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A Nitroimidazole Derivative, PR-350, Enhances the Killing of Pancreatic Cancer Cells Exposed to High-dose Irradiation under Hypoxia

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The radiosensitizing effects of PR-350, a nitroimidazole derivative, were examined concerning the cell killing of human pancreatic cancer cell lines exposed to high doses of gamma-ray irradiation *in vitro*. The percentages of dead cells were analyzed with a multiwell plate reader to measure the fluorescence intensity of propidium iodide before and after a digitonin treatment. The sensitizing effect of PR-350 on cell killing by high-dose irradiation was confirmed by time-course, dose-dependency, and microscopic observations. In five of seven pancreatic cancer cell lines in which the number of dead cells was determined 5 days after 30 Gy irradiation in the presence of PR-350, the number was significantly increased under hypoxic conditions, but not under aerobic conditions. The selective radiosensitive effect of PR-350 on hypoxic cells was also confirmed by flow cytometry. The results indicate that PR-350 can enhance the killing of pancreatic cancer cells by high-dose irradiation under hypoxia, which supports its clinical radiosensitizing effects when administered during intraoperative irradiation to pancreatic cancer.

INTRODUCTION

Due to a lack of useful diagnostic procedures, many pancreatic cancers have been detected at a late stage of the disease when major vessels are involved and the tumors are unresectable (1). Even if these tumors are resected, the 5-year survival rates of pancreatic cancer are only 10% to 20% with a high incidence of local recurrence (2, 3). To overcome the biological aggressiveness of pancreatic cancer at the primary site, radiation has been applied as a major supportive therapy for advanced pancreatic cancer (4, 5). In particular, intraoperative radiation has the great advantage of delivering high doses of irradiation

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directly to a locally advanced pancreatic cancer (6, 7). However, the radiation response is limited because of the presence of radio-resistant hypoxic cells within tumors (8).

Several members of the family of hypoxic radiosensitizers have been extensively explored for over the past 20 years (9), and nitroimidazol derivatives are major compounds examined in clinical studies (10). However, most clinical trials have failed to document a significant sensitizing effect (11, 12). In these clinical studies, low doses of drugs were given repeatedly with fractionated irradiation because of their side effects, such as neurotoxicity. High doses of a radiosensitizer with high doses of irradiation are probably needed to produce the definitive effects of radiosensitizers.

PR-350, 2-nitroimidazole nucleoside derivative, was synthesized to reduce the lipophilicity and to prevent the accumulation of the drug in the central and peripheral nervous systems (13). PR-350 can be given at high doses during intraoperative high-dose irradiation. In fact, PR-350 suppressed the re-growth of a subcutaneously implanted mouse tumor after 20 Gy irradiation (14). The effects of radiosensitizers have been tested with a colony-forming assay after low-dose irradiation *in vitro* (15,16). Because over 10 Gy irradiation completely inhibits the growth of cancer cells (17,18), the sensitizing effects with high-dose irradiation must be examined by a direct cell-killing assay, such as a dye exclusion test (19); unfortunately, this is a time-consuming method.

We recently reported that cell death could be rapidly determined with a multi-well plate reader in a large number of samples by measuring the fluorescence intensity of propidium iodide before and after a digitonin treatment (20). Using this technique, in the present study, we examined the effect of PR-350 on cell killing by high-dose irradiation in human pancreatic cancer cells *in vitro*.

METHODS AND MATERIALS

Cell culturing conditions

Seven human pancreatic cancer cell lines were used in this study: Panc-1, Suit-2, KP-3, and KP-1N were generously provided by Dr. H. Iguchi (National Kyushu Cancer Center, Fukuoka, Japan); MIA-PaCa-2 was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan); Capan-1 and Capan-2 were obtained from American Type Culture Collection (Rockville, MD, USA). KP-3 and Capan-1 cells express wild-type p53, and the other cells have point mutations of p53. Human pancreatic fibroblasts were obtained during surgery, and were used during 10–15 passages.

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum, streptomycin (100 mg/ml), and penicillin (100 U/ml) at 37°C in a humidified atmosphere containing 90% air and 10% CO_2. All experiments were performed 24 hr after plating cells in Falcon Flat-bottom 24-well plates (Becton-Dickinson, Lincoln Park, NJ, USA) when the cells were multiplying exponentially. For the cell-killing assay, $2 - 3 \times 10^5$ cells per well were used at the beginning of the experiment. The number of cells was counted with a particle distribution counter, CDA500 (Sysmex, Kobe, Japan). All experiments were performed on triplicate wells, and each experiment was repeated at least three times.

Chemicals

PR-350, kindly provided by POLA Chemical Industry Inc. (Yokohama, Japan), was dissolved in phosphate-buffered saline (PBS). Cells were treated with PR-350 for 1 hr during irradiation at various concentrations (indicated below). After irradiation, the cells were washed with PBS and replaced with a fresh medium. Anticancer agents were obtained from the following suppliers: VP-16 and cisplatin were from Nippon Kayaku Co., Ltd. (Osaka, Japan); 5-FU was from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan); SN-38 was from Yakult Honsha Co., Ltd. (Tokyo, Japan). The anticancer agents were applied to the cells continuously during the entire experimental period.

Irradiation

Cells in 24-well plates were irradiated with various doses (10, 30, and 50 Gy) at room temperature with a ^{137}Cs source (Gamma Cell 40, Atomic Energy of Canada Ltd., Ontario, Canada) delivering 1.0 Gy/min. During irradiation, hypoxia was maintained in cells in 24-well plates, which were placed in a closed chamber, by flushing with 100% N_2 30 min prior to the treatment.

Determination of cell death

The cell death was evaluated by determining the fluorescence intensity of propidium iodide (PI), as described previously (20), with minor modifications. Briefly, PI was added to each well to reach a concentration of 30 mM, and the initial fluorescence intensity from dead cells was measured in a multi-well plate reader, CYTOFLUOR II (PerSeptive Biosystems Inc., Framingham, MA, USA) with 530-nm excitation and 645-nm emission filters. After the intensity was read, digitonin (600 mM) was added to each well to permeabilize all cells and label all nuclei with PI. After a 30-min incubation, the fluorescence intensity was measured again to obtain a value corresponding to the total cells. The percentage of dead cells was calculated as the proportion of the fluorescence intensity corresponding to dead cells to that corresponding to the total cells. Over 10 Gy irradiation completely inhibited cell proliferation; a total fluorescence intensity corresponding to the total cells was maintained at the initial fluorescence intensity during an experimental period. When the total fluorescence was decreased concomitant with significant cell killing, a decrease in the fluorescence intensity was regarded as representing dead cells.

Microscopic observation

Cells were plated on glass coverslips in a well and irradiated in the absence or presence of PR-350. The cells attached to the coverslips were stained with hematoxylin and eosin and observed under a microscope.

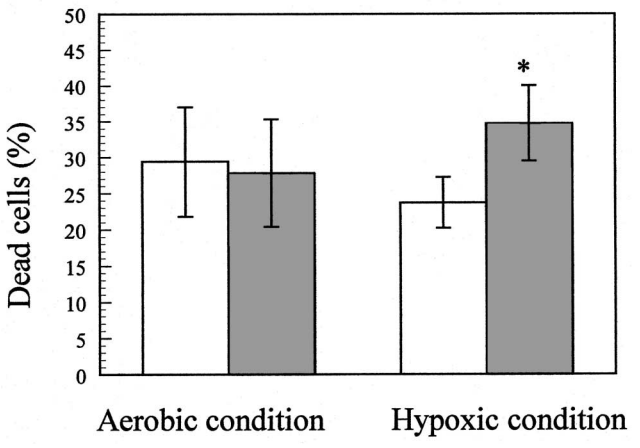

Fig. 1. Killing of a human pancreatic cancer cell line, Panc-1, exposed to 30 Gy irradiation under aerobic or hypoxic conditions. Five days after irradiation, the percentage of dead cells was determined with propidium iodide and digitonin, as described in methods and materials. Open bar, 30 Gy irradiation alone; closed bar; 30 Gy irradiation in the presence of 5 mM PR-350.
* Significantly different from 30 Gy irradiation alone, $p < 0.05$.

Table 1. Effect of PR-350 on the cell killing of pancreatic cancer cell lines by 30 Gy irradiation under hypoxia.

	Dead cells (%)[a]						
Cell line	Panc-1	Suit-2	MIA Paca-2	Capan-2	KP-3	KP-1N	Capan-1
P53 status	mt	mt	mt	mt	wt	mt	wt
Control	29.7 ± 2.4	21.9 ± 3.7	28.7 ± 1.9	12.7 ± 1.6	12.5 ± 0.9	24.2 ± 1.5	23.9 ± 4.6
PR-350 (5 mM)	43.4 ± 1.9[b]	36.8 ± 4.0[b]	38.9 ± 3.5[c]	19.2 ± 2.9[c]	17.0 ± 1.9[c]	24.8 ± 1.4	18.6 ± 1.8

[a] Dead cells were determined 5 days after a treatment.
[b] Significantly different from control, $P < 0.01$.
[c] Significantly different from control, $P < 0.05$.
mt = mutant type; wt = wild type

Morphological observation of apoptosis with a fluorescence microscope

After 30 Gy radiation with or without a hypoxic treatment, the PR-350 containing medium was displaced with a fresh one. The cells were cultured for 72 h, and the scraper-detached cells were fixed with 1% glutaraldehyde for 30 min at room temperature, washed twice with PBS, and stained for nuclear DNA with 1 mM Hoechst 33258. The nuclear morphology of stained cells was visualized using a fluorescence

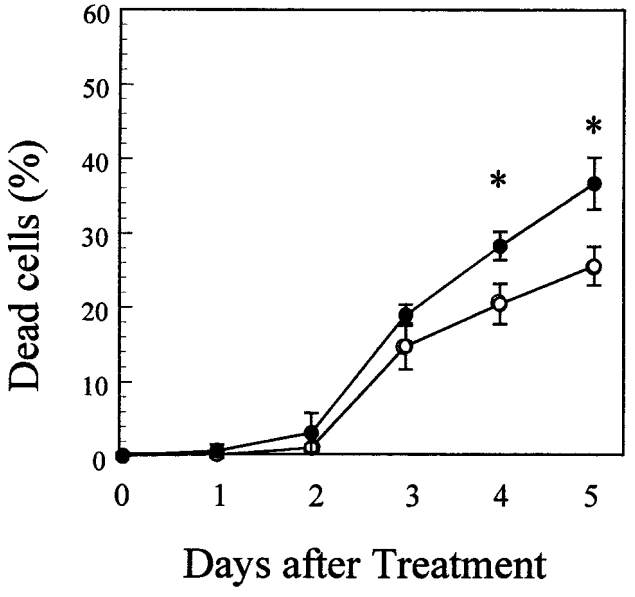

Fig. 2. Serial measurement of killing of a human pancreatic cancer cell line, Panc-1, after 30 Gy irradiation under hypoxia in the absence (open circle) or presence (closed circle) of 5 mM PR-350.
* Significantly different from 30 Gy irradiation alone, $p < 0.05$.

Table 2. Dose-dependent effect of PR-350 on the cell killing of Panc-1 cells by 30 Gy irradiation under hypoxia.

Dose of PR-350 (mM)	Dead cells (%)[a]
0	30.1 ± 3.3
1	33.4 ± 2.1
5	42.7 ± 2.3[b]
10	55.6 ± 2.0[c]
20	66.5 ± 2.2[c]

[a] Dead cells were determined 5 days after a treatment.
[b] Significantly different from a 30 Gy irradiation alone, $P < 0.01$.
[b] Significantly different from a 30 Gy irradiation alone, $P < 0.001$.

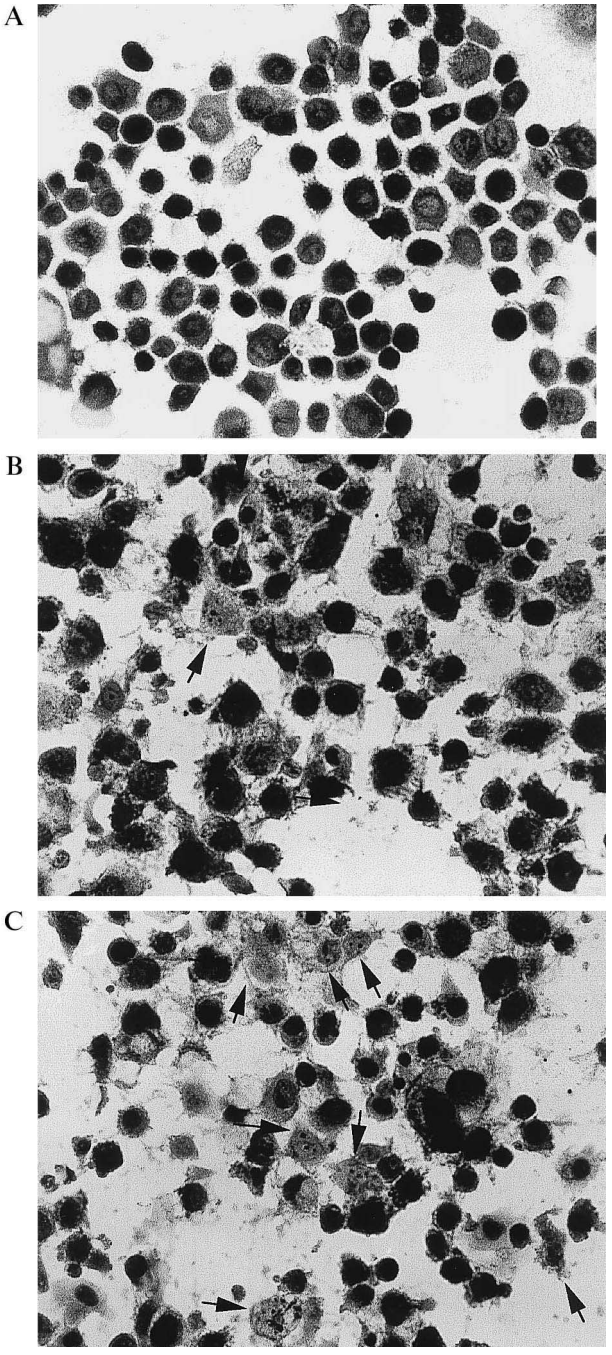

Fig. 3. Microscopic appearance of human pancreatic cancer cells, Panc-1, exposed to 30 Gy irradiation under hypoxia. Cells were stained with hematoxyline and eosin. Untreated controls (A). Cells 5 days after 30 Gy irradiation (B). Cells 5 days after 30 Gy irradiation in the presence of 5 mM of PR-350 (C). The arrows indicate the apoptotic cells with a fragmented nucleus. Magnification, × 200.

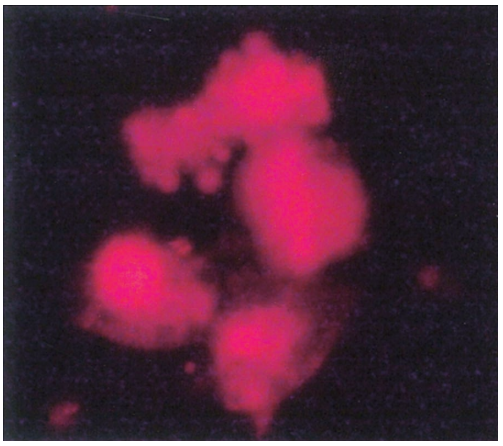

Fig. 4. Nuclear DNA fluorescent staining by Hoechst 33258 also exhibited a characteristic morphology of apoptosis, nuclear fragmentation. Magnification, × 400

microscope (Nikon DIAPHOT, Tokyo, Japan).

DNA degradation analysis by flow cytometry

Cultured Panc-1 cells were irradiated at a dose of 30 Gy in the absence or presence of PR-350 under an aerobic or nitrogenized circumstance, respectively; the medium was then replaced by a fresh culture medium. The flow cytometry for DNA degradation was undertaken 20 h after finishing the radiation. Trypsinized cells were collected, washed twice with PBS, and fixed in cold 70% ethanol for 4 h at 37°C. The cells were then washed with PBS, stained with a hypotonic fluorochrome solution [0.1% Triton X-100, 0.1% Tri-sodium citrate (pH 7.0) and 50 μ/ml propid-

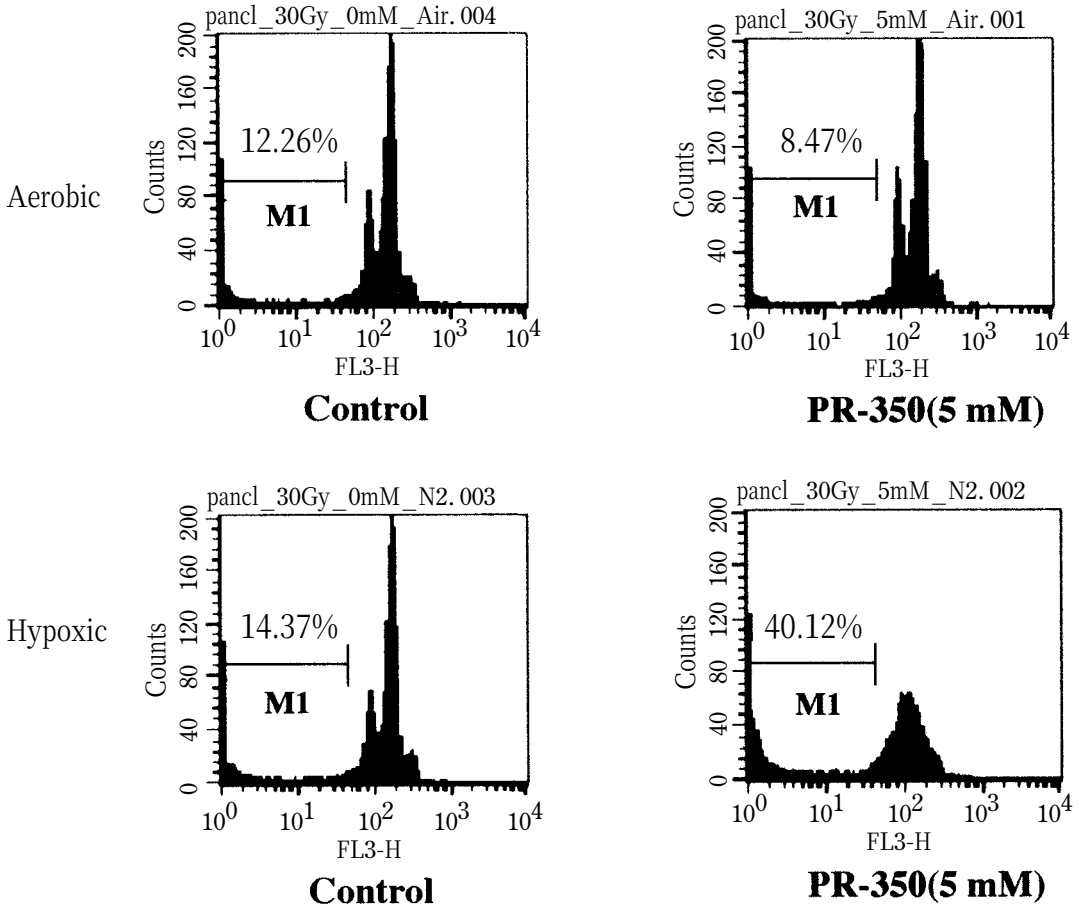

Fig. 5. PR-350 enhances radiation-induced cell apoptosis. Control or PR-350 treated Panc-1 cells were irradiated with a single dose of 30 Gy under either the aerobic or hypoxic condition. Aerobically, PR-350 failed to show any radiosensitive effect. In cells irradiated under hypoxic circumstance, PR-350 significantly increased the apoptotic rate from14.4% to 40.1%. The percentages of apoptosis were determined based on the sub-G_0 DNA content (M1) of the histogram.

ium iodide] and analyzed with a flow cytometer (FACSCaliber, Becton Dickinson, Bedford, MA, U.S.A). The DNA content was evaluated using Cell Quest software (Becton Dickinson).

Statistical analysis

The statistical significance was analyzed by the Mann-Whitney U test. The difference was considered to be significant at $p < 0.05$.

RESULTS

The effects of PR-350 on cell-killing enhancement under aerobic or hypoxic conditions were examined in pancreatic cancer cell lines after exposure to 30 Gy irradiation. Under aerobic culturing conditions, 29.4% of the Panc-1 cells were killed in 5 days, and that rate was not enhanced by 5 mM PR-350 (Fig. 1). Simultaneous PR-350 use during irradiation could not promote cell killing in two other pancreatic cancer cell lines (Suit-2 and Capan-2) under the aerobic condition (data not shown). Under hypoxia, 23.7% of the Panc-1 cells were killed in 5 days and 5 mM of PR-350 significantly increased the cell killing to 34.7% ($p < 0.05$). Similarly, with the addition of 5 mM PR-350, the cell killing by 30 Gy irradiation under hypoxia was significantly increased by 5% to 15% in five of seven pancreatic cancer cell lines (Table 1). The radiosensitizing effects of PR-350 were not significantly different in those cells harboring different types of p53 status, regardless of being mutated or wild.

Time-course and dose-dependency experiments confirmed the enhancing effects of PR-350 in Panc-1. As shown in Fig. 2, significant cell killing was observed 3 days after 30 Gy irradiation, and on days 4 and 5 the rate was significantly higher in the presence of 5 mM PR-350 than in its absence. A dose-dependent analysis of PR-350 revealed that cell killing by 30 Gy irradiation increased with increasing doses of PR-350 (Table 2). A statistically significant increase in cell death was obtained at doses of PR-350 over 5 mM. With a microscopic observation, Panc-1 cells exposed to 30 Gy irradiation showed nuclear condensation or fragmentation, indicating apoptotic cell death (Fig. 3B) ; the addition of 5 mM PR-350 increased the percentage of apoptotic cells (Fig. 3C). Nuclear DNA staining with Hoechst 33258 further provided a clearer image of radiation-induced apoptotic cell death (Fig. 4). The radiosensitizing effects of PR-350 were also confirmed by flow cytometry assayed for a DNA degradation analysis. As shown in Fig. 5, PR-350 characteristically enhanced the radiation-induced apoptotic effect on hypoxic Panc-1 cells.

The effects of PR-350 were examined in different cell-killing doses of irradiation (Fig. 6). At day 5 after exposure, the percentage of dead cells showed increases correlated with increasing doses of irradiation: 20% with 10 Gy, 28% with 30 Gy, and 46% with 50 Gy. The addition of 5 mM PR-350 significantly increased the cell death by approximately 10% at every dose level.

The cytotoxic effect of PR-350 was also investigated. A one-hour treatment of PR-350 did not show any cell killing or any cell-proliferation inhibitory effects on Panc-1 cells or the cultured human pancreatic fibroblasts at doses ranging from 1 mM to 20 mM (data not shown).

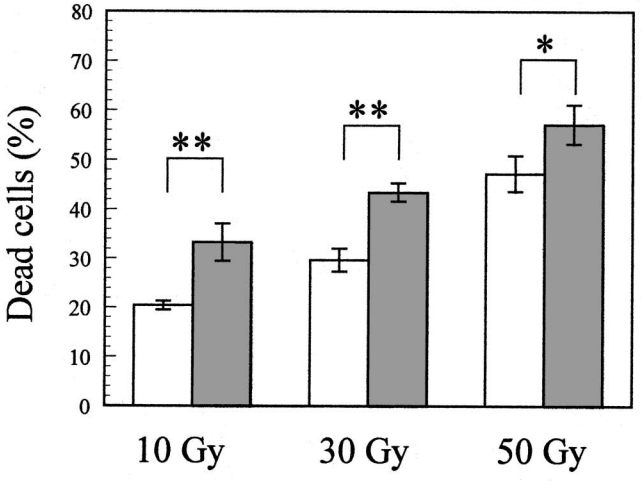

Fig. 6. Killing of a human pancreatic cancer cell line, Panc-1, exposed to 10 Gy, 30 Gy or 50 Gy irradiation under hypoxia. Open bar, irradiation only; Closed bar, irradiation in the presence of 5 mM PR-350.
* Significantly different from irradiation alone, $p < 0.05$.
** Significantly different from irradiation alone, $p < 0.01$.

Table 3. Effect of PR-350 and anticancer agents on the cell Killing of Panc-1 cells exposed to 30 Gy irradiation under hypoxia.

Treatment	Dead cells (%)[a]	
	Without irradiation	With 30 Gy irradiation
Control	0.2 ± 0.3	24.3 ± 3.4
PR-350 (5 μM)	−0.6 ± 6.4	33.1 ± 1.8[b]
VP-16 (5 μM)	24.4 ± 1.7	44.1 ± 3.9[b]
Cisplatin (10 μM)	27.4 ± 2.3	40.3 ± 9.5[b]
SN-38 (10 μM)	48.5 ± 7.9	61.9 ± 6.2[b]
5-FU (50 μM)	14.2 ± 5.0	27.1 ± 8.0

[a] Dead cells were determined 5 days after a treatment.
[b] Significantly different from 30 Gy irradiation alone, $P < 0.01$.

The radiosensitizing effects of four anticancer agents on pancreatic cancer cells were compared to that of PR-350 (Table 3). The use of 30 Gy irradiation with VP-16, cisplatin, SN-38, or PR-350 significantly increased the rate of cell killing comparing to that with irradiation alone. Treatments with the anticancer agents alone at the radiosensitizing doses significantly killed Panc-1 cells, which contrasted with the noncytotoxic effects of PR-350.

DISCUSSION

Our present results clearly indicate that PR-350 enhances the killing of human pancreatic cancer cells exposed to high-dose irradiation under hypoxic conditions. The results were confirmed by cell-killing assay, apoptosis analysis and morphologic observations. The cell killing doses of irradiation adopted in the present study are comparable to those used clinically during intraoperative irradiation for the treatment of advanced pancreatic cancer. The doses of PR-350 in the present experiment were achievable concentrations in patients when administered intravenously. Taking together, PR-350 will work as a potential sensitizer of intraoperative radiotherapy for advanced pancreatic cancer.

Since the enforced cell apoptotic event detected by flow cytometry appeared earlier than that of the cell-killing enhancement determined by the PI assay, cell apoptosis is reasonably regarded as being the main component of the cell-killing effect which is caused by irradiation and is enhanced by PR-350.

As a radiosensitizer, the clinical benefit of PR-350 is different from that of anticancer agents. As shown in the present study, anticancer agents enhance the cell killing caused by high-dose irradiation, but they also kill cells without irradiation. Therefore, anticancer agents at radiosensitizing doses must have side effects on non-irradiated organs, including bone marrow and intestines. In contrast, the treatment of PR-350 alone did not show even cytostatic effects to either normal fibroblast or malignant pancreatic cells at very high doses of up to 20 mM. High-dose irradiation is administered intraoperatively to a restricted area of the tumors, and a covered cylinder is used to avoid irradiation of surrounding normal tissues. Therefore, systemically administered PR-350 will show enhancing effects only on the irradiated tumor and will not harm surrounding non-irradiated normal tissues.

The present results are obviously different from those of previous studies which used a colonogenic assay to examine the effects of a radiosensitizer on the cell-proliferating ability of cancer cells (21, 22). Because irreversible cell death and reversible cell proliferating inhibition are completely different in terms of their molecular signaling mechanisms (23, 24), discrimination of the two cytotoxic effects is necessary (25). When evaluating the clinical antitumor effects of the treatments, cell killing is more important than cell proliferation inhibition (26, 27). The sensitizing effects of PR-350 for cell killing may promise success in the on-going clinical trials of PR-350 in intraoperative irradiation to locally advanced pancreatic cancer in Japan.

REFERENCES

1. Warshaw, A. L. and Fernandez-del Castillo C. (1992) Pancreatic carcinoma. *N. Engl. J. Med.* **326**: 455–465.

2. Tepper, J., Nardi G. and Sutt H. (1976) Carcinoma of the pancreas: review of MGH experience from 1963 to 1973. Analysis of surgical failure and implications for radiation therapy. *Cancer* **37**: 1519–1524.
3. Griffin, J. F., Smalley S. R., Jewell W., Paradelo J. C., Reymond R. D., Hassanein R. E. and Evans R. G. (1990) Patterns of failure after curative resection of pancreatic carcinoma. *Cancer* **66**: 56–61.
4. Johnstone, P.A. and Sindelar W.F. (1993) Patterns of disease recurrence following definitive therapy of adenocarcinoma of the pancreas using surgery and adjuvant radiotherapy:correlations of a clinical trial. *Int. J. Radiat. Oncol. Biol. Phys.* **27**: 831–834.
5. Gastrointestinal Tumor Study Group. (1988) Treatment of locally unresectable carcinoma of the pancreas: comparison of combined-modality therapy (chemotherapy plus radiotherapy) to chemotherapy alone. *J. Natl. Cancer Inst.* **80**: 751–755.
6. Staley, C. A., Lee J. E., Cleary K. R., Abbruzzese J. L., Fenoglio C. J., Rich T. A. and Evans D. B. (1996) Preoperative chemoradiation, pancreaticoduodenectomy, and intraoperative radiation therapy for adenocarcinoma of the pancreatic head. *Am. J. Surg.* **171**: 118–124.
7. Shibamoto, Y., Manabe T., Baba N., Sasai K., Takahashi M., Tobe T. and Abe M. (1990) High dose, external beam and intraoperative radiotherapy in the treatment of resectable and unresectable pancreatic cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **19**: 605–611.
8. Coleman, C. N. (1996) Conference summary: the ninth international conference on the Chemical Modifiers of Cancer Treatment. *Br. J. Cancer - Supplement* **27**: S297–304.
9. Rauth, A. M., Melo T. and Misra V. (1998) Bioreductive therapies: an overview of drugs and their mechanisms of action. *Int. J. Radiat. Oncol. Biol. Phys.* **42**: 755–762.
10. Denny, W. A., Wilson W. R. and Hay M. P. (1996) Recent developments in the design of bioreductive drugs. *Br. J. Cancer - Supplement* **27**: S32–38.
11. Dische, S. (1989) Keynote address: hypoxic cell sensitizers: clinical developments. *Int. J. Radiat. Oncol. Biol. Phys.* **16**: 1057–1060.
12. Lee, D.J., Cosmatos D., Marcial V.A., Fu K.K., Rotman M., Cooper J.S., Ortiz H.G., Beitler J.J., Abrams R.A., Curran W.J. and et al. (1995) Results of an RTOG phase III trial (RTOG 85-27) comparing radiotherapy plus etanidazole with radiotherapy alone for locally advanced head and neck carcinomas. *Int. J. Radiat. Oncol. Biol. Phys.* **32**: 567–576.
13. Murayama, C., Suzuki A., Sato C., Tanabe Y., Shoji T., Miyata Y., Nishio A., Suzuki T., Sakaguchi M. and Mori T. (1993) Radiosensitization by a new potent nucleoside analog: 1-(1',3',4'-trihydroxy-2'-butoxy)methyl-2-nitroimidazole(RP-343). *Int. J. Radiat. Oncol. Biol. Phys.* **26**: 433–443.
14. Oya, N., Shibamoto Y., Sasai K., Shibata T., Murata R., Takagi T., Iwai H., Suzuki T. and Abe M. (1995) Optical isomers of a new 2-nitroimidazole nucleoside analog (PR-350 series): radiosensitization efficiency and toxicity. *Int. J. Radiat. Oncol. Biol. Phys.* **33**: 119–127.
15. Courtenay, V. D., Smith I. E. and Steel G. G. (1978) The effect of misonidazole on the radiation response of clonogenic human pancreatic carcinoma cells. *Br. J. Cancer - Supplement* **37**: 225–227.
16. Lawrence, T. S., Chang E. Y., Hahn T. M., Hertel L. W. and Shewach D. S. (1996) Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine. *Int. J. Radiat. Oncol. Biol. Phys.* **34**: 867–872.
17. Verovski, V. N., Van den Berge D. L., Soete G. A., Bols B. L. and Storme G.A. (1996) Intrinsic radiosensitivity of human pancreatic tumour cells and the radiosensitising potency of the nitric oxide donor sodium nitroprusside. *Br. J. Cancer* **74**: 1734–1742.
18. Coucke, P. A., Decosterd L. A., Li Y. X., Cottin E., Chen X., Sun L. Q., Stern S., Paschoud N. and Denekamp J. (1999) The ribonucleoside diphosphate reductase inhibitor (E)-2'-deoxy-(fluoromethylene) cytidine as a cytotoxic radiosensitizer in vitro. *Cancer Res.* **59**: 5219–5226.
19. Chmura, S. J., Mauceri H. J., Advani S., Heimann R., Beckett M. A., Nodzenski E., Quintans J., Kufe D. W. and Weichselbaum R. R. (1997) Decreasing the apoptotic threshold of tumor cells through protein kinase C inhibition and sphingomyelinase activation increases tumor killing by ionizing radiation. *Cancer Res* **57**: 4340–4347.
20. Zhang, L., Mizumoto K., Sato N., Ogawa T., Kusumoto M., Niiyama H. and Tanaka M. (1999) Quantitative determination of apoptotic death in cultured human pancreatic cancer cells by propidium iodide and digitonin. *Cancer Lett.* **142**: 129–137.
21. Bernhard, E. J., McKenna W. G., Hamilton A. D., Sebti S. M., Qian Y., Wu J. M. and Muschel R. J. (1998) Inhibiting Ras prenylation increases the radiosensitivity of human tumor cell lines with activating mutations of ras oncogenes. *Cancer Res.* **58**: 1754–1761.
22. Robertson, J. M., Shewach D. S. and Lawrence T. S. (1996) Preclinical studies of chemotherapy and radiation therapy for pancreatic carcinoma. *Cancer* **78**: 674–679.
23. Samejima, K., Tone S., Kottke T. J., Enari M., Sakahira H., Cooke C. A., Durrieu F., Martins L. M., Nagata S., Kaufmann S. H. and Earnshaw W.C. (1998) Transition from caspase-dependent to caspase-independent mecha-

nisms at the onset of apoptotic execution. *J. Cell. Biol.* **143**: 225–239.

24. Kagawa, S., Fujiwara T., Hizuta A., Yasuda T., Zhang W.W., Roth J.A. and Tanaka N. (1997) p53 expression overcomes p21WAF1/CIP1 mediated G1 arrest and induces apoptosis in human cancer cells. *Oncogene* **15**: 1903–1909.
25. Waldman, T., Zhang Y., Dillehay L., Yu J., Kinzler K., Vogelstein B. and Williams J. (1997) Cell-cycle arrest versus cell death in cancer therapy . *Nature Med.* **3**: 1034–1036.
26. Kurdoglu, B., Cheong N., Guan J., Corn B. W., Curran W. J., Jr. and Iliakis G. (1999) Apoptosis as a predictor of paclitaxel-induced radiosensitization in human tumor cell lines. *Clin. Cancer Res.* **5**: 2580–2587.
27. Coco Martin, J. M., Balkenende A., Verschoor T., Lallemand F. and Michalides R. (1999) Cyclin D1 overexpression enhances radiation-induced apoptosis and radiosensitivity in a breast tumor cell line. *Cancer Res.* **59**: 1134–1140.