophagic cells was significantly greater when treated in combination with GEM + IR, suggesting that GEM combined with IR induces autophagy synergistically (Fig. 6B).

**Growth inhibitory effects of GEM and/or IR in PC xenografts.** The effect of IR combined with GEM on tumor growth was further investigated with an in vivo MIAPaCa-2 mouse model. All animal experiments were performed strictly according to the institutional ethics guidelines. GEM was injected intraperitoneally into the mice, and IR was locally administered to the tumor (Fig. 7A). Representative results from each mouse group are shown in Fig. 7B. Xenograft growth speed was moderately reduced when the mice were treated with either IR or GEM alone. In contrast, the tumor
size significantly decreased after combined GEM + IR treatment (P<0.05; Fig. 7C). As shown in Fig. 7D, the median survival time of mice was 53 days in the sham group, whereas it was significantly increased to 107, 119, and 153 days in the GEM (P=0.0034), IR (P=0.0025), and GEM + IR (P<0.0001) groups, respectively. Survival time in the GEM + IR group was prolonged by 289% when compared with the sham group.

**Immunohistochemical analyses of autophagy-related proteins by each treatment.** Immunohistochemical analysis of LC3 and beclin-1 was performed using tumors obtained after each treatment (Fig. 8A). In comparison to the cells treated with GEM or IR alone, a synergistic effect was observed when GEM was combined with IR (P<0.01). Quantitative evaluation by counting positive cells showed that the synergistic effects were induced by the combination, as shown in Fig. 8B.

**Discussion**

It is generally agreed that autophagic activity contributes to the development of cancers. However, there is continuing controversy as to whether induced autophagy results in cancer suppression or progression. Kondo et al noted that there is no consensus as to whether autophagy is a cause of cell death or whether autophagy occurs in an effort to avoid cell death (14). Yang et al reported that autophagy has different effects on cell survival depending on the stage of cell death (15). Ogier-Denis et al suggested that suppression of autophagy may contribute to the initial rapid growth of tumors, however, in more advanced stages of cancer, autophagy may be required to provide essential nutrients to the cells in the inner part of solid tumors that do not have direct access to circulation (16). Therefore, some of the recently suggested strategies for cancer treatment include inducing autophagy in early developed cancers while inhibiting autophagy in advanced tumor cells with intact autophagy responses to sensitize the cells to a variety of anti-cancer agents (10,17).

Some studies have shown that activated autophagy is a tumor suppressor. The overexpression of beclin-1, a mammalian autophagy protein, induces autophagy and contributes to cancer suppression in various cancer cells (8,18,19,20). Decreased expression of autophagy proteins may contribute to the development or progression of breast and other human malignancies (19). Studies of beclin-1 specifically, provided genetic evidence that autophagy is a novel mechanism of cell-growth control and tumor suppression (20). Additionally, Scarfatti et al reported that tamoxifen causes cell death by autophagy in mammary tumor cells and that the inhibition of autophagy by 3-MA decreased cell death (21). In the present study, we examined the contribution of autophagy in PC cells treated with GEM by applying 3-MA. As shown Fig. 5, the 3-MA-use group required a significantly higher concentration of GEM to suppress PC cells compared with the 3-MA-non-use group. In addition, as shown in Fig. 6, electron microscopy confirmed the presence of autophagy vacuoles, which disrupt mitochondria, within the cytoplasm in PC cells in the presence of GEM, IR and the combination of the two. Taken together, these results clearly indicate that GEM induces autophagy and that induced autophagy acts on cancer suppression.

In contrast, some reports concluded that autophagy acts on cancer progression. Li et al reported that SFU-induced apoptosis in colon cancer cells can be enhanced by the inhibitor of autophagy (3-MA) and autophagy may act as a self-defense mechanism in SFU-treated colon cancer cells (22). Sato et al reported that both the autolysosome inhibitors and 3-MA induced marked apoptotic death of all colorectal cancer cells examined, suggesting that autophagy directly contributes to the survival of cancer cells under nutrient starvation (23). In both reports, however, larger doses of 3-MA were applied for longer exposure times compared to those used in our study (Li et al, 5 mM x 36 h, and Sato et al, 10 mM x 12 h, vs. our study, 1 mM x 1 h). The apoptotic death reported in these studies may be cytotoxicity caused by high concentrations of 3-MA. As shown in Fig. 4, our results indicated that 3-MA concentrations greater than 1 mM x 1 h were cytotoxic to both PANC-1 and MiaPaCa-2 cells. At a 1 mM concentration, our results showed that 3-MA could inhibit autophagy and decrease the antitumor effect without inducing apoptosis.

Only a few attempts have been made so far to study the phenomena of autophagy and apoptosis induced by the combination of chemotherapy and radiotherapy. IR alone induced autophagy for breast, prostate, and colon cancer (24). Similarly, IR inhibited cell proliferation and increases autophagy activity in various glioblastoma cells (25). We have examined the phenomena of autophagy and apoptosis with the combined treatment of GEM and IR on PC cells. The expression of LC3-II was high in the GEM and IR groups and was even higher in the combination group in comparison to the control group (Fig. 3). Immunohistochemical assessment of the tumor grafts showed that, while the expression of LC3 and beclin-1 was remarkably enhanced in the GEM and IR groups, it was further enhanced in the combination group (Fig. 8). The proportion of apoptotic cells also increased synergistically following treatment with GEM and IR in combination compared to the use of GEM or IR alone (Fig. 2).

In conclusion, autophagy is induced by the administration of GEM and/or IR to PC cells and that induced autophagy acts to suppress cancer. Further studies are needed to analyze the molecular mechanism of autophagy, and may lead to the development of new treatment strategies for the use of GEM and IR for PC.

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