Radiation-induced Increase in Invasive Potential of Human Pancreatic Cancer Cells and Its Blockade by a Matrix Metalloproteinase Inhibitor, CGS27023

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ABSTRACT

Purpose: Radiotherapy remains a major therapeutic option for patients with advanced pancreatic cancer. Nevertheless, the effects of irradiation on malignant biological behaviors (e.g., migration and invasion of cancer cells) have yet to be clarified. Thus, we conducted an in vitro study to investigate the radiation-induced alterations around cell migration and invasion capacity.

Experiment design: Three cell lines from human pancreatic cancer were included in the study. γ-radiation was used for irradiation treatment. Cell migration and invasion ability were evaluated by Transwell migration assay and Matrigel invasion assay. The activity of MMP-2 and 9, and expression of urokinase-type plasminogen activator were investigated with gelatin zymography and immunoblot, respectively.

Results: Irradiation enhances invasive potential in some pancreatic cancer cells, whereas it significantly inhibits cell proliferation and migration. This hitherto unknown biological effect of irradiation involves enhanced matrix metalloproteinase (MMP)-2 activity. Consequently, simultaneous administration of an MMP inhibitor, CGS27023A, suppresses the radiation-enhanced invasion through blockade of transition of MMP-2 from latent type to active type.

Conclusion: Because radiation may increase invasion ability through activating MMP proteolytic system, simultaneous administration of the MMP inhibitor during radiotherapy could be a potential adjuvant therapeutic approach to improve the efficacy of radiotherapy for pancreatic cancer.

INTRODUCTION

Pancreatic cancer is one of the most aggressive and lethal neoplasms with an extremely low 5-year survival rate (1, 2).

Because most patients with this disease miss the opportunity for complete surgical resection at the time of diagnosis, radiotherapy remains as a major component of treatment modalities for controlling tumor progression. However, pancreatic cancer often shows resistance to radiation (3). The precise mechanism underlying this radio-resistance remains unknown, but several factors have been reported to be involved including cell types, degree of differentiations (4), cell cycle (5), DNA ploidy (6), p53/p21 status (7), and DNA-dependent protein kinase activity (8).

Malignant progression of pancreatic cancer depends not only on rapid proliferation of tumor cells but also on other biological behaviors including motility, invasiveness, and metastatic potential. In this respect, conventional colony formation assay or cell viability assay may be insufficient to evaluate the therapeutic effect of radiotherapy. Changes in motility or invasive potential after irradiation have been poorly understood.

In the present study, we examined the effects of irradiation on proliferation, motility, and invasiveness of pancreatic cancer cells. On the basis of the finding of enhancement of invasive potential by irradiation in a subset of pancreatic cancer cells, we expanded the investigations into two proteolytic systems known as crucially relevant to tumor invasive phenotypes, the uPA2 and MMPs. Because the increased expression/activity of MMP-2 was shown as a causative event of the enhanced invasion, a synthetic MMP inhibitor was used to block the radiation-induced increase in invasive potential. Thus, we try to provide evidence that the concomitant use of MMP inhibitors during radiotherapy could be a potential therapeutic approach to improve the efficacy of radiotherapy for pancreatic cancer.

MATERIALS AND METHODS

Cell Culture and Reagents. Three human pancreatic cancer cell lines were used in this study. Panc-1 and Suit-2 were generously provided by Dr. H. Iguchi (National Kyushu Cancer Center, Fukuoka, Japan), Hs766T was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C with humidified 90% air and 10% CO2. The number of cells was counted with a

Received 10/19/2001; revised 12/28/2001; accepted 1/7/2002.

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2 The abbreviations used are: uPA, urokinase-type plasminogen activator; MMP, matrix metalloproteinase; CGS27023A, N-hydroxy-2(R)-(4-methoxy)sulfonyl)[3-picolyl] amino]-3-methylbutaneamide hydrochloride monohydrate; PI, propidium iodide.

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Radiation-induced Pancreatic Cancer Cell Invasion

Fig. 1 Irradiation inhibits the growth of pancreatic cancer cells in a dose-dependent manner. Panc-1 cells were irradiated with 3 Gy (△), 5 Gy (□), or 10 Gy (●), and the relative proliferation rates were determined at 1, 2, 3, and 4 days after irradiation; bars, ± SD.

particle distribution counter, CDA500 (Sysmex, Kobe, Japan). The MMP inhibitor CGS27023A was kindly provided by Novartis Pharma, Inc., Tsukuba, Japan.

Irradiation. The cells were irradiated with doses of 3, 5, or 10 Gy at room temperature using a 137Cs source (Gamma Cell 40; Atomic Energy of Canada Ltd., Ontario, Canada) delivering 1 Gy/min.

Cell Proliferation Assay. Cell proliferation was evaluated by measuring the fluorescence intensity of PI as described previously with minor modifications (9). Briefly, cells were seeded in 24-well plates at a density of 3 × 10^4 cells/well. After overnight cultivation, cells were irradiated and cultured for 4 days. PI (30 μM) and digitonin (600 μM) were added to each well to label all nuclei of the cells with PI. Fluorescence intensity corresponding to total cells in each well was measured by a multiwell plate-reader, CYTOFLUOR II (PerSeptive Biosystems Inc., Framingham, MA) with 530-nm excitation and 645-nm emission filters. The cell proliferation rate was calculated as the proportion of fluorescence intensity of each well at the time point indicated in the text to that at the day of irradiation.

Migration Assay. Migration of pancreatic cancer cells through 8-μm pores was assessed using the transwell cell culture chamber (6.5-mm diameter; Corning Costar, Tokyo, Japan) as described in detail in our previous publications (10, 11). Cells at a density of 1 × 10^4 were seeded in the upper chamber with 100 μl of medium supplemented with 10% fetal bovine serum. Same medium of 750 μl were placed in the lower wells. After seeding, the cells were subjected to irradiation and then cultured for 24 h. The filter membranes were removed and fixed with 70% ethanol and stained with H&E, and counted in five random fields under a light microscope.

Matrigel Invasion Assay. Invasion of pancreatic cancer cells was measured by the invasion of cells through Matrigel-coated transwell inserts (Becton Dickinson, Franklin Lakes, NJ) as reported previously (10, 11). Briefly, transwell inserts with 8-μm pores were coated with Matrigel (40 μg/well; Becton Dickinson, Bedford, MA). Cell suspension (500 μl; 1 × 10^7/ml) was added to the upper chambers. Same medium of 750 μl were placed in the lower wells. Thereafter, the cells were irradiated and incubated for 24 h. Cells that had invaded to lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with H&E, and counted in five random fields under a light microscope.

Gelatin Zymography. The conditioned medium either from nonirradiated or irradiated Panc-1 cells was concentrated to 10-fold with Centricon-10 (Amico, Beverly, MA). Samples were added to each lane and subjected to 10% SDS-PAGE using 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gel was washed in 2.5% Triton X-100, and incubated in 50 μl Tris-HCl buffer (pH 8.0) containing 0.5 mM CaCl2 and 1 mM ZnCl2 for 20 h at 37°C. The gel was stained with 1% Coomassie Brilliant Blue R-250 and destained with destaining buffer (5% acetic acid and 10% methanol).

Western Blotting. The proteins (80 μg/lane) from the soluble fraction of Panc-1 cells were fractionated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with 1:500 dilutions of polyclonal antibody for human uPA (Santa Cruz Biotechnology, Santa Cruz, CA) and then probed with antigoat IgG conjugated with horseradish peroxides (Santa Cruz Biotechnology). Immunoblots were detected by the enhanced chemiluminescence (Amersham International, Buckinghamshire, United Kingdom).

Statistical Analysis. Statistical analyses were performed by using ANOVA and unpaired Student’s t test. All of the statistics were performed on two-sided test. P < 0.05 was considered as significant. Each experiment was repeated at least three times.

Table 1 Result of Transwell migration assay of three human pancreatic cancer cell lines under graded doses of γ irradiation

<table>
<thead>
<tr>
<th>Cancer cell lines</th>
<th>Control</th>
<th>3 Gy</th>
<th>5 Gy</th>
<th>10 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panc-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.1 ± 1.5</td>
<td>22.1 ± 2.7</td>
<td>25.6 ± 2.4</td>
<td>29.2 ± 3.0</td>
</tr>
<tr>
<td>Suit-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.8 ± 3.5</td>
<td>19.6 ± 3.5</td>
<td>23.5 ± 3.3</td>
<td>25.4 ± 2.5</td>
</tr>
<tr>
<td>Hs766T&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.6 ± 3.3</td>
<td>24 ± 4.3</td>
<td>22.4 ± 3.0</td>
<td>20.2 ± 2.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values shown are mean ± SD of the cell number per ×400 field counting Matrigel and micropore membranes.
<sup>b</sup> P < 0.01, significant difference while comparing with control, ANOVA test.
<sup>c</sup> P < 0.01, significant difference while comparing with control, ANOVA test.

Table 2 Results of Matrigel invasion assay in three pancreatic cancer cell lines under graded doses of γ irradiation

<table>
<thead>
<tr>
<th>Cancer cell lines</th>
<th>Control</th>
<th>3 Gy</th>
<th>5 Gy</th>
<th>10 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panc-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1 ± 1.3</td>
<td>3.3 ± 2.3</td>
<td>4.5 ± 3.4</td>
<td>7.6 ± 2.6</td>
</tr>
<tr>
<td>Suit-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0 ± 2.4</td>
<td>5.7 ± 2.7</td>
<td>8.5 ± 3.0</td>
<td>11.4 ± 3.0</td>
</tr>
<tr>
<td>Hs766T&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.0 ± 2.4</td>
<td>6.2 ± 3.0</td>
<td>8.7 ± 3.2</td>
<td>11.1 ± 3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values shown are mean ± SD of the cell number per ×400 field crossing Matrigel and micropore membranes.
<sup>b</sup> P < 0.01, significant difference among groups, unpaired t test.
<sup>c</sup> P < 0.01, significant difference while comparing with control, ANOVA test.
RESULTS

Irradiation Inhibits Proliferation of Pancreatic Cancer Cells. First, we examined the proliferation of pancreatic cancer cells after irradiation. Irradiation suppressed the proliferation of Panc-1 cells in a dose-dependent manner, and an almost complete inhibition was observed at a dose of 10 Gy (Fig. 1). Similar results were obtained in Suit-2 at the same dose range. However, in Hs766T cells whereas dose reached to 5 Gy, radiation had already entirely inhibited the cell growth (data not shown).

Irradiation Promotes Invasive Potential but Inhibits Migration Ability in a Subset of Pancreatic Cancer Cells. To determine the effect of radiation on cell motility, we analyzed the migration of human pancreatic cancer cells before and after irradiation using the Transwell cell migration assay. Compared with untreated controls, Panc-1 and Suit-2 cells irradiated at doses of 3, 5, and 10 Gy showed significantly lower numbers of migrated cells ($P < 0.01$; Table 1). There was no significant change in migration potential after irradiation in Hs766T cells, which showed a relatively low basal migration activity.

We next examined changes in the invasive potentials of pancreatic cancer cells after irradiation using the Matrigel invasion assay. In contrast to the decline in migration ability, invasive potentials in both Panc-1 and Suit-2 cells were significantly increased after irradiation at doses of 3, 5, and 10 Gy (Table 2). This increase in invasive potential appeared to be dose-dependent. Remarkably, the average number of invaded cells in Panc-1 was increased by >2-fold after irradiation at 10 Gy (Fig. 2). We found no significant change in invasive potential in irradiated Hs766T cells (Table 2).

Irradiation Increases MMP-2 Activity. To determine the role of gelatinases in the radiation-induced changes in invasive potential, we examined MMPs activity in Panc-1 cells before and after irradiation. Cells were incubated 24 h after irradiation, and the conditioned medium was subjected to the gelatin zymography. Untreated Panc-1 cells secreted both latent and active forms of MMP-2 ($M_r$ 72,000 and 62,000 gelatinases). After irradiation, MMP-2 activity of either latent or activated type was significantly increased (Fig. 3), thus suggesting that the increased MMP-2 activity may play an important role in the enhanced invasiveness after irradiation.

An MMP Inhibitor Blocks the Radiation-enhanced Invasion of Pancreatic Cancer Cells. Finally, we tested whether a synthetic MMP inhibitor, CGS27023A, could prevent the radiation-enhanced invasiveness. CGS27023A was added to invasion chambers at final concentrations of 1, 5, and 10 $\mu M$ just before irradiation. After irradiation at 5 Gy, the number of invaded cells in Panc-1 increased from 14.6 cells/field to 24.4 cells/field, whereas concomitant treatment with CGS27023A at concentrations of 5 and 10 $\mu M$ significantly blocked the increase in invaded cells after irradiation (Fig. 4). Treatment with CGS27023A did not affect the growth and viability of Panc-1 cells at concentrations up to 10 $\mu M$ (data not shown). Furthermore, gelatin zymography revealed that treatment with CGS27023A at 5 $\mu M$ markedly decreased the active type MMP-2 without affecting the enzymatic activity of latent type MMP-2 (Fig. 5).
Expression of uPA Decreases after Irradiation.

To determine the possible involvement of uPA in the changes in cell motility after irradiation, we examined the expression of uPA in Panc-1 cells by Western blotting. The uPA expression in cell lysate that represents the constituent portion of uPA was suppressed by irradiation (Fig. 6).

DISCUSSION

In the present study, we examined the effects of irradiation on multiple biological behaviors that determine the malignant progression of pancreatic cancer. The results have shown that irradiation promotes invasive potential at least in some pancreatic cancer cells, whereas cell proliferation and motility were significantly suppressed; and this radiation-enhanced invasiveness is associated with an increased expression/activity of MMP-2. Furthermore, simultaneous administration of an MMP inhibitor, CGS27023A, blocks the radiation-induced increase in invasive potential. Taken together, these findings suggest that the concomitant use of MMP inhibitors during radiotherapy could be a potential therapeutic approach to improve the efficacy of radiotherapy for pancreatic cancer.
Importantly, radiation-induced enhancement in invasiveness was observed at doses used clinically as a fractionated irradiation for pancreatic carcinoma. This implies that the feasibility of the current radiotherapy needs to be reconsidered. Recently, similar findings were reported by other investigators. Wild-Bode et al. (12) showed that sublethal doses of irradiation enhanced the migration and invasiveness of human glioblastoma cells in association with enhanced expression/activity of MMP-2 and MMP-9. Furthermore, Camphausen et al. (13) reported that radiation therapy to a primary Lewis lung carcinoma accelerated metastatic growth in mice. These findings support our present results in pancreatic cancer cells. Notably, however, of three lines of pancreatic cancer cells studied, there is one line (Hs766T) displaying no response to irradiation in regard to cell migration or invasion statuses. Additional investigations are needed for elucidating the relevant molecular mechanisms behind the different responses toward irradiation.

We found that the increased invasiveness after irradiation was associated with increased expression and activity of MMP-2. Previous reports also described the increased expression of MMP-2 after irradiation (12, 14). MMP-2 has two types, latent type and active type. Through a complex series of biochemical interactions, $M_r \approx 72,000$ of latent MMP-2 is cleaved to form $M_r \approx 62,000$ active type (15). Consistent with our present results, several studies have shown that synthetic MMP inhibitors block the invasion and metastasis of pancreatic cancer cells by inhibiting MMP activation process from latent type to active type (16, 17).

The present study demonstrated that irradiation suppressed the motility in some pancreatic cancer cells. It has been suggested that uPA and its uPA receptor may play a key role in the regulation of cell motility as well as proteolysis (18, 19). Carriero et al. (20) reported that the ligation between uPA and uPA receptor initiated remarkable cytoskeletal rearrangements and promoted cell migration in breast cancer. After irradiation, uPA expression was reduced in association with a decreased motility. Thus, we suggest that the decreased motility after irradiation may account, at least in part, for the decreased expression of uPA.

We also demonstrated that a synthetic MMP inhibitor, CGS27023A, blocked the activation of MMP-2 and, subsequently, radiation-enhanced invasiveness. Several MMP inhibitors have been developed, and some of them have already been evaluated in clinical trials for treatment of aggressive tumors including pancreatic cancer (21). MMP inhibitors inherently do not cause leucopenia, a universal side effect of conventional anticancer agents. In the present study, CGS27023A showed no effect on cell viability and proliferation. CGS27023A is a potent inhibitor of several MMPs including collagenase, gelatinase, and stromelysin, and, importantly, this drug can be administered p.o. We believe that concomitant administration of CGS27023A during radiotherapy could be a novel approach to enhance the efficacy of radiotherapy for pancreatic cancer.

REFERENCES

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