**Loss of Mitochondrial DNA by Gemcitabine Triggers Mitophagy and Cell Death**

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Gemcitabine (2,2-difluorodeoxycytidine nucleic acid), an anticancer drug exhibiting a potent ability to kill cancer cells, is a frontline chemotherapy drug. Although some chemotherapeutic medicines are known to induce nuclear DNA damage, no investigation into mitochondrial DNA (mtDNA) damage currently exists. When we treated insulinoma pancreatic β-cells (line INS-1) with high mitochondrial activity with gemcitabine for 24h, the mtDNA contents were decreased. Gemcitabine induced a decrease in the number of mitochondria and the average potential of mitochondrial membrane in the cell but increased the superoxide anion radical levels. We observed that treatment with gemcitabine to induce cell death accompanied by autophagy-related protein markers, Atg5 and Atg7; these were significantly prevented by the autophagy inhibitors. The localization of Atg5 co-occurred with the location of mitochondria with membranes having high potential and mitophagy in cells treated with gemcitabine. The occurrence of mitophagy was inhibited by the inhibitors of the phosphatidylinositol 3-kinase/Akt pathway. Our results led us to the conclusion that gemcitabine induced cell death through mitophagy with the loss of mtDNA. These findings may provide a rationale for the combination of mtDNA damage with mitophagy in future clinical applications for cancer cells.

**Key words** gemcitabine; mitochondrial DNA; mitophagy; cell death; mitochondrial membrane potential

**INTRODUCTION**

Gemcitabine, known as 2,2-difluorodeoxycytidine nucleic acid, is an anticancer drug used to treat a wide range of tumors, such as pancreatic ductal adenocarcinoma and breast cancer, by inhibiting the extension reaction of DNA strands in the cell nucleus.1–4) Nuclear DNA (nDNA) damage initiates the generation of apoptosis, one of the fundamental forms of cell death.5) Gemcitabine induces the inhibition of ribonucleotide reductase, thereby depleting the cellular pool of deoxyribonucleotides; it is also metabolized by deoxycytidine kinase into gemcitabine–triphasate, which is subsequently incorporated into the growing DNA strand, resulting in the termination of the extension reaction.6,7) Gemcitabine may consequently lead to cell death. Although some chemotherapeutics are known to induce nDNA damage directly or indirectly, few investigations address mitochondrial DNA (mtDNA) damage.

In most eukaryotes, mitochondria are organelles in which oxidative phosphorylation couples the electron transfer system to ATP synthesis through a proton gradient to regulate energy homeostasis. Pathological stimuli such as mtDNA damage, hypoxia, and oxidative stress can disturb the normal mitochondrial functions, potentially leading to energy deficiency and the modulation of nutrient metabolism.8) In response to the blood glucose level, mitochondria can synthesize ATP for the release of insulin in pancreatic β-cells. In addition, mitochondria act as the central executioners of cell death.9,10) Therefore, to avoid the disruption of cell function, there is an absolute requirement for both the quality and the quantity of mitochondria to be high. From an alternative point of view, eukaryotic mitochondria contain a circular structure of 16569 base pairs encoding 2 ribosomal RNAs, 22 tRNAs, 13 genes for electron transport subunits, and at least two hormone-like peptides.11,12)

Since all 13 proteins are components of the electron transfer system, mtDNA damage can induce cell death resulting from dysfunction of energy synthesis. Mitochondrial damage is also known to be involved in the early phase of apoptosis and to control its occurrence.13) Previous studies have demonstrated that mitochondrial damage, through the retrograde signaling activation of the apoptosis process, caused resistance to apoptosis.14) We, therefore, undertook the present study to assess the participation of the dysfunction of mitochondria with mtDNA damage in cell death induced by gemcitabine.

Damage to mtDNA in only a few genes easily induces serious dysfunctions and is likely to contribute to some human diseases.15,16) The onset of cancer, neurodegenerative disorder, diabetes mellitus, and aging has all been associated with mutations of mtDNA.17–19) The depletion of mtDNA can also impair mitochondrial function, leading to a diseased state and cell death.20) Such a reduction of mtDNA may result from treatment with drugs, such as Zidovudine and 2,4-dioxycytidine, both of which are nucleoside reverse transcriptase inhibitors used as antiviral agents.21,22) To address this assumption, we aimed to determine the participation of mtDNA loss in cell death induced by gemcitabine.

Here, we used insulinoma pancreatic β-cells (INS-1) cells retaining an ability to release glucose-stimulated insulin similar to that of native β-cells by X-ray-induced rat transplantable insulinoma.23) In pancreatic β-cells containing constitutively activated mitochondria, mitochondrial metabolism is tightly coupled with insulin secretion evoked by ATP production-enhancing mitochondrial metabolism.24–26) In addition, in a patient with advanced pancreatic cancer, treatment with gemcitabine may cause an increase in urine glucose concentration.27) It is, therefore, reasonable to use INS-1 cells containing constitutively activated mitochondria for the elucidation of...
gemcitabine-induced mitochondrial damage.

The breakage of DNA strands is among the stimuli initiating apoptotic cell death.\(^{28}\) We previously reported gemcitabine to promote apoptotic cell death in INS-1 cells through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway for cell proliferation.\(^{29}\) PI3K-activated serine/threonine kinase Akt is upstream of mammalian target of rapamycin (mTOR), when phosphorylated can inhibit the downstream target of pro-autophagy signaling, ULK1.\(^{30,31}\) This suggests that the activation of the PI3K/Akt pathway abrogated the ability of autophagy. Autophagy has recently been proposed as a participant in the promotion of the death of tumor cells or resistance to chemotherapy.\(^{32,33}\) Autophagy is a conserved lysosomal degradation pathway that degrades and recycles damaged organelles and aggregated or misfolded proteins. Therefore, autophagy is practically a common occurrence at low levels in normal cells. Though starvation- and rapamycin-induced autophagy is non-selective, the dysfunction of mitochondria can induce mitophagy, a form of selective autophagy, to maintain the quality of mitochondria. Few studies into the participation of autophagy and mitochondrial damage in mammalian cells treated with gemcitabine currently exist.

Although numerous studies have demonstrated an association between mtDNA damage and cell death in cancer cells, few have demonstrated the involvement of mtDNA damage and autophagy in the death of cells treated with gemcitabine. We undertook the present study to assess the participation of mitochondrial dysfunction in gemcitabine-induced cell death and that of low mtDNA content in the occurrence of autophagy. Here, we aim to evaluate whether the loss of mtDNA evokes cell death.

MATERIALS AND METHODS

Materials  We used the following chemicals: gemcitabine hydrochloride (LKT laboratories, St. Paul, MN, U.S.A.), Cyto-ID® Autophagy Detection Kit (Enzo Life Science, Farmingdale, NY, U.S.A.), RPMI-1640 MEDIUM Modified, spautin-I, sodium pyruvate, SYBR® green I and albumin bovine serum radioimmunoassay Grade Fraction V (Sigma-Aldrich, St. Louis, MO, U.S.A.), fetal bovine serum (FPS, MB Biomedicals, LLC, Santa Ana, CA, U.S.A.), 0.25% Trypsin–ethylenediaminetetraacetic acid 1X (Gibco-Invitrogen Corp., U.K.), penicillin/streptomycin MitoTracker® Red CMXRos, FM MitoTracker® Green FM, and Pierce® BCA Protein Assay Kit \(K_i\) (Thermo Fisher Scientific, Waltham, MA, U.S.A.), phosphat buffered salts (PBS, TaKaRa Bio Inc., Shiga, Japan), MitoSOX® Red mitochondrial superoxide indicator (Invitrogen Life Technologies Corporation, Eugene, OR, U.S.A.), Hoechst 33258 solution and Hoechst 33342 solution (Dojindo Laboratories, Kumamoto, Japan), RIPA buffer, 2-mercaptoethanol, chloroquine (CQ), 3-methyladenine (3-MA), (+)-wortmannin and LY294002 (Wako, Osaka, Japan), MitoSOX® Green I (MitoTracker®) and a dye solution of MTT (7.5 mg) for 2h and then in a solubilization/stop solution (50 µL) for an additional 2h. We recorded the absorbance at 570 nm (formation of formazan) using a microplate reader (Bio-Rad, Tokyo, Japan). We calculated the percentage survival fraction as follows: Survival fraction (%) = amount of a sample/amount of a control ×100.

Determination of mtDNA  After treating the INS-1 cells with gemcitabine for 24h, we detached them from the plates using trypsin and rinsed them twice with PBS. We homogenized the cells resuspended in 250 µL of the homogenizing buffer with a homogenizer (BIOUPTOR® UCD-300, COSMO BIO, Tokyo, Japan) at 20kHz, 300W, and 30s three times in cold water. We centrifuged the homogenate at 1000×g at 4°C for 1 min, transferred the supernatant, and then removed the resultant supernatant from the extranuclear site to another microtube by papering. We determined the protein content in the supernatant of the extranuclear site using the Pierce® BCA protein assay kit and used it to normalize the mtDNA content. After these processes, we extracted mtDNA according to the manufacturer’s instructions. We stained the mtDNA with SYBR Green I at a 1:10000 dilution for 2 h and then in a solubilization/stop solution (50 µL) for an additional 2h. We recorded the absorbance at 485/535 nm using a SPECTRAFLUOR PLUS (TECAN, Zürich, Switzerland).

We selectively visualized mtDNA using SYBR® green I fluorescent staining, which is 50- to 100-fold more sensitive to DNA than other reagents, such as Hoechst 33258 and Midori green.\(^{30}\) Using this procedure, we confirmed, using a confocal laser scanning microscope, that mtDNA existed in the extranuclear region and coincided with mitochondrial localization.

Total DNA in INS-1 cells treated with or without gem-
citabine for 24h was isolated using NucleoSpin Tissue (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the manufacturer’s protocol. The quantity of mtDNA was corrected by simultaneous measurement of a single-copy nDNA. The primers were specific, respectively, for the rat mitochondrial-encoded gene cytochrome oxidase II, and for nuclear-encoded gene β-actin as previously described by Cioffi et al. 33) The primer sequences used were as follows: Cytochrome oxidase II 5′-TGGACGCCATCCTTTACATGG3′ (sense) and 5′-TGGAGCCGCAATTTCAGAG3′ (anti-sense). β-Actin, 5′-CTGCTTTTCCAGATGAGG3′ (sense) and 5′-CCA CAG CACTGTAGGGTTT3′ (anti-sense). Mitochondrial DNA copy number was measured using quantitative real time PCR (qRT-PCR). RT-PCR amplification reactions were performed on 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). Each sample was analyzed in triplicate in 25 μL of final volume containing: 0.12 μg total DNA, TB Green™ Premix EX Taq II (TaKaRa Bio Inc., Shiga, Japan) and 80 nM of specific gene primers. After 30 s of denaturation at 95°C, amplification proceeded for 40 cycles, each consisting of denaturation at 95°C for 5 s, annealing and extension at 58°C for 34 s. In our study, the threshold cycle (Ct) values of mtDNA and nDNA were obtained for each of the two target genes. We determined the difference in the Ct values for cytochrome oxidase II/β-actin pair and find 2ΔCt as mtDNA copy number (the copy number of mtDNA = 2ΔCt for β-actin-Ct for cytochrome oxidase II).

**Autophagy and Mitophagy** After treating the INS-1 cells with gemcitabine for 24 or 48 h, we stained them with Hoechst 33342 to detect nuclei at 1:1000 dilution for 30 min in the dark. We determined autophagy activation by analyzing the fluorescent point formation to the Cyto®-ID detection reagent, using a confocal laser scanning microscope. Other conditions were the same as described above. We imaged the prepared cells at λex/λem: 405/420–460 nm for detection of nuclei by Hoechst 33258 and at λex/λem: 488/518 nm for Atg5 or Atg7 by immunostaining using a confocal laser scanning microscope and then analyzed them at λex/λem: 473/512.5 nm for Atg5 or Atg7 using a Cytell Cell Imaging System to quantize fluorescence intensity.

**Detection of mΔψ and Mitochondrial Number** We used MitoTracker® Red and MitoTracker® Green to detect mΔψ and the number of mitochondria, respectively. After treating the INS-1 cells with gemcitabine for 24 or 48 h, we stained them with Hoechst 33342 to detect nuclei with the MitoTracker® Red or MitoTracker® Green for 15 min. We imaged the prepared cells at λex/λem: 405/420–460 nm for nuclei detected by Hoechst 33342, at λex/λem: 488/518 nm for the number of mitochondria by the MitoTracker® Green, and at λex/λem: 473/512.5 nm for the mΔψ by MitoTracker® Red using a confocal laser scanning microscope and then analyzed them at λex/λem: 390/430 nm for detection of nuclei, at λex/λem: 473/512.5 nm for the number of mitochondria, and at λex/λem: 544/588 nm for mΔψ using the Cytell Cell Imaging System to quantize fluorescence.

**Western Blot Assay** To determine the expression level of target proteins, Western blotting was performed. Briefly, cells (2 × 10⁶) were scraped off from the 6 wells plate and lysed with RIPA buffer containing a cocktail protease inhibitor. After measuring protein concentration using Pierce® BCA Protein Assay Kit, cell lysate was added to the protein sample buffer and was boiled at 100°C for 20 min. An equal amount of protein (20 μg) was loaded in each lane. Protein samples were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride blotting membrane (ATTO CORPORATION). After blocking with EzBlock Chemi for 1 h, it was incubated with primary antibodies at 4°C overnight. Then it was incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The protein bands were visualized by using ECL Western blotting Detection Reagents with Ez-Capture MG (ATTO CORPORATION). Band intensity was measured by using CS Analyzer (ATTO CORPORATION). The primary antibody used in this study was as follows: ATG5 Polyclonal ANTIBODY (1:1000), Anti-ATG7 pAb (1:1000), and anti-β-actin antibody (proteintek) (1:10000). The corresponding secondary antibody was Anti-RABBIT IgG (H&L) (GOAT) Antibody Peroxidase Conjugated (1:5000) for anti-ATG5 and anti-ATG7 and Anti-Mouse IgG, HRP-Linked Whole Ab Sheep (1:5000) for β-actin.

**Statistical Analysis** Data are represented as the mean ± standard deviation (S.D.) from at least three independent experiments. Student’s t-test was used for unpaired comparisons. ANOVA followed by Dunnett’s test was used to determine differences among three groups. We considered p < 0.05 to be statistically significant.
RESULTS

Loss of Mitochondrial DNA by Gemcitabine  We conducted the first analysis to determine whether gemcitabine can decrease the mtDNA content. We observed cells labeled “SYBR® green I” for the determination of DNA in combination with MitoTracker® Red indicator for mitochondrial staining, using a confocal laser scanning microscope. In Fig. 1A, we illustrate the strong green fluorescence signal for nuclear and extranuclear DNA and the red fluorescence signal for mitochondria observed in the control INS-1 cells untreated with gemcitabine. As indicated by arrow heads, the yellow area of the merged image represents the co-localization of the DNA probe and the mitochondrial probe. When we treated INS-1 cells with gemcitabine 500 nM for 24 h, it appeared that the fluorescence intensity of the DNA probe in the yellow area was decreased in comparison with that of the control cells. We analyzed the intensity profiles of the green and the red fluorescence signals with 3D-image stacks and determined that the green fluorescence signal for mtDNA was co-localized with the red signal for mitochondria (Fig. 1B). Concurrently, as we illustrate in Fig. 1C, the content of mtDNA extracted using the mtDNA extraction kit was significantly decreased in cells treated with gemcitabine (to approximately 35% of that extracted from the control cells). The extracted sample contained very few nDNA (β-actin gene) detected by qRT-PCR as compared to mtDNA (data not provided). Quantitative PCR was

Fig. 1. Loss of mtDNA by Gemcitabine

INS-1 cells were incubated with or without 500 nM gemcitabine for 24 h. (A) Intracellular DNA and mitochondria were stained with SYBR® green I and MitoTracker® Red, respectively, and observed them using a confocal laser scanning microscope. Co-localization of DNA and mitochondria is indicated by white arrow heads. We present a typical result of experiments; we obtained similar results with at least three different preparations of cells. (B) Intensity profile measured along the white arrow indicated in the merged image in panel A. (C) mtDNA was extracted from the cells using an extraction kit and stained using SYBER green I. Fluorescence intensity was normalized by the protein content in the extranuclear site. Mitochondrial DNA copy number was determined by measuring amplification of the ratio of mtDNA to nDNA using qRT-PCR. Other conditions were the same as described above and in Materials and Methods. Data are expressed as an average of triplicate experiments ± standard deviation (S.D.).
used to calculate the mtDNA copy number (mitochondrial-to-nuclear DNA ratio) in INS-1 cells. It was observed that the mtDNA copy number was significantly decreased in the cells treated with 500 nM gemcitabine for 24 h. Furthermore, nDNA copy number in cells treated with gemcitabine at 500 nM for 24 h (Ct value = 21.33, n = 6) is almost same the copy number in control cells (Ct value = 21.42, n = 10). These results suggest the gemcitabine-induced loss of mtDNA in INS-1 cells.

Mitochondrial Dysfunction To specifically clarify the gemcitabine-induced mitochondrial dysfunction, we determined the mitochondrial membrane potential (mΔψ), ATP contents and reactive oxygen species (ROS) generation in the INS-1 cells treated with gemcitabine. As we illustrate in Fig. 2A, when we treated the cells with gemcitabine at 500 nM for 24 or 48 h, mΔψ was significantly reduced. Used as a positive control, a depolarizing agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) strongly disrupted mΔψ by incubation for 20 min. As we illustrate in Fig. 2B, when we incubated the cells with gemcitabine for 24, 36, and 48 h, the mitochondrial mass was significantly decreased in a time-dependent manner. We illustrate in Fig. 2C that the treatment with gemcitabine for 24 h induced an increase in the red signal by staining MitoSOX® Red for the superoxide anion radical (O_2·−) in mitochondria but did not influence the intensity of the blue signal for nDNA as compared to the control cells not treated with gemcitabine. In Fig. 2D, we illustrate that the O_2·− generation/cell was markedly increased by treatment with gemcitabine in a dose-dependent manner. These results suggest that treatment with gemcitabine induced the dysfunction of mitochondria in INS-1 cells.

Induction of Autophagy and Cell Death We next investigated whether autophagy is involved in gemcitabine-induced cell death. In Fig. 3A, we illustrate a clear increase in the fluorescence intensity of the dye for the formation of autophagosomes and for the expression of Atg5 protein, which is related to the formation of autophagy vacuoles, in cells treated with
gemcitabine, as compared to that of the control cells. In Figs. 3B and 3C, we also illustrate a significant increase in the fluorescence intensity for both Atg5 and Atg7 autophagy-related proteins after treatment with gemcitabine for 24, 36, and 48 h in a time-dependent manner. We examined the protein levels of Atg5 and Atg7 in cell treated with gemcitabine. In Fig. 3D, we observed an increase in the protein level of Atg5 after treatment with 500 nM gemcitabine for 30 h. Similarly, in Fig. 3E, we also observed an increase in the protein level of Atg7. The present data indicate that gemcitabine induced the occurrence of autophagy in INS-1 cells.

To clarify the contribution of autophagy in cell death, we investigated the time course of gemcitabine-induced cell death and the effect of autophagy inhibitors on cell death. In Fig. 4A, we illustrate an increase in cell number observed over 48 h in the control cells but only over 18 h in gemcitabine-treated cells, after which the cell number was reduced over 24–48 h. In Figs. 4B, C and D, we illustrate that the autophagy inhibitors, CQ, 3-MA, and spautin-1 significantly inhibited the reduction of the nuclear number induced by the treatment with gemcitabine, suggesting that gemcitabine-induced autophagy might be related to cell death.

To evaluate the localization of mitochondria and autophagy, we subjected INS-1 cells to double staining by fluorescence reagents for mΔψ and Atg5 (left column) and analyzed these localization (right column) in Fig. 5. We easily observed red fluorescence for mΔψ but observed green fluorescence for Atg5 an index of autophagy only poorly in control cells. We gated the control cells so that about 95% of the total cell population was negative for the occurrence of autophagy. An inducer of autophagy rapamycin induced a strong green fluorescence; however, the observed yellow signal was not significant.

Fig. 4. Effect of Autophagy Inhibitors on Cell Death by Gemcitabine

(A) After adding 250 nM (▲) or 500 nM (●) gemcitabine, INS-1 cells were incubated for the indicated times. The nuclear number in cells stained by Hoechst 33342 was enumerated using the Cytell Cell Imaging System. Data represent the mean ± S.D. of five experiments. *p < 0.05 vs. corresponding control cells. (B–D) Cells were treated with or without 500 nM gemcitabine or the combination of gemcitabine and 50 µM CQ (B), 1 mM 3-MA (C), or 1 µM spautin-1 (D) for 48 h. Other conditions were the same as described above (A). Data represent the mean ± S.D. of 3–5 experiments.
in the merged image. The population of cells belonging to the region of autophagy was approximately 60%, comprising 43.5% in area 1 as indicated low $m\Delta \psi$. At 24 h after the addition of 500 nM gemcitabine, we observed the obvious yellow fluorescence caused by co-localization of mitochondria and autophagy. The population of cells belonging to the region of autophagy was approximately 77%; 38% in area 3, indicating autophagy with high $m\Delta \psi$; and 39% in area 2 with low $m\Delta \psi$. These results indicate that gemcitabine treatment induced autophagy with high $m\Delta \psi$ in INS-1 cells.

We had previously provided direct evidence for the involvement of the PI3K/Akt pathway in gemcitabine-induced cell death. We thus investigated the effect of LY294002 and wortmannin (inhibitors of the PI3K/Akt pathway) on the occurrence of autophagy in Fig. 5. The strong intensity of fluorescence green induced by gemcitabine treatment was significantly inhibited by LY294002 and wortmannin. The population of cells belonging to the region of autophagy was approximately 4.2 and 10.4% in autophagic area (areas 1 and 3) in the presence of LY294002 and wortmannin, respectively. These results suggest that the activation of the PI3K/Akt pathway is involved in the occurrence of autophagy with high and low $m\Delta \psi$. These results suggest the possibility that the activation of PI3K/Akt pathway is involved in the occurrence of autophagy.

Involvement of the PI3K/Akt Pathway in Occurrence of Mitophagy

We next aimed to determine whether gemcitabine induces mitophagy. In Fig. 6, we present images of INS-1 cells that were subjected to double staining by reagents for the detection of mitophagy and lysosomes. We observed a strong intensity of fluorescence points and its co-localization for mitophagy and lysosomes in gemcitabine-treated cells using confocal fluorescence microscopy. However, we scarcely observed mitophagy in the control (Fig. 6) and rapamycin-treated cells (data not provided). The strong intensity of fluorescence points was significantly inhibited by LY294002 and wortmannin. These results suggest that gemcitabine induced...
mitophagy, which can be participated in the activation of the PI3K/Akt pathway inhibitors.

**DISCUSSION**

The present data indicated that the anticancer drug gemcitabine, even at lower concentrations, induced loss of mtDNA, mitophagy, and cell death in INS-1 cells. In our study, we used a concentration of gemcitabine less than half of that circulating in gemcitabine-treated cancer patients and of between approximately 5 and 0.25% of other anticancer drugs such as cisplatin, methotrexate, cyclophosphamide and chloroacetaldehyde, implying that the mechanism of cell death induced by gemcitabine may be different to that of other anticancer drugs. Ewing sarcoma cells may be known to be more sensitive to gemcitabine than other types of cancer cells, suggesting that certain cells with a high mitochondrial activity may have high susceptibility to gemcitabine.

We found that treatment with gemcitabine caused the loss of mtDNA within 24h, followed by the collapse of mΔψ, the production of O₂⁻, and the decrease of mitochondrial mass in cells. However, we confirmed that nDNA contents did not decrease in cells treated with gemcitabine treatment at 24h using qRT-PCR, suggesting that the effect of drug was specific to mtDNA. Since mtDNA encodes the components comprising the electron transfer chain in mitochondria, the loss of mtDNA would lead to the defect of respiratory function with the collapse of mΔψ and the production of ROS. The treatment with gemcitabine resulted in a delayed onset of dysfunction compared to that of direct inhibitors of the respiratory chain, such as rotenone, azide or an uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (data not provided). Gemcitabine depletes the cellular pool of deoxyribonucleotides and inhibits the growing genome DNA strands. The content of mtDNA was modulated by the expression of nDNA genes encoding enzymes of mtDNA synthesis. Although gemcitabine induces the S-phase arrest, it was not observed the loss of mtDNA in INS-1 cells treated with camptothecin which is topoisomerase I inhibitor or with nocodazole which is microtubule inhibitor (data not provided). These findings suggest that gemcitabine inhibits the synthesis of mtDNA rather than having a direct inhibitory effect on the components of the chain or arresting the cell cycle.

When compared with those of the nDNA, the decrease in the mtDNA content and cell death were clearly detected in INS-1 cells treated with gemcitabine. Mitochondria are widely known as the main source of intracellular ROS, and an abnormal respiratory chain is understood to be the mechanism responsible for an increase in ROS generation in mitochondria. Since the loss of mtDNA induces a deficiency of proteins in the electron transfer chain, the degradation of mtDNA would result in the acceleration of ROS generation in mitochondria. Furthermore, mtDNA was found to be highly susceptible to damage by oxidative stress and to be located in proximity to the electron transfer chain, which is the main site of ROS generation. The mtDNA and the transcription factor A abundant in mitochondria are formed in the nucleoid. The number of mitochondrion is regulated through the balance between fission and fusion, so as to retain the quality and quantity of mitochondria in response to the demand in intracellular environments; this results in the frequent synthesis and degradation of mtDNA. These findings explain why gemcitabine easily induced the decrease in mtDNA content, followed by the generation of ROS, which further accelerated the loss of mtDNA in the cells.

Although multiple repair pathways exist for persistent damage of mtDNA and include a base excision repair, which normally repairs 8-oxo-guanin, abasic sites, and single strand breaks, double strand breaks and rapid mtDNA damage remain poorly understood. Our study demonstrated that gemcitabine induced the loss of mtDNA, mitophagy, and cell death. From these findings, we assume the possibility that cells depleted of mtDNA are rapidly eliminated by apoptosis through the occurrence of mitophagy. Concurrent with the occurrence of mitophagy, the specific autophagy with high mΔψ was observed after treatment with gemcitabine, but not with rapamycin. Some studies have demonstrated the relationship between mtDNA damage and cell death. Shokolenko et al. indicated that damaged mtDNA molecules may be degraded if not rapidly repaired. Pickrell et al. demonstrated the transient induction of mtDNA double strand breaks in a hepatocyte-driven cell line stably expressing a mielopine-trux-inducible mitochondrial-targeted endonuclease mito-Scal. Ignatenco et al. demonstrated that the loss of mtDNA led to spongiotic encephalopathy. While the effects of total loss of mtDNA (ρ0 phenotype) on cellular physiology have been relatively well described, the effects and consequences of rapid mtDNA damage remain poorly understood. Yeo et al. demonstrated that sublethal concentrations of gemcitabine in human cancer cell lines did not reduce mtDNA content but caused mitochondrial dysfunctions. In contrast, 2',3'-dideoxyctydine significantly reduced mtDNA content after 6d of exposure, but did not cause cytotoxicity. Rapid declines in the mtDNA copy number are followed by mitochondrial dysfunction, which decreases mitochondrial respiratory activity and contributes to ROS production. Because abnormal mitochondria lead to energy deficiency and are a source of ROS generation, cell survival critically depends on selective removal of dysfunctional mitochondria. From these results, we concluded that mitochondria in which mtDNA have been lost are removed by mitophagy, followed by cell death. Since we were unable to fully characterize the kinetics and the extent of mtDNA loss by gemcitabine, a further study is necessary to clarify the mechanism of mtDNA loss.

In almost every aspect of physiological and pathological cell function, including survival, proliferation, differentiation, apoptosis, and autophagy, the survival signaling PI3K/mTOR/Akt pathway plays a critical role. Gemcitabine is known to easily induce the activation of the PI3K/Akt pathway involved in the resistance to cancer cells. We previously reported that in INS-1 cells treated with gemcitabine, which induced apoptotic death, this pathway was active. The present study indicated that inhibitors of this pathway also inhibited the accumulation of autophagy, the occurrence of mitophagy and cell death in gemcitabine-treated INS-1 cells. Recently, studies of some drugs targeting the autophagy pathway to promote the autophagic death of tumor cells have proposed that apoptosis and autophagy could act as partners to induce cell death in a cooperative manner. Thus, the activation of the PI3K/Akt pathway by gemcitabine might be considered to induce mitophagy, causing the degradation of mitochondria, followed by cell death. Since rapamycin which caused autophagy with low...
Gemcitabine
↓
mtDNA defect
↓
Mitochondrial dysfunction
↓
ROS generation
↓
mtDNA loss
↓
Mitophagy
↓
Cell death

Chart 1. Hypothetical Scheme for mtDNA Loss, Mitophagy, and Cell Death Induced by Gemcitabine

mΔγ did not induce death in INS-1 cells under the present conditions, the autophagy with high mΔγ induced by gemcitabine is likely to be related to mitophagy and to contribute to cell death. However, it was unable to confirm that the effect of on PI3K/Akt pathway inhibitors on autophagy was not observed using the method of Western blot which is a common method to detect protein. An analysis by fluorescence imaging is more sensitive than Western blotting and can specifically detect the protein which maintained stereoscopic structure. Another study is required to elucidate the precise underlying mechanisms of the potential relationship among the PI3K/Akt pathway, mitophagy, and cell death.

In Chart 1, we outline the findings of the present study. Gemcitabine-treated INS-1 cells appear to undergo as follows. Defects in mtDNA through DNA synthesis inhibition lead to electron transfer system dysfunction and ROS generation. The oxidative stress induced by ROS leads to the loss of mtDNA and subsequent mitophagy-mediated death of INS-1 cells. To the best of our knowledge, the current data indicate for the first time that the rapid loss of mtDNA can trigger mitophagy and induce cell death. Based on these findings, we believe mtDNA to be an important target for cancer treatment. We regard this strategy as an attractive candidate for therapeutic interventions and support the development of medicines targeting mtDNA engaged in various stages of clinical development.

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Conflict of Interest The authors declare no conflict of interest.

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