Cyclopamine increases the radiosensitivity of human pancreatic cancer cells by regulating the DNA repair signal pathway through an epidermal growth factor receptor-dependent pathway

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Received December 16, 2012; Accepted July 5, 2013

DOI: 10.3892/mmr.2013.1605

Abstract. Pancreatic cancer is an aggressive malignancy with a characteristic metastatic course of disease and resistance to conventional radiotherapy. As a result, the continual development of novel therapeutic agents is required to improve the current situation. In the present study, the effect of the hedgehog pathway inhibitor, cyclopamine, on cellular radiosensitivity was determined in K-RASwt Colo-357 and K-RASmt SW-1990 human pancreatic cancer cell lines using the clonogenic survival assay. Apoptosis and cell cycle distribution were detected using flow cytometry assay. Following irradiation (30 mins), residual double-strand breaks were quantified by identification of γ-H2AX foci of micronuclei and radiation-induced γ-H2AX, p-ATM, DNA-PKcs and Ku70 expression was analyzed using western blot analysis. The epidermal growth factor (EGF) and EGF receptor (EGFR) inhibitor, gefitinib, were utilized to determine the related mechanisms. The results revealed that cyclopamine treatment significantly reduced cell clonogenic survival but failed to induce apoptosis and radiation-induced G2 arrest. Flow cytometry revealed that cyclopamine treatment enhanced γ-H2AX foci in Colo-357 and SW-1990 cells exposed to irradiation. In addition, radiation-induced p-ATM, DNA-PKcs and Ku70 were all inhibited. EGF also rescued pancreatic cancer cells from cyclopamine-induced H2AX phosphorylation following irradiation. Thus, cyclopamine enhanced the radiosensitivity of human pancreatic cancer cells, in part, through an EGFR-dependent pathway, indicating a rational approach in combination with radiotherapy.

Introduction

Pancreatic cancer is a lethal disease that is notoriously difficult to treat (1). Only a small proportion of cases are curative through surgical resection and standard chemoradiotherapy for patients with advanced disease only has modest effects with substantial toxicity (2,3). Clearly, the continual development of novel therapeutic agents is required to improve the current situation.

Several studies on biological approaches targeting the molecular abnormalities of pancreatic cancer are available (4-6). One such pathway is the hedgehog (Hh) signaling pathway, which specifies patterns of cell growth and differentiation during embryogenesis in a wide range of tissues (7). In addition to its function in developmental patterning, the Hh pathway is also important in maintaining the homeostasis of mature tissues and the number of somatic cells in various organs. This pathway represents an attractive target for drug development and has shown promise in clinical trials of cancer treatments. The specificity of cyclopamine for the Hh pathway is demonstrated by the absence of cytotoxicity in cells that lack Hh signaling.

The K-ras oncogene mutation occurs in 75-90% of pancreatic cancers (8,9). The gene encodes a 21-kDa membrane-bound guanosine triphosphate-binding protein involved in growth factor-mediated signal transduction pathways. K-ras is activated through the overexpression or activation of ras-activating signaling partners, including the epidermal growth factor receptor (EGFR) (10). In the present study, the effects of cyclopamine on pancreatic cancer radiosensitivity were investigated in vitro using K-RASwt Colo-357 and K-RASmt SW-1990 human pancreatic cancer cell lines.

Materials and methods

Cell culture and reagents. Human pancreatic cancer cell lines, Colo-357 and SW-1990, were purchased from the American...
Type Culture Collection (Manassas, VA, USA). Colo-357 cells were maintained in Dulbecco's modified Eagle's medium and SW-1990 cells were seeded onto tissue culture dishes containing RPMI-1640 medium supplemented with 10% fetal calf serum, L-glutamine (5 mM), non-essential amino acids (5 mM), penicillin (100 U/ml) and streptomycin (100 U/ml; Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified 5% CO₂ atmosphere. Cyclopamine and EGF were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The EGFR inhibitor, gefitinib (Iressa), was purchased from AstraZeneca (Macclesfield, UK).

Inhibitor treatment. Stock solutions of the cyclopamine Hh pathway inhibitor, gefitinib EGFR inhibitor and EGF were prepared at appropriate concentrations in dimethyl sulfoxide (DMSO) and then stored at -70°C. For treatment, inhibitor solutions were diluted 1:1,000 to appropriate working concentrations (20 or 40 µmol/l cyclopamine, 10 µmol/l gefitinib and 3 µmol/l EGF) in serum-free medium. Control cultures received medium containing the solvent DMSO at a concentration of 0.1%. Gefitinib and EGF were supplemented to the culture media 0.5 h before irradiation and 24 h of preirradiation treatment with cyclopamine was conducted.

Ionizing radiation. A Siemens 6 MV X-ray linear accelerator (Siemens, Munich, Germany) was used to deliver a single dose of ionizing radiation (IR) with a dose rate of 200 cGy/min at room temperature.

Clonogenic assay. Cells were plated at various cell densities and irradiated with 0.5, 1, 2, 4 and 6 Gy X-ray 24 h later. Following 12-14 days incubation at 37°C, cells were stained with Giemsa. The number of colonies per dish was counted with the FACS Calibur (Becton-Dickinson, San Jose, CA, USA). The plating efficiencies for irradiated and unirradiated cells. Experiments were conducted in triplicate and data from three independent experiments are presented as the means ± SD. All survival fractions were fitted into the linear quadratic model.

Apoptosis assay. Cells were removed with trypsin and collected into centrifuge tubes together with the culture medium. Flow cytometry and Annexin V-fluorescein isothiocyanate (FITC) apoptosis analysis were performed as previously described (11). Cell cycle distribution and apoptotic rate were calculated from 1x10⁶ cells using ModFit LT software with the FACS Calibur (both Becton-Dickinson, San Jose, CA, USA).

Cell cycle assays. Cells were removed with trypsin and collected into centrifuge tubes together with the culture medium. Detailed methods for flow cytometry analysis were previously described (12). Cell cycle distribution was calculated from 1x10⁶ cells using ModFit LT software with the FACS Calibur.

Immunofluorescence assay. Detection of γ-H2AX foci immunofluorescence was performed to determine residual DNA double-strand breaks (DSBs). Cells grown on coverslips (Fisher Scientific, Loughborough, UK) were fixed in ice-cold 4% paraformaldehyde for 30 min, blocked with 3% bovine serum albumin in phosphate buffer solution (PBS) and then incubated with an antibody against γ-H2AX (ser139; 1:500; Cell Signaling Technology, Inc.) for 2 h at 4°C. After washing with PBS, secondary FITC-conjugated antibody was added for 1 h. The slides were washed with PBS and then mounted with mounting medium containing 4',6-diamino-2-phenylindole.

Western blot analysis. Cell lysates were prepared and western blot analysis was performed as previously described (13). Equal aliquots of total cell protein (50 µg/lane) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, transferred onto polyvinylidene fluoride membranes and then probed with β-actin (C-4), DNA-PKcs, Ku70 (A-9) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,000 dilution), γ-H2AX or p-ATM (Cell Signaling Technology, Inc.; 1:1,000) primary antibodies, followed by horseradish peroxidase-labeled goat anti-mouse (GAM-007) and goat anti-rabbit (SC-2004) IgG secondary antibodies. The protein bands were visualized using an enhanced chemiluminescence system (Union Bioscience Corporation, Hangzhou, China) with prestained markers as molecular size standards. The densitometry of the protein bands was quantified with Quantity One (Bio-Rad, Hercules, CA, USA) and the values were expressed relative to β-actin (control for loading and transfer). At least three independent experiments were performed for each cell type studied.

Statistical comparisons. Data are presented as the mean ± SD. Experimental results of the treated and control groups were compared using the two-tailed Student's t-test. All statistical tests were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cyclopamine enhances radiosensitivity of pancreatic cancer cells. Given that cyclopamine modulates the Hh pathway, the effect of cyclopamine on the radiation clonogenic survival of K-RAS wt (Colo-357) and K-RAS mut (SW-1990) was analyzed in human pancreatic cancer cell lines. The radiosensitizing effect of cyclopamine was confirmed by single-dose irradiation with doses up to 6 Gy. The results revealed that cyclopamine treatment exerted significant radiosensitization of Colo-357 and SW-1990 cells to the clinically relevant radiation dose per fraction of 2 Gy relative to DMSO controls (Fig. 1).

Cyclopamine does not enhance radiation-induced apoptosis. Previous studies have reported induction of apoptosis by cyclopamine (14). Therefore, the rate of apoptosis was examined upon cyclopamine treatment with irradiation compared with DMSO controls. Cyclopamine (40 µmol/l) alone significantly induced apoptosis. However, in combination with irradiation, it failed to induce apoptosis in K-RAS wt and K-RAS mut pancreatic cancer cells (Fig. 2A).

Cyclopamine treatment does not affect cell cycle redistribution. Cell cycle phases are associated with various degrees of
radiosensitivity. Thus, the percentage of cells in the radiosensitive G2 cell cycle phase was determined upon treatment with cyclopamine alone or in combination with irradiation (15). A significant G2 cell cycle arrest was noted following irradiation. However, cyclopamine (40 µmol/l) treatment failed to abrogate radiation-induced G2 arrest as compared with DMSO controls. This effect was observed in K-RAS<sup>wt</sup> and K-RAS<sup>mt</sup> pancreatic cancer cells (Fig. 2B).

**Effects on DNA-DSB repair by cyclopamine.** To analyze how cyclopamine affects radiation-induced H2AX phosphorylation as an indicator of DNA damage signaling, Colo-357 and SW-1990 cells were irradiated with a single dose of ionizing radiation (6 Gy). H2AX phosphorylation at Ser139 reached a maximum at 0.5 h following ionizing radiation (Fig. 3A). Using western blot analysis, a dose-dependent increase in γ-H2AX following ionizing radiation was observed, particularly at the 0.5 h time point (Fig. 3B). The formation of γ-H2AX foci was measured 0.5 h after irradiation of pancreatic cancer cells. This procedure was conducted in order to recognize the molecular mechanisms of cyclopamine radiosensitization. Radiation-induced γ-H2AX foci were significantly increased in cyclopamine-treated Colo-357 and SW-1990 cells (Fig. 4A), indicative of DNA repair inhibition. Fig. 4B shows the effect of cyclopamine on the expression of DNA repair-related proteins. For the two cell lines, H2AX phosphorylation was enhanced following irradiation. In

Figure 1. Cyclopamine enhances the radiosensitivity of K-RAS<sup>wt</sup> Colo-357 and K-RAS<sup>mt</sup> SW-1990 cells. Log-phase (A) Colo-357 and (B) SW-1990 cells were pretreated for 24 h with cyclopamine (20 or 40 µmol/l) and cells were plated at various cell densities and irradiated with 0.5, 1, 2, 4 and 6 Gy X-ray 24 h later. Following 14 days, the number of colonies formed was counted and survival fractions were calculated. Data points represent the mean surviving fraction (mean ± SD) of three parallel experiments.

Figure 2. Cyclopamine-treatment does not affect radiation-induced apoptosis and cell cycle redistribution. (A) Following treatment with cyclopamine for 24 h, cells remained unirradiated or received a single dose of 6 Gy. After 48 h, cells were treated as indicated, fixed and then stained with 4',6-diamino-2-phenylindole to microscopically determine cells with typically apoptotic nuclear morphology. (B) Cell cycle distribution was assayed in cells after 24 h of cyclopamine treatment plus 6 Gy X-rays. The student's t-test was used to compare cyclopamine/irradiated and DMSO/irradiated cells. *P<0.05, vs. control. DMSO, dimethyl sulfoxide.

Figure 3. Time kinetics of radiation-induced H2AX phosphorylation. K-RAS<sup>wt</sup> Colo-357 and K-RAS<sup>mt</sup> SW-1990 cells were exposed to ionizing radiation at single doses of 3, 6 or 9 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE and then incubated with antibodies against γ-H2AX and β-actin.

Figure 4. Time of irradiation and phosphorylation of H2AX after irradiation.
contrast to γ-H2AX, radiation-induced p-ATM, Ku70 and DNA-PKcs were all inhibited (Fig. 4B).

**Cyclopamine inhibits DNA-DSB repair in an EGFR-dependent pathway.** To verify the effect of EGFR in cyclopamine-induced radiosensitivity, cells were treated with gefitinib prior to irradiation. The expression levels of γ-H2AX showed that the inhibitory effect of gefitinib blocked cyclopamine-induced H2AX phosphorylation, which is consistent with the results discussed (Fig. 5A). Furthermore, EGF markedly inhibited cyclopamine-induced phosphorylation of H2AX following irradiation, indicating the dependence of H2AX phosphorylation partly through an EGFR-dependent pathway (Fig. 5B).
Discussion

Pancreatic cancer is the fourth leading cause of cancer-related mortality and is associated with multiple aberrations in cellular signaling cascades (16). The treatment of pancreatic cancer is frequently met with poor outcomes due to the development of resistance to therapy (17). Currently, an effective treatment for pancreatic cancer is lacking and conventional therapy has shown limited success in improving patient survival. Therefore, understanding the mechanisms regulating the molecular changes that drive the refractoriness to therapy is a prerequisite for the development of effective interventions for pancreatic cancer.

In the present study, the effect of cyclopamine on the radiation clonogenic survival of pancreatic cancer cell lines was analyzed. Cyclopamine treatment exerted significant radiosensitization on K-RAS wt and K-RAS mut pancreatic cancer cell lines, indicating cyclopamine-induced radiosensitivity through a K-RAS-independent pathway. Apoptosis and cell cycle assays showed that cyclopamine failed to affect radiation-induced apoptosis and cell cycle redistribution. In addition, phospho-H2AX was analyzed following ionizing radiation to determine the underlying mechanisms. Radiation-induced γ-H2AX foci were significantly increased in cyclopamine-treated cells. Western blot analysis showed that radiation-induced p-ATM, Ku70 and DNA-PKcs were all inhibited in cyclopamine-treated cells. To verify the underlying mechanisms in cyclopamine-induced DNA-DSB repair, cells were treated with gefitinib or EGF prior to irradiation. The results indicated that the cyclopamine-induced activity of H2AX occurred, in part, through an EGFR-dependent pathway.

The results of the present study provide convincing evidence for the function of inhibited Hh pathway in pancreatic cancer. This study may serve as a basis for clinical studies identifying the role of cyclopamine in pancreatic cancer radiotherapy. In conclusion, these observations indicate that the role of cyclopamine in the radiosensitivity of pancreatic cancer may be important for translational research on the development of more effective and targeted therapeutic strategies for pancreatic cancer.

Acknowledgements

This study was supported by grants from the Hospital Center Technology Development Fund of Wuxi City (no. YGM1101) and the Social Development Project of Kunshan City (no. KS1224).

References