Pergamon

European Journal of Cancer, Vol. 35, No. 9, pp. 1374–1380, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00134-3

Original Paper

Role of Bcl-2 Family Proteins (Bax, Bcl-2 and Bcl-X) on Cellular Susceptibility to Radiation in Pancreatic Cancer Cells

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The aim of this study was to examine Bax, Bcl-2 and Bcl-X_L proteins in human pancreatic cancer cell lines and to clarify the mechanism of radiation resistance. PANC-1 and AsPC-1 pancreatic cell lines were used, both having mutated *p53*. Radioresistant PANC-1/Rad cells and AsPC-1/Rad cells were obtained by repeated 5 Gy irradiation of PANC-1 cells and AsPC-1 cells, respectively. Radiation was found to inhibit the growth of PANC-1 cells and AsPC-1 cells. After exposure to radiation, detached cells were subjected to FITC-TUNEL staining to calculate the ratio of apoptosis. TUNEL positive ratios increased dose-dependently in both cell lines. Western blotting showed that the basal level of the Bax/Bcl-2 ratio reflected the radiosensitivity of these cell lines, and Bax expression was obviously upregulated after irradiation in the presence of mutated *p53*, but Bcl-2 expression remained almost constant. Both PANC-1/Rad and AsPC-1/Rad cells had greater Bcl-X_L expression than the parental cells, and the basal level of the Bax/Bcl-2 ratio was no longer predictive of radiosensitivity. Upregulated expression of Bax protein after irradiation was not related to induction of apoptosis in these cells, suggesting that overexpression of Bcl-X_L and functional reconstruction of Bcl-2 family proteins are important factors in acquired radioresistance.

Key words: apoptosis, pancreatic cancer, radiation, cell line, oncoproteins
Eur J Cancer, Vol. 35, No. 9, pp. 1374–1380, 1999

INTRODUCTION

Apoptosis is a form of cell death that occurs as the result of a variety of insults including radiation. Apoptosis is characterised by fragmentation of DNA caused by enzymatic cleavage of DNA into 180 to 200 bp segments, which correspond to nucleosomes in size [1]. It has also been reported that the fragmentation is initiated by endonucleolytic activity that cleaves DNA into 50 to 300 kb fragments, which correspond to chromatin loop domains, and that subsequent cleavage at internucleosomal sites is not always involved in the apoptotic process [2–4]. Much interest has been devoted to the understanding of the mechanism of apoptosis in relation to Bcl-2 family proteins such as Bax, Bcl-2 and Bcl-X_L. Bax, Bcl-2 and Bcl-X_L.

Bax and Bcl-2 are members of a family of intracellular proteins that are thought to be important regulators of apoptosis [5]. It has been reported that Bax inactivates Bcl-2 proteins through heterodimerisation and that the ratio of Bax to Bcl-2 proteins increases during the apoptosis induction [5, 6]. The ratio of Bax to Bcl-2 proteins is involved in the susceptibility to apoptotic stimuli in normal haematopoietic cell lines [7] and in a variety of cancer cell lines such as leukaemia, breast cancer, and lung cancer [6, 8, 9]. The Bcl-X_L protein is also a member of the Bcl-2 family and is thought to be a negative regulator of apoptosis [7, 10]. The induction of Bcl-X_L depends on normal p53 functioning [10], and overexpression of Bcl-X_L is associated with a high tumour grade [11] and with acquired resistance to chemotherapy [12] and radiation [10].

It is widely accepted that the bax promoter contains a typical *p53* tumour suppressor gene-binding site and that *p53* exerts its role as inducer of apoptosis partly by transactivating the expression of bax gene [13]. Wild-type *p53* gene products can inhibit bcl-2 expression through their interaction with a *p53*-dependent negative response in the *bcl-2* gene [6]. It has

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Received 26 Oct. 1998; revised 7 May 1999; accepted 7 May 1999.

been reported that the Bax protein is not upregulated in cells with a mutated *p53* after apoptotic stimuli [14]. It is, however, not clear whether the presence of any type of *p53* mutation prevents an upregulation of Bax protein and whether a *p53* independent pathway for upregulating Bax protein exists. In fact, we showed that in PANC-1 cells, a well-known human pancreatic cancer cell line which harbours mutated *p53*, Bax protein was upregulated following 2 h exposure to cisplatin [15]. This study showed that not only in PANC-1 cells but also in AsPC-1 cells, a human pancreatic cancer cell line with mutated *p53*, Bax proteins were upregulated following X-irradiation.

In this study, we focused on Bax, Bcl-2 and Bcl-X_L proteins and used human pancreatic cancer cell lines to clarify the mechanism of radiation-induced apoptosis and radiation resistance.

MATERIALS AND METHODS

Cells

The human pancreatic cancer cell lines, PANC-1 and AsPC-1, were purchased from American Type Culture Collection. These cell lines have mutated *p53*. PANC-1 cells were maintained as a monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C, in a humid atmosphere of 5% CO_2/95% air. AsPC-1 cells were maintained in RPMI1640 medium supplemented with 20% FBS and under the same conditions as for PANC-1 cells. All experiments were performed during the exponential growth phase.

The radiation-resistant cell lines, PANC-1/Rad and AsPC-1/Rad, were obtained by repeated irradiation at 5 Gy once a week and passage between the irradiations. PANC-1/Rad-N and AsPC-1/Rad-N refer to cells that were irradiated at 5 Gy for N times during the exponential growth phase. PANC-1/Rad-17, PANC-1/Rad-24 and AsPC-1/Rad-13 cells were employed to examine growth inhibition by irradiation in order to identify the radioresistance. PANC-1/Rad-20 and AsPC-1-15 were employed to study the relationship between acquired radioresistance and the expressions of Bax, Bcl-2 and Bcl-X_L.

X-irradiation

PANTAK X radiation system (Shimadzu Co., Kyoto, Japan) was employed to irradiate cells. X-irradiation was performed at room temperature at a dose rate of 0.97 Gy/min by using a 160 kV X-ray generator unit operating at 25 mA and equipped with an external 0.5 mm Cu filter.

Evaluation of growth inhibition

Cells were seeded into 60 mm culture dishes (3×10^5 cells/dish) in 5 ml of medium and incubated for 48 h. After incubation, the cells were exposed to X-radiation at doses of 2, 5 and 10 Gy. The medium was replaced with fresh medium immediately after irradiation. 48 h later, the number of attached cells was determined in triplicate with a Coulter-counter (Coulter Electronics, Beds, London).

Dose–response of apoptosis induction by X-radiation

PANC-1 cells and AsPC-1 cells were seeded into 25 cm^2 culture flasks (5×10^4 cells/flask) in 5 ml of medium and incubated for 48 h. After incubation, the cells were exposed to X-radiation at doses of 2, 5 and 10 Gy. 24 h later, the medium was collected and centrifuged at $150\times g$ for 5 min. The resultant cell pellets (referred to as detached cells) were washed with PBS once, centrifuged as described above and fixed with 4% paraformaldehyde (PFA) buffered with 0.1 M sodium phosphate (pH 7.4) for 15 min. The fixed cells were centrifuged again and smeared on to slides pretreated with 3-amino-propyltriethoxysilane (Matsunami Co., Osaka, Japan). The slides were then dried for 48 h at room temperature and subjected to modified FITC-TUNEL staining. The monolayer cells in the flasks (referred to as attached cells) were trypsinised at each time point and then examined by TUNEL staining in the same manner as used for the detached cells.

Modified FITC-TUNEL staining and its assessment

Details of this procedure, which is a modified version of the method of Gavrieli and colleagues have been described previously [16–18]. For counter-staining of the intact nuclei, 0.5 mg/L propidium iodide in 1.12% sodium citrate was employed. The TUNEL positive nuclei were counted on the photoslides and expressed as a percentage of the total nuclei. The methods for smearing cells on to slides and counting cells with TUNEL positive nuclei have been previously described [15].

Hoechst staining

To verify that apoptosis was the contributing form of death, detached cells were harvested from the 10 Gy irradiated cultures at 24 h and subjected to Hoechst 33342 staining, which can discriminate cells with normal nuclei from cells with shrunken or fragmented nuclei. Detached cells were washed with PBS once and centrifuged as described above and the pellets were fixed with 4% PFA for 5 min. The pellets were then washed again with PBS and stained with Hoechst 33342 (final concentration of 2%).

Protein extraction and Western blotting

PANC-1, AsPC-1, PANC-1/Rad and AsPC-1/Rad cells were irradiated at 5 Gy and the medium was replaced with fresh medium. At 4, 8, 12, 18 and 24 h, the attached cells were lysed and the supernatants collected and resuspended with the same volume of gel-loading buffer as previously described by Wada and colleagues [19]. For Western blotting, proteins in quantities equal to those of the lysates were separated by SDS-PAGE (12% gels) and transblotted on to nitrocellulose filters. Proteins were detected using anti-Bcl-2 monoclonal antibody (MAb) (clone 124, DAKO A/S, Denmark), anti-Bax MAb (clone 4F11, MBL, Nagoya, Japan) and anti-Bcl-X polyclonal antibody (clone SC634, Santa Cruz, California, U.S.A.).

Data analysis

Means ± SEM are shown. The comparative statistical evaluations among groups were first performed by a two-way analysis of variance (ANOVA) for repeated measures, followed by a *post-hoc* test using a Tukey-Kramer HDS for multiple comparisons for means. A probability level of <0.05 was considered significant.

RESULTS

Growth inhibition

Growth inhibition curves of PANC-1 cells and AsPC-1 cells after irradiation are shown in Figure 1(a). Radiation inhibited the growth of PANC-1 cells and AsPC-1 cells

dose-dependently. The doses required for 50% growth inhibition (ID_{50}) were 5.0 Gy for PANC-1 cells and 3.0 Gy for AsPC-1 cells. PANC-1/Rad cells were less sensitive to radiation than PANC-1 cells (Figure 1c), and AsPC-1/Rad cells less sensitive than AsPC-1 cells (Figure 1d). This sensitivity decreased as irradiation was repeated for both cell lines (illustrated for PANC-1 cells, Figure 1c). The degree of inhibition was approximately 20% of the PANC-1 cells in PANC-1/Rad-13 and 5% in PANC-1/Rad-24, and approximately 8% of the AsPC-1 cells in AsPC-1/Rad-17.

TUNEL and Hoechst staining

After the irradiation, TUNEL positive nuclei were observed as light-green signals in fluorescence microscope as previously described by Lee and colleagues [15]. The TUNEL positive ratio of attached cells was less than 1% for

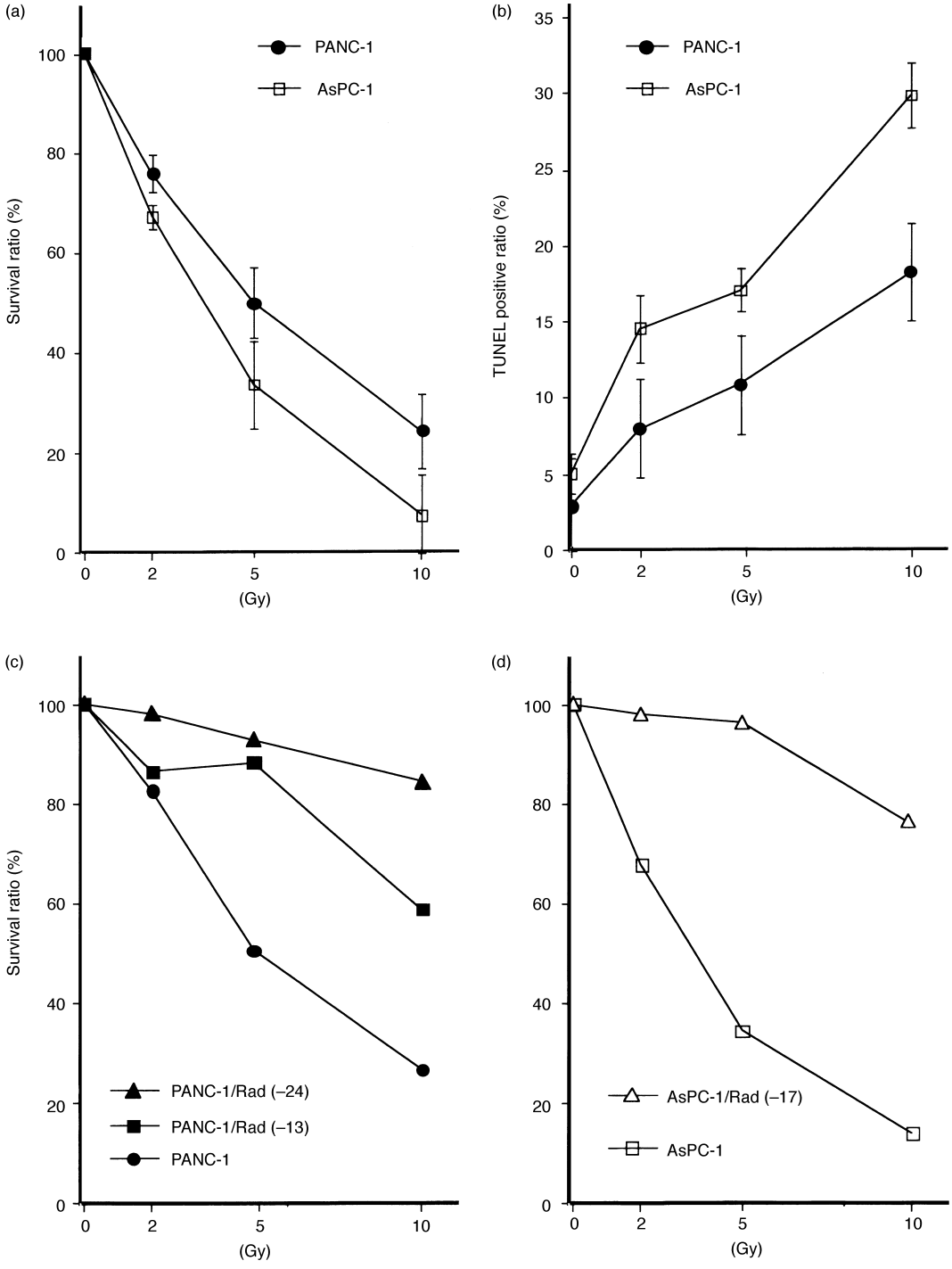

Figure 1. The effects of radiation on PANC-1 cells and AsPC-1 cells. (a) Dose-inhibition curves of PANC-1 and AsPC-1 cells. Mean ± SEM (three experiments). (b) Dose-response changes of TUNEL positive ratio in PANC-1 and AsPC-1 cells. Mean ± SEM (three experiments). The dose-inhibition curves of PANC-1/Rad cells (c) and AsPC-1/Rad cells (d). Inhibition of cell growth was significantly attenuated in PANC-1/Rad-13, PANC-1/Rad-24, and AsPC-1/Rad-17 cells compared with their parental cells.

both PANC-1 cells and AsPC-1 cells regardless of the dose of radiation. Hoechst 33342 staining of the attached cells showed that the ratio of cells with nuclear shrinkage or nuclear fragmentation was less than 1%.

For PANC-1 cells and AsPC-1 cells, the TUNEL positive ratios of detached cells obtained at doses of 0, 2, 5 and 10 Gy are shown in Figure 1(b). TUNEL positive ratios at 10 Gy in PANC-1 cells and AsPC-1 cells were consistent with the ratios of cells with nuclear changes observed under Hoechst staining (data not shown). TUNEL positive ratios of detached cells increased in dose-dependently in both cell lines, while the ratios were significantly lower in PANC-1 than in AsPC-1 cells ($P<0.05$, ANOVA).

Western blot analysis

The extracted proteins from untreated cells or cells treated with 5 Gy radiation were examined for Bax, Bcl-2 and Bcl-X expressions in PANC-1, AsPC-1, PANC-1/Rad-20 and AsPC-1/Rad-15 cells. When untreated, AsPC-1 cells showed higher levels of Bax expression and lower levels of Bcl-2 expression than did PANC-1 cells (Figure 2a). PANC-1/Rad-20 cells and AsPC-1/Rad-15 cells showed trace levels of Bax, slightly lower levels of Bcl-2 and much higher levels of Bcl-X_L than their parental cells (Figure 2b). Although the anti-Bcl-X antibody used in this study recognises both larger and smaller molecular forms of Bcl-X, the Bcl-X_L protein was found to have migrated as a doublet of proteins with an apparent molecular mass of 29–32 kDa; a 20 kDa band compatible with Bcl-X_S protein was detected as only a trace band.

Following 5 Gy irradiation, expression of Bax increased in a time-dependent manner, whilst that of Bcl-2 and Bcl-X_L remained almost unchanged in our four cell lines (Figure 3). The intensity of each band was measured by an image analyser, and the Bax to Bcl-2 ratio at each time point was calculated relative to that at time 0 of PANC-1 cells. Figure 4 shows the changes in relative Bax/Bcl-2 ratio after 5 Gy irradiation in the four cell lines. The Bax/Bcl-2 ratio of AsPC-1 cell at time 0 was approximately 5-fold greater than PANC-1 cells. PANC-1/Rad-20 cells and AsPC-1/Rad-15 cells showed lower Bax/Bcl-2 ratio at time 0 and smaller increases after irradiation than their parental cells.

DISCUSSION

Bax, a potent inducer of apoptosis, is a 21-kDa protein with 43% homology to Bcl-2, which is a suppressor of apoptosis [5]. Bax interacts with the Bcl-2 protein, a suppressor of apoptosis, and this interaction results in acceleration of the rate of cell death, probably through altering the ratio of Bax to Bcl-2 [13, 20]. In addition, there is mounting evidence that Bcl-X_L, another anti-apoptotic member of the Bcl-2 family, also exerts an important effect on the regulation of apoptotic cell death in advanced cancer [10] and in chemoresistant

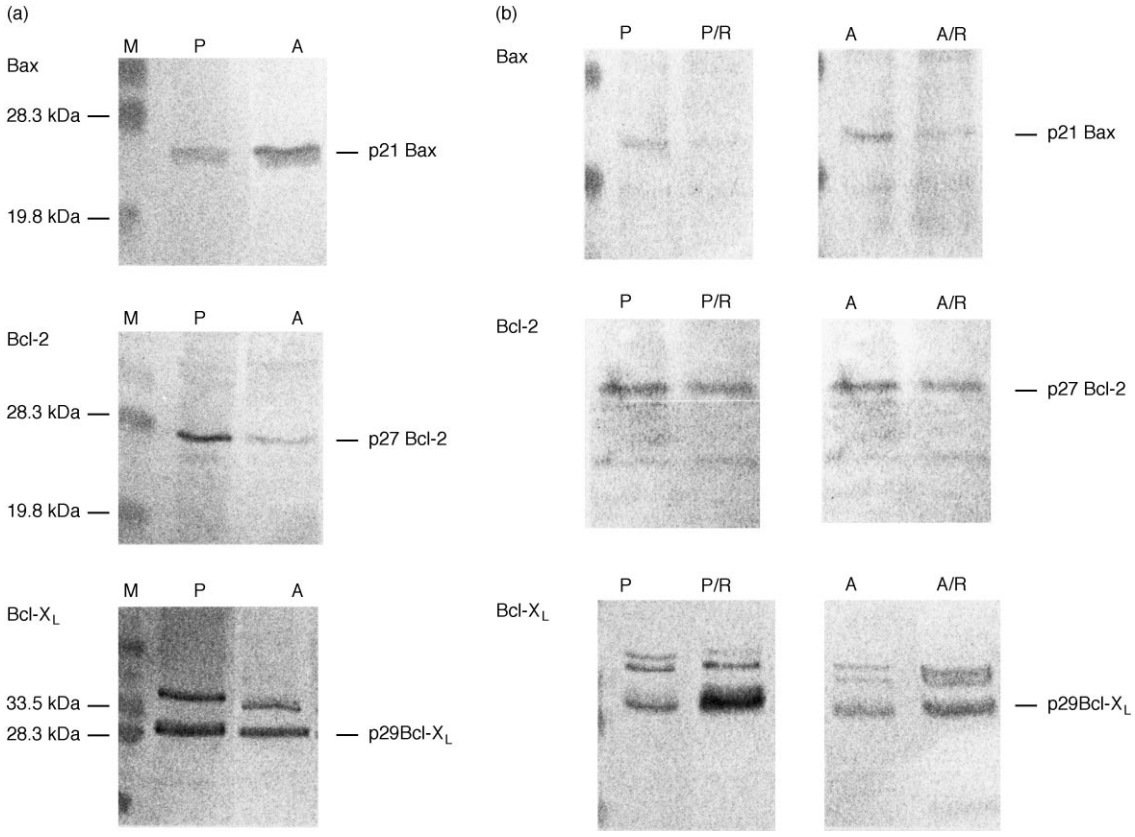

Figure 2. Western blot analysis. (a) Basal level of Bax and Bcl-2 proteins in PANC-1 (P) and AsPC-1 (A) cells. AsPC-1 cells showed 1.5-fold higher level of Bax expression and 0.3-fold lower level of Bcl-2 expression than did PANC-1 cells on the same blotted membrane (NIH image analyser). M, marker ladder. (b) Comparison of basal level of Bax, Bcl-2 and Bcl-X_L between radioresistant cells and their parental cells. PANC-1/Rad (P/R) cells showed a 0.3-fold lower level of Bax expression and 4-fold higher expression of Bcl-X_L expression than their parental cells. AsPC-1/Rad (A/R) cells showed 0.4-fold lower level of Bax expression and 3-fold higher expression of Bcl-X_L expression than their parental cells (NIH image analyser). Different colour developing times were used for ASPC-1 and PANC-1 cells for Bcl-2 and Bax expression.

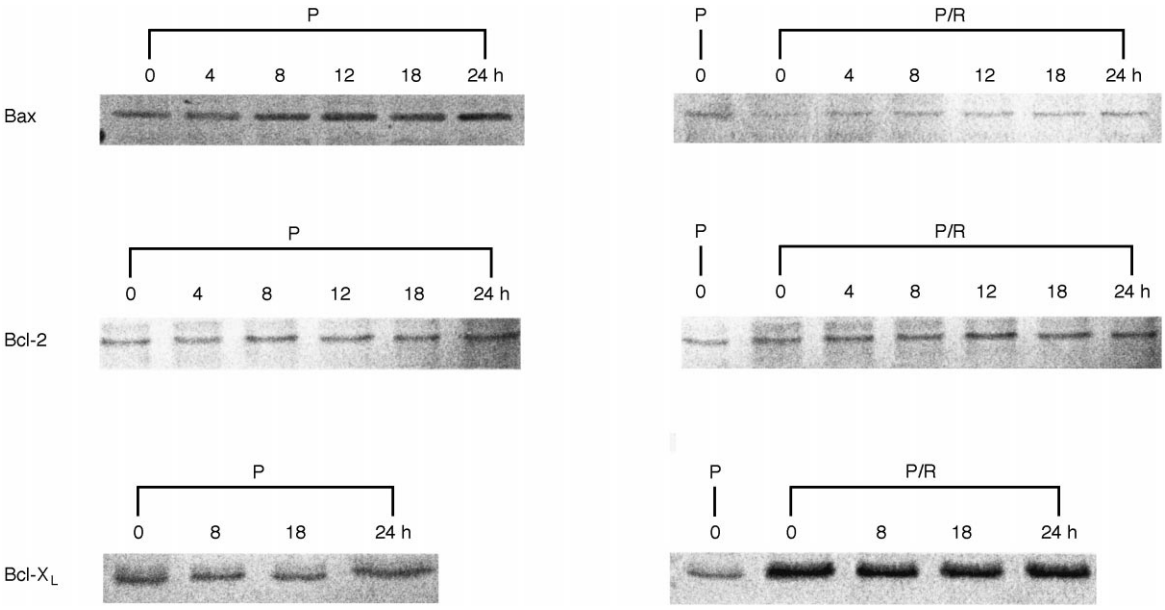

Figure 3. Changes in expressions of Bax, Bcl-2 and Bcl-X_L for PANC-1 (P) and PANC-1/Rad (P/R) cells following 5 Gy irradiation. Expression of Bax increased in a time-dependent manner, whilst that of Bcl-2 and Bcl-X_L remained almost the same as their own control (time 0). Note the high level of basal expression of Bcl-X_L, which was maintained throughout the time-course, in radioresistant PANC-1/Rad cells.

cancer [12]. In the current study, human pancreatic cancer cell lines were employed and the apoptotic cell death caused by radiation was verified in terms of expression of these Bcl-2 family proteins as detected by Western blot analysis.

PANC-1 cells and AsPC-1 cells are established human pancreatic cancer cell lines with mutated *p53*. The growth of PANC-1 cells and AsPC-1 cells attached to the monolayer was inhibited by irradiation dose-dependently. The cells detached from the monolayer were stained with the modified FITC (fluorescein isothiocyanate)-TUNEL (TdT-mediated dUTP-biotin Nick End Labeling) method, which can detect DNA fragmentation occurring in a single cell in a tissue specimen. This modified FITC-TUNEL (TUNEL) staining of cells in culture is not widely accepted as a method for detection of apoptosis, but less than 1% of the attached cells in culture flasks were stained by either the trypan blue (TB) or the TUNEL method, and somewhat less than 1% of the cells positively stained with TB were stained with the TUNEL method (data not shown). It can be said that neither live cells nor necrotic cells were stained with TUNEL method. Furthermore, the ratio of TUNEL positive cells showed good accordance with the ratio obtained with Hoechst 33342 staining. It can, therefore, be concluded that TUNEL staining detected apoptosis with enough accuracy for quantitative assessment of apoptotic cell death.

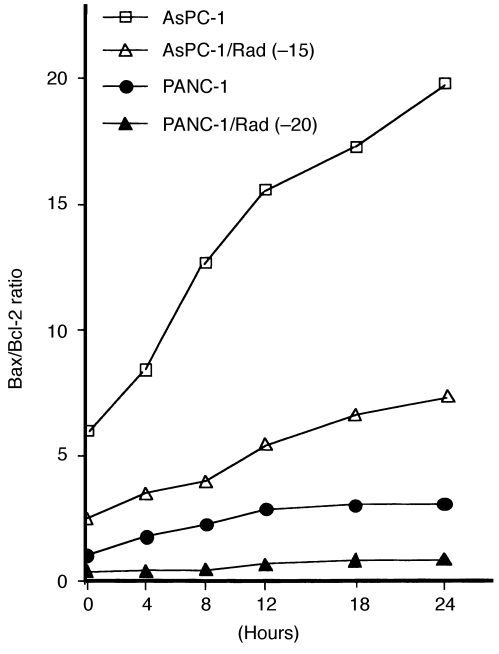

Figure 4. Changes in Bax/Bcl-2 ratio for PANC-1, AsPC-1, PANC-1/Rad-20 and AsPC-1/Rad-15 cells after 5 Gy irradiation. The intensity ratios were calculated in comparison to that of control (PANC-1, time 0), which was adjusted to 1.

The growth of both PANC-1 and AsPC-1 cells was inhibited by irradiation dose-dependently, and the degree of inhibition was lower for PANC-1 than for AsPC-1 at any given dose. TUNEL positive ratios increased dose-dependently for both PANC-1 and AsPC-1 cells, and the ratio was again lower for PANC-1 than for AsPC-1 cells at any given dose (Figure 1). Apoptosis was clearly involved in cell death caused by irradiation. The basal level of the Bax/Bcl-2 ratio was lower in PANC-1 than in AsPC-1 cells, and the levels of Bcl-X_L expression were almost the same for these two cell lines (Figure 2). These results suggest that the basal level of the Bax/Bcl-2 ratio might reflect the susceptibility to apoptotic cell death by radiation in PANC-1 cells and AsPC-1 cells.

It is widely accepted that, in a variety of cell lines with wild-type *p53*, cell death triggered by apoptotic stimuli is accompanied by an increase in the Bax/Bcl-2 ratio [6, 21]. There have been a number of studies demonstrating that wild-type *p53* exerts its role as an inducer of apoptosis partly by transactivating the expression of the *bax* gene [13]. However, Ludwig and colleagues analysed the transcriptional activities of various *p53* mutants, which were obtained by

plasmid transfection to *p53*-deleted cell lines, and showed that expression of p21 Waf-1 and bax depends on the type of *p53* mutations [22]. Shao and colleagues demonstrated in MCF-7 cells, which harbour wild-type *p53*, that apoptosis induced by a synthetic retinoid was accompanied by p53-independent regulation of Waf-1 as well as of bax mRNA levels, whilst bcl-2 mRNA levels decreased [23]. In addition, we have previously shown in PANC-1 cells that cisplatin or VP-16 causes apoptosis via a significant activation of Bax protein in the presence of *p53* mutation [15]. We further investigated whether the change in the Bax/Bcl-2 ratio was also involved in the radiation-induced apoptosis of cells with mutated *p53*. The Bax/Bcl-2 ratios were increased after 5 Gy irradiation in a time-dependent manner in both PANC-1 and AsPC-1 cells (Figures 3 and 4). The level of Bcl-X_L remained the same after 5 Gy irradiation, while the increase in the Bax/Bcl-2 ratio correlated with the induction of apoptotic cell death in these cell lines. This study demonstrated that radiation also induces apoptotic cell death in *p53*-mutated cell lines, accompanied by significant activation of Bax protein.

It is commonly found that repeated apoptosis-inducing stimuli to cell lines results in the introduction to the cell lines of resistance against such stimuli [12, 24, 25]. PANC-1/Rad-24 cells and AsPC-1/Rad-17 cells were obtained after repeated 5 Gy irradiations of their parental cells, and the radiosensitivity in these cells significantly decreased, while the basal levels of Bax expression dramatically decreased in these radioresistant cells. The expression of Bax was up-regulated over time in AsPC-1/Rad cells, although the degree of up-regulation in PANC-1/Rad cells was lower than that in PANC-1 and AsPC-1 cells. The expressions of Bcl-2 remained almost constant in PANC-1/Rad and AsPC-1/Rad cells. The increase in the Bax/Bcl-2 ratio did not lead to massive cell death in radioresistant AsPC-1/Rad cells as expected from the results obtained for PANC-1 and AsPC-1 cells. The difference between the radioresistant cells and their parental cells was the high level of basal expression of Bcl-X_L in the former, and this high level was maintained throughout the time-course after irradiation. The Bax/Bcl-2 ratio did not seem to be so important in the cells with high levels of the Bcl-X_L protein. Han-Z and colleagues reported that HCW-2 cells, a human promyelocytic leukaemia cell line with a high level of Bax expression and no expression of Bcl-2, showed radioresistance, and the cells displayed high level of Bcl-X_L [26]. Our results, taken together with those of other investigators, indicate that Bax/Bcl-2 status no longer correlates with the induction of apoptosis in the presence of high levels of Bcl-X_L expression. A high level of Bcl-X_L expression might be involved in the acquired resistance of these cells to radiation-induced apoptosis, probably through heterodimerisation of Bcl-X_L with Bax.

In conclusion, apoptosis was found to be involved in radiation-induced cell death, which is related to an increase in the Bax/Bcl-2 ratio in pancreatic cancer cells with mutated *p53*. The basal level of the Bax/Bcl-2 ratio correlated with radiosensitivity in PANC-1 cells and AsPC-1 cells, with a level of Bcl-X_L proteins less elevated than that of Bax or Bcl-2 proteins. In radioresistant PANC-1/Rad and AsPC-1/Rad cells, an increase in the Bax/Bcl-2 ratio might not be predictive of cellular susceptibility to radiation possibly because of basal and sustained overexpression of Bcl-X_L. The development of acquired radioresistance might involve a dynamic functional reconstruction of oncoproteins and include a high level of Bcl-X_L expression, and this is in part a reason why radioresistant AsPC-1 cells can have a higher increased Bax/Bcl-2 ratio than parent PANC-1 cells.

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Acknowledgement—This study was supported in part by a grant from the Japanese Ministry of Education.