Original Article

Gemcitabine induces apoptosis via JAK2/STAT3 signaling pathways in lung cancer

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Abstract: Background: This study was aimed to elucidate the antitumor effect of gemcitabine in lung cancer SPC-A-1 cells and the possible molecular mechanism involved. Materials and methods: Cell proliferation was measured by the Cell Count Kit-8 (CCK-8). Cell cycle, apoptosis, reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) level were assessed by flow cytometry analysis. Real-time PCR and Western blot analysis were used to detect the ratio of Bax/Bcl-2 and the activation of JAK2 and STAT3 after cells were treated with different concentrations of gemcitabine. Results: The results revealed that gemcitabine could inhibit the growth of SPC-A-1 cells significantly in both a time and dose-dependent manner. The cell cycle was blocked and ROS production was increased, while MMP level was decreased after the cells were treated with different concentrations of gemcitabine. Bcl-2 expression was down-regulated remarkably while Bax expression was increased after apoptosis occurred. Additionally, the activation of JAK2 and STAT3 was significantly inhibited in gemcitabine treated SPC-A-1 cells. Conclusions: Gemcitabine may serve as a potential therapeutic agent for lung cancer.

Keywords: Lung cancer, SPC-A-1, gemcitabine, apoptosis, JAK2/STAT3

Introduction

Lung cancer is one of the most common leading causes of death worldwide [1] and nonsmall cell lung cancer (NSCLC) represents the most frequent type of lung cancer [2]. Although a combination of surgery, chemotherapy and radiotherapy is available, the prognostics are generally poor [3]. Upon this reason, it is currently extensive to screen new bioactive compounds from either natural and synthetic sources for potential antitumor activity. In recent years, many studies have observed that gemcitabine, a novel nucleoside analog that is incorporated into DNA as a fraudulent base, inhibits the activity of DNA polymerases responsible for DNA repair and synthesis [4]. These characteristics suggest that gemcitabine may be a good candidate for cancer treatment. In clinical study, gemcitabine has demonstrated important antitumor activity in many human tumor xenografts, including lung cancer [5].

The proliferation inhibitory effect of gemcitabine is strongly related to cancer research. Recently, it was demonstrated that gemcitabine possessed an anti-proliferative effect on human oral squamous cell carcinoma [6] and breast cancer in vitro [7]. In cancer, cell cycle homeostasis is generally disrupted by mutations, leading to uncontrolled cell division and tumor growth. For example, gemcitabine induced arrest of pancreatic cancer cells in S phase both in vivo and in vitro [8]. Model predictions and experimental data showed that gemcitabine induced cell cycle arrest in the S phase at low concentrations, whereas higher concentrations induced arrest in all cell cycle phases [9].

The abnormal production of reactive oxygen species (ROS) by exogenous sources or endogenous mechanisms is among the main causes of cancer-related mutations [10]. ROS also stimulate apoptosis through mitochondrial signal mechanisms associated with cancer progression. It was previously reported that gemcitabine-induced apoptosis in breast cancer cells was associated with the mitochondrial apoptotic signaling pathway and induced mitochondrial membrane potential (MMP) loss in pancreatic cancer cells [11, 12].
Signal transducer and activator of transcription 3 (STAT3) protein is a member of a family of latent cytoplasmic transcription factors transmitting signals from the cell surface to the nucleus activated by cytokines and growth factors, which results in dimerization of their cognate receptors and activation of tyrosine kinases such as Janus kinase (JAK) [13]. Numerous experiments have demonstrated that normal physical functions of STATs are critical in regulating many aspects of cellular proliferation, apoptosis and migration. STAT3 is the most intimately linked to cell proliferation and tumorigenesis, and indispensable for apoptosis in numbers of cancer cells, including breast, colon and lung cancer cells [14-16].

In this study, we examined the effects of gemcitabine on proliferation, apoptosis and JAK2/STAT3 signaling in lung cancer cells. We found that Gemcitabine inhibited the proliferation and induced apoptosis, resulting in increase of Bax/Bcl-2 ratio and inhibition of JAK2 and STAT3 activation, thus suggesting a potential utility for development of preclinical and clinical investigations.

Materials and methods

Cell culture

Lung cancer cell line SPC-A-1 (Academia Sinica Cell Bank, Shanghai, China) were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA, USA), and incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cell proliferation assay

The cell proliferation was evaluated by Cell Counting Kit 8 (CCK-8, Dojindo Molecular Technologies, Rockville, USA). SPC-A-1 cells were plated in the 96-well plates (5×10³ cells/well) and incubated for 24 h. Gemcitabine (10, 20 and 40 mM) was introduced to the cells and incubated for another 4 h. A549 cells were collected, washed twice with cold D-hanks buffer solution, and re-suspended in binding buffer (1×10⁶ cells/mL). After 100 µl of SPC-A-1 cells was transferred to a tube, 5 µl of FITC-conjugated Annexin V and 5 µl of propidium iodide were added followed by incubation for 15 min at room temperature in the dark. The stained SPC-A-1 cells were diluted by the binding buffer and directly analyzed by flow cytometry using Cell Quest software.

Cell apoptosis detection

Apoptosis kit (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences) was employed to detect apoptotic cells, following the manufacturer’s instructions. Briefly, SPC-A-1 cells were plated in the 6-well plates (1×10⁶ cells/well) and incubated for 24 h. Gemcitabine (10, 20 and 40 mM) were introduced to the cells and incubated for another 48 h. A549 cells were collected, washed twice with cold D-hanks buffer solution, and re-suspended in binding buffer (1×10⁶ cells/mL). After 100 µl of SPC-A-1 cells was transferred to a tube, 5 µl of FITC-conjugated Annexin V and 5 µl of propidium iodide were added followed by incubation for 15 min at room temperature in the dark. The stained SPC-A-1 cells were diluted by the binding buffer and directly analyzed by flow cytometry using Cell Quest software.

Mitochondria membrane potential (MMP)

Mitochondria Membrane Potential (MMP) was detected by using Tetramethylrhodamine, methyl ester (TMRM) dye. Cells (1×10⁶ cells/well) were cultured in 6-well plate. After a period of treatment (48 h) with various concentrations of gemcitabine (10, 20 and 40 mM), cells were washed with PBS, incubated with TMRM (10 mM) and subsequently subjected to flow cytometry.
Detection of reactive oxygen species (ROS)

Detection of ROS was performed by flow cytometry analysis as described previously. In brief, (5×10^4 cells/well) were cultured in 6-well plate, after a period of treatment (48 h) with various concentrations of gemcitabine (10, 20 and 40 mM), cells were washed with PBS and resuspended in complete medium followed by incubation with 10 μM DCFH-DA for 20 min at 37°C. ROS fluorescence intensity was determined by cytometry with excitation at 480 nm and emission at 525 nm.

Real-time PCR

Total RNAs were extracted from SPC-A-1 cells using TRIZOL reagent (Invitrogen Life Technologies) and stored at -80°C. The DyNAmo Flash SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) was used according to the manufacturer’s instructions. The primers sequences (sense/antisense) used were as follows: Bax, forward: 5'-GTCATCTCGCTCTGGTGAGGCG-3' and reverse: 5'-CACACACACAAAGCTGCTCC-3'; Bcl-2, forward: 5'-CCACCTGCGTCTTGACTCGAC-3' and reverse: 5'-CAATCCTCCCAGGTTCACC-3'; GAPDH, forward: 5'-GGATCCACTGCGTCTCTCA-3' and reverse: 5'-GGTTCCACGCCCATCACAAC-3'. Relative quantification of the signals was performed by normalizing the signals of different genes with the GAPDH signal.

Western blot

Following treatment with gemcitabine at the desired concentrations and time, SPC-A-1 cells were harvested. The expression of apoptosis-related proteins was detected by Western blot according to the manufacturer’s instructions and previous reports [17]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used as an internal control for whole cell lysates. The experiment was repeated three times independently.

Statistical analysis

Experimental data were presented as mean ± SD of at least three independent replicates through analyzing with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The paired, two-tailed Student’s t-test was used to analyze the significance of difference between groups.

Differences were considered significant at values of P<0.05.

Results

Gemcitabine causes growth inhibitory and cell cycle arrest in lung cancer cells

Human lung cancer cell lines SPC-A-1 was employed as a model system to investigate the effect of gemcitabine on lung cancer cell proliferation. We quantified the cytotoxic effects of gemcitabine by measuring percent proliferation using CCK-8 assay. Our data demonstrated that gemcitabine induced a dose- and time-dependent decrease in proliferation of the lung cancer cell lines SPC-A-1 with 60%, 35% and 18% of control at the concentration of 40 mM after 24, 48 and 72 h treatments, respectively (Figure 1A). Suppression of cancer cell proliferation can be caused by arrest of cell cycle progression. Our findings on cell cycle distribution demonstrated that treatment with Gemcitabine resulted in enrichment of lung cancer cells in G1 and G2 phase with a concomitant decrease in number of cells in S phase. We observed a 1.3, 1.9 and 6.4 folds decrease in number of cells in S phase at 10, 20 and 40 mM concentrations of gemcitabine, respectively (Figure 1B and 1C). Together, these data indicate that gemcitabine has proliferation inhibitory effects on lung cancer cells through arresting cell cycle.

Gemcitabine induces apoptosis in lung cancer cells

Induction of apoptosis is another cause of cancer cell growth suppression. In apoptosis assay, our data demonstrated a considerable increase in apoptotic index in a dose-dependent manner after 48 h of gemcitabine treatment (Figure 2). At 10, 20 and 40 mM concentrations of gemcitabine, we observed 3.0, 4.8 and 8.2 folds increase in apoptotic indices of SPC-A-1 cells, respectively. Altogether, our findings demonstrate that gemcitabine has both cytostatic and cytotoxic properties against lung cancer cells.

Gemcitabine induces apoptosis in the mitochondrial pathway

To assess the effect of gemcitabine on the changes of MMP in SPC-A-1 cells, flow cytometry analysis was carried out to detect the fluo-
Gemcitabine blocks JAK2/STAT3 pathways

Figure 1. Gemcitabine inhibited the proliferation and arrested cell cycle of SPC-A-1 cells. The cells were treated with gemcitabine (10, 20 and 40 mM) for 24, 48 and 72 h. (A) Cell proliferation was determined using CCK-8 assay and (B, C) cell cycle was analyzed using a propidium iodide staining assay and flow cytometry. *P<0.05, **P<0.01, ***P<0.001 compared with control cells.
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Figure 2. Gemcitabine promoted the apoptosis of SPC-A-1 cells. The cells were treated with gemcitabine (10, 20 and 40 mM) for 48 h. A, B. Cell apoptosis was determined using Annexin-V/PI double stain assay and flow cytometry. ***P<0.001 compared with control cells.

rescence intensity of TMRM. As shown in Figure 3A and 3B, treatment of SPC-A-1 cells with gemcitabine at the concentrations of 10, 20 and 40 μM for 48 h caused a moderate depolarization of MMP in a dose-dependent manner. We observed 1.2, 1.9 and 2.4-folds decrease in MMP level of SPC-A-1 cells, respectively. On the other hand, ROS generation is also linked to mitochondria. Fluorescence probe DCFH-DA was used to determine the levels of ROS production in SPC-A-1 cells. As shown in Figure 3C and 3D, SPC-A-1 cells exposed to gemcitabine at 10, 20 and 40 μM for 48 h caused a significant increase in the intracellular accumulation of ROS in a dose-dependent manner. We observed 1.2, 1.4 and 1.7-folds increase in ROS accumulation of SPC-A-1 cells, respectively. Taken together, these data suggest that gemcitabine induces lung cancer cells apoptosis through the mitochondrial pathway.

Gemcitabine alters the expression of proteins in lung cancer cells

To investigate the mechanistic basis of proliferation inhibitory effects of gemcitabine, we next examined its effect on the expression of key proteins involved in cell apoptosis and signaling pathway. Our data revealed a dose-dependent increase in the ratio of Bax/Bcl-2 both in mRNA and protein levels (Figure 4A-C). Among the signaling pathway proteins, we observed a dose-dependent reduction in the levels the phosphorylation of JAK2 and STAT3, whereas the expression of total JAK2 and STAT3 was not changed (Figure 4D and 4E). These findings demonstrate that Gemcitabine alters the expression of proteins involved in the regulation of cell apoptosis and signaling pathway to confer its proliferation inhibitory effect.

Discussion

Lung cancer remains a devastating malignancy due to lack of effective therapy for treatment. The present study demonstrated that gemcitabine is effective in suppressing the proliferation and arresting cell cycle of human lung cancer cells SPC-A-1 due to its cytostatic and cytotoxic properties. Furthermore, our studies provided evidence for a role of gemcitabine in
Figure 3. Gemcitabine decreased the MMP level and increased the ROS production of SPC-A-1 cells. The cells were treated with gemcitabine (10, 20 and 40 mM) for 48 h. A, B. Cell MMP level was determined using TMRM and flow cytometry. C, D. Cell ROS production was determined using DCFH-DA and flow cytometry. ***P<0.001 compared with control cells.
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inducing the lung cancer cells apoptosis and in suppressing activation of JAK2 and STAT3.

Deregulated growth in cancer cells is often attributed to loss of control in proliferation and apoptosis [18]. In this study, we observed that cells were incubated with different concentrations of gemcitabine significantly declining cell proliferation in a time- and dose-dependent manner. Similar concentrations of gemcitabine were found to exert the same effects with different cancer cell lines by other groups [19]. In fact, molecular studies have revealed that cell cycle associated with cell survival is frequently altered in multiple human cancers. We observed that cells were incubated with different concentrations of gemcitabine significantly increased the number of cells in G1 and G2 phase, while decreased the number of cells in S phase. However, low concentration of gemcitabine (<0.5 mM) showed cell cycle arrest in S and G2 phases, which similar to the report of Hamed and colleagues [9, 20]. These results suggest that gemcitabine arrest cell cycle dependent on its concentrations and targeted cancer cells.

Following cell cycle arrest, cells may either undergo repair or enter the apoptotic pathway to maintain cellular integrity [21]. Thus, induction of apoptosis is one of the protective mechanisms against cancer development and progression. In the present study, we observed significant induction of apoptosis in gemcitabine-treated lung cancer cells. Cell survival is maintained by a balance of the ratio of anti-apoptotic and pro-apoptotic protein. The upregulation of Bax and downregulation of Bcl-2 were observed in a dose-dependent manner in lung cancer cells after treated with different concentrations of gemcitabine. The permeabilization of the mitochondrial outer membrane is tightly controlled by Bcl-2 [22] and activation of Bax can trigger a sequence of events that leads to alterations in mitochondrial permeability transition [23]. Therefore, we found that reduction of
MMP level emerged in lung cancer cells after treated with gemcitabine. Besides, mitochondria are major sources of ROS, and the reduction in the level of Bcl-2 would promote the generation of ROS [24]. An increase of ROS production was observed in lung cancer cells treated with gemcitabine. These results indicate that gemcitabine induce lung cancer cells apoptosis through the mitochondria pathway.

Abnormal JAK/STAT signaling pathways are involved in the pathogenesis of several cancers including apoptosis [25]. However, the molecular mechanism by which disordered JAK2/STAT3 signaling pathways contributes to apoptosis has not been clarified in lung cancer cells. Therefore, our work found that gemcitabine treatment could inhibit the activation of JAK2 and STAT3 in a dose-dependent manner evidenced by decreased p-JAK2 and p-STAT3 levels. Previous study found that the expression of Bcl-2 that is known to be downstream target of the STATs pathway is decreased and the MMP level is reduced after suppression of JAK2/STAT3 signaling pathways [15]. These findings suggest that abnormal expression of Bcl-2 may be the trigger involved in JAK2/STAT3 signaling pathways found in pancreatic cancer [26].

In conclusion, several factors involving the modulation of cell cycle, apoptosis and expression of critical genes involved in drug activity may contribute to the effect of gemcitabine against lung cancer cells in vitro. Here, we have shown, for the first time, the growth inhibitory and apoptosis induction of gemcitabine in lung cancer cells. Gemcitabine causes G1 and G2 phase cell cycle arrest and induction of apoptosis by altering the expression of Bax and Bcl-2 and phosphorylation of JAK2 and STAT3. We therefore believe that gemcitabine could be a novel promising agent for the treatment of lung cancer.

Disclosure of conflict of interest

None.

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