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APOPTOSIS OF LUNG CANCER CELLS CAUSED BY SOME ANTI-CANCER AGENTS (MMC, CPT-11, ADM) IS INHIBITED BY *BCL-2*

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Summary: To determine whether the apoptotic cell death induced by anti-cancer agents could be inhibited by *bcl-2*, we established a *bcl-2*-transfected human small cell lung cancer cell line, SBC-3/Bcl2. SBC-3/Bcl2 showed higher resistance to ADM, CPT-11 and MMC compared with the parental line SBC-3, with relative resistance values of 3.4, 7.6 and 5.7, respectively. However, there was no difference in sensitivity to CDDP, VP-16, ACNU, MTX and taxol between SBC-3 and SBC-3/Bcl2. Agarose gel electrophoresis showed typical DNA fragmentation of SBC-3 following treatment with CPT-11 or MMC, in a concentration-dependent manner. In contrast, the same concentration of the drugs did not induce DNA fragmentation in SBC-3/Bcl2. Treatment with CDDP resulted in the same degree of DNA fragmentation in SBC-3 and SBC-3/Bcl2. These studies indicate that *bcl-2* can modulate the cytotoxicity of some anti-cancer agents by inhibiting the process of apoptosis.

Wyllie et al. characterized two mechanisms of cell death: necrosis and apoptosis. These two processes differ both morphologically and biochemically (1). The morphological changes associated with necrosis are swelling, followed by rupture of membranes and dissolution of organized structure. In contrast, apoptosis is characterized by chromatin condensation, cytoplasmic blebbing and fragmentation of genomic DNA into oligo-nucleosomal-sized units, which often depends on RNA and protein synthesis by the dying cell. It has been reported that several anti-cancer agents cause apoptosis in some of cancer cell lines. The *bcl-2* gene, originally identified by studies of the t(14;18) chromosomal translocation associated with human follicular B-cell lymphoma, encode a protein localized

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The abbreviations used are: ADM; Adriamycin, CPT-11; 7-ethyl-10-(4-(1-piperdino)-1-piperidino) carbonyloxy camptothecin, MMC; mitomycin C, CDDP; cis-diamminedichloroplatinum (II), VP-16; etoposide, ACNU; 3-((4-amino-2-methyl-5-pyrimidinyl)methyl)-1-(2-chloroethyl)-1-nitrosourea, MTX; methotrexate.

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to the inner mitochondrial membrane (2) and to the nuclear membrane (3). Expression of the *bcl-2* gene enhances the survival of B-cell precursors by preventing the onset of apoptosis and contributes to B-cell memory. In addition *bcl-2* has been found to promote survival in response to a wide variety of stresses (4). In this study we demonstrate that anticancer drugs can cause two types of apoptotic cell death in a human small cell lung carcinoma, one which is *bcl-2* inhibitable and a second which is *bcl-2* independent.

Materials and Methods

Cell Line and Culture: The human small cell lung cancer cell line, SBC-3, originally established at Okayama University School of Medicine, was donated by the Japanese Cancer Research Resources Bank (JCRB)-Cell. SBC-3 cells and their transfectants were grown as attached cultures in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were routinely harvested by trypsinization and diluted with the medium to the appropriate concentrations. Cell size was measured by a Coulter Channalyzer C-256 system (Coulter Electronics, Hialeah, FL).

Expression Vector for Transfection of *bcl-2* cDNA: The full-length coding region of murine bcl-2 was obtained by PCR of plasmid DNA from double strand cDNA of DC1, a mouse chronic T-cell leukemia cell line. The primer sequences were: 5'-TCTCGAGA GGATGGCGCAAGCCGGGA-3' and 5'-TCTCGAGACCTCACTTGAGGCCCAG GT-3'. The 0.7 kb PCR product was cloned in the XhoI site of the eukaryotic expression vector pBMGNeo.

Transfection: BMGNeo and BMGNeo-*bcl-2*, containing BMGNeo and the *bcl-2* PCR product, were transfected into SBC-3 cells using the Lipofectin reagent (Gibco BRL, MD, USA) according to the manufacture's instructions (5). Twenty micrograms of plasmid DNA was used for transfection. Five million SBC-3 cells were plated in a 60-mm tissue culture dish (Falcon 3002) in RPMI-1640 with 10% FBS and cultured overnight. The cells were washed twice with PBS (-) and resuspended in RPMI-1640 without FBS. Lipofectin Reagent-DNA complex was then added, and the cells were incubated for 8 h at 37°C in a humidified atmosphere, followed by addition of 3 ml of RPMI-1640 with 20% FBS. After an additional 48 h of incubation, the cells were washed and resuspended with RPMI-1640 with 10% FBS containing 1 mg/ml G418 (Sigma, MO, USA) for selection. The selected cell lines, transfected pBMGNeo-*bcl-2* and pBMGNeo, were designated SBC-3/Bcl-2 and SBC-3/Neo, respectively.

Northern Blot Hybridization Analysis: Total RNA was prepared from the wild-type SBC-3, SBC-3/Bcl-2, and SBC-3/Neo by the acid guanidium thiocyanate-phenol-chloroform extraction method (6). Approximately 20 µg of total RNA was electrophoresed and transferred to a positively charged nylon membrane (Hybond-N+, Amersham Japan Co.). We used *bcl-2* and *c-myc* probes (Oncorgene Science Inc., NY, USA) for Northern blot hybridization analysis. *Bcl-2* probes labelled with [α-^{32}P]dCTP to a specific activity of 5 x 10^7 cpm/µg DNA using the Multiprime DNA Labelling System (Amersham, Japan). *C-myc* probes labelled with [γ-^{32}P]dATP to a specific activity of 2 x 10^8 cpm/µg DNA using the Megalabel System (Takara, Japan). Details of hybridization and autoradiography have been described previously (7).

Doubling Time and Growth Inhibition Assay: Cytotoxic effects of chemotherapeutic agents were measured by the tetrazolium dye assay (8). Briefly, 200 µl/well of the cell suspension (1 x 10^4/ml) was plated in 96-well microculture plates (Falcon 3072). For the growth inhibition assay, the culture plates were treated with 20 µl/well of drugs at various concentrations, and incubated at 37°C in a humidified atmosphere for 3 days. After the incubation period, 20 µl/well of the MTT solution (5 mg/ml, Sigma) was added. The produced formazan was dissolved in 200 µl/well dimethyl sulfoxide (DMSO) and the optical density was measured at 562 and 630 nm using Δ soft ELISA analysis for a Macintosh computer interfaced to a Bio-Tek Microplate Reader (EL-340, Bio Metallics, Princeton, NJ). For the study of doubling times, cell

numbers were counted daily as above for 6 days, and the doubling time of each cell line in its logarithmic phase was calculated using linear regression curves.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis: Three million cells were treated with various drugs for 3 days. DNA fragmentation analysis was fundamentally the same as that reported previously by Smith C. A. (9), except that the sample treatment solution did not contain bromophenol blue. 123-bp DNA ladder (Gibco BRL, MD, USA) was used as a molecular weight marker.

Percentage DNA Fragmentation: Two million cells were prelabeled with [^{14}C]thymidine for 24 h and then treated with various concentrations of drugs for 3 to 4 days. After the drug treatment, the cells were washed and lysed in 0.5 ml of extraction buffer (5 mM Tris [pH 8.0], 20 mM EDTA, 0.5% Triton X-100) for 30 min at 4°C. The cellular lysates were then centrifuged at 27,000 g for 30 min at 4°C in order to separate the fragmental DNA from intact chromatin. The pellets were resuspended in 0.5 ml of extraction buffer. Radioactivity was then measured, and percentage DNA fragmentation was expressed as the ratio of DNA in the supernatant to the total DNA recovered in the supernatant plus pellet (10).

Results and Discussion

To determine whether *bcl-2* gene plays a role in preventing the cytotoxic effect of anti-cancer agents, we transfected DNA encoding the full-length murine *bcl-2* into the human small cell lung cancer cell line, SBC-3, by the lipofection method. The transfected subline was designated SBC-3/Bcl2. As a control, we also established the cell line, SBC-3/Neo, contaning the plasmid vector without *bcl-2*. There was no difference in cell size or protein content of these cell lines. Doubling times were slightly longer for SBC-3/Bcl2 (76.7±3.5 h) and SBC-3/Neo (66.3±9.5 h) than for SBC-3 (56.5±0.7 h). The expression of *bcl-2* RNA was evaluated by Northern blot analysis, as shown in Fig. 1. *Bcl-2* mRNA was not detectable in the SBC-3 cell line with the bcl-2 RNA probe.

In order to elucidate whether *bcl-2* transfection induced resistance to anti-cancer agents, we examined the sensitivity of these cell lines to various anti-cancer agents using the MTT assay . The SBC-3/Bcl2 cell line showed resistance to ADM, CPT-11, and MMC, with relative resistance values of 3.4-, 7.6- and 5.7-fold, respectively (Table I). However, the SBC-3/Bcl2 cell line did not show resistance to CDDP, VP-16, ACNU, MTX, or taxol. SBC-3/Neo did not show any resistance to any of the tested drugs,

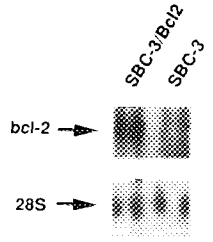

Fig. 1. Expression of bcl-2 RNA in SBC-3 and SBC-3/Bcl2 cell lines. Twenty micrograms of total RNA was analyzed as described in Materials and Methods. As a control, 28S probe demonstrated that an equivalent amount of total RNA from each cell line was loaded in each lane.

Table I

Drug	IC50 values (μM) SBC-3		SBC-3/Bcl2		SBC-3/Neo	
CDDP	1.65 ± 0.24***	(1.0)*	2.00 ± 0.30	(1.2)	1.48 ± 0.08	(0.9)
VP-16	0.67 ± 0.34	(1.0)	1.18 ± 0.10	(1.8)	0.57 ± 0.09	(0.9)
ADM	0.027 ± 0.004	(1.0)	0.093 ± 0.004	(3.4)	0.034 ± 0.005	(1.3)
CPT-11	1.15 ± 0.207	(1.0)	8.80 ± 2.40	(7.6)	1.27 ± 0.10	(1.1)
ACNU	241.2 ± 62.4	(1.0)	226.9 ± 23.3	(0.9)	173.7 ±32.5	(0.7)
MMC	0.24 ± 0.08	(1.0)	1.35 ± 0.14	(5.7)	0.17 ± 0.05	(0.7)
MTX	0.019 ± 0.008	(1.0)	0.017 ± 0.002	(0.9)	0.018 ± 0.004	(0.9)
Taxol (nM)	22.6 ± 1.4	(1.0)	25.2 ± 1.1	(1.1)	N.D.**	

Sensitivities of SBC-3 and its sublines to various anti-cancer agents. Each cell line was cultured for 3 days in the continuous presence of drugs. The cytotoxic effect of anti-cancer agents was measured by the MTT assay. IC50 values mean drug concentration that inhibits cell growth by 50%. * Value in parentheses means relative reisitance value, ** N. D. means not determined. *** Each value is mean ± S.D. of the three independent experiments.

suggesting the expression of the *bcl-2* gene selectively influence the cytotoxic effect of ADM, CPT-11, and MMC in the SBC-3/Bcl2 cell line.

In order to clarify whether the overexpression of *bcl-2* could inhibit the apoptotic process caused by these anti-cancer agents, we evaluated DNA fragmentation using gel electrophoresis and calculated the percentage DNA fragmentation using [^{14}C] thymidine-labeled cells . The results of gel electrophoresis are shown in Fig. 2. In SBC-3, after 3

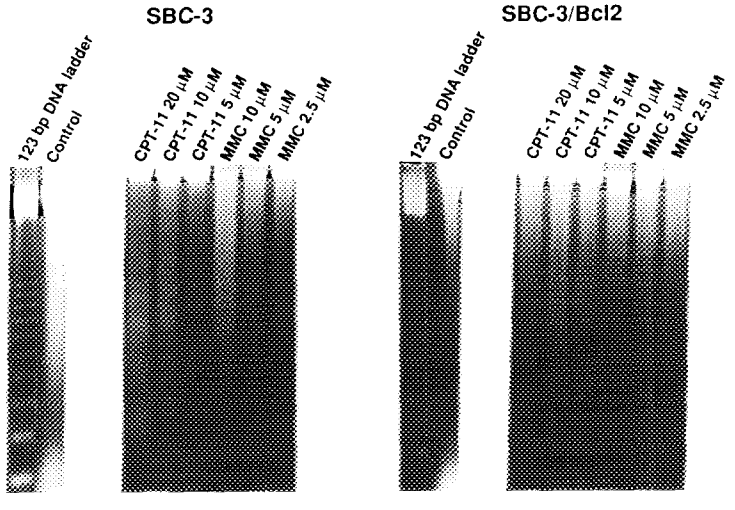

Fig. 2. DNA fragmentation caused by CPT-11 and MMC in SBC-3 and SBC-3/Bcl2. SBC-3 and SBC-3/Bcl2 cells were cultured for 3 days in the continuous presence of the indicated concentrations of drugs. DNA fragmentation ($3x10^6$ cells/lane) was then assessed by a gel electrophoresis method as described in Materials and Methods. A 123-bp DNA ladder was used as a molecular weight marker.

days of continuous exposure to drugs, DNA fragmentation due to treatment with CPT-11 and MMC was observed. A concentration-dependency of this effect on this cell line was detected. On the other hand, in SBC-3/Bcl2, no clear DNA fragmentation was observed after treatment with CPT-11 and MMC. These results suggest that DNA fragmentation and apoptosis might correlate with the cytotoxic effects of drugs on these cell lines, and that the expression of the *bcl-2* may inhibit the apoptosis caused by CPT-11 or MMC in SBC-3/Bcl2. The same results were also obtained with quantitative DNA fragmentation assays using [^{14}C] thymidine. As shown in Fig. 3, after 3 days of continuous exposure to CPT-11, DNA fragmentation was significantly lower in SBC-3/Bcl2 than in SBC-3. On the other hand, there was no difference in percentage DNA fragmentation between SBC-3 and SBC-3/Bcl2 due to CDDP treatment.

It has been reported that CDDP (11), VP-16 (12), ADM (13), camptothecin (14), nitrosourea compound (15), MTX (16) and taxol (17) cause apoptotic changes in some cell lines. We demonstrated that all of these drugs induced apoptotic changes in the SBC-3 cell line using microscopy or DNA gel electrophoresis (data not shown). As is well known that individual anti-cancer agents have different cellular targets. Therefore, in small cell lung carcinoma apoptosis may be a late and common process of cancer cell death caused by these anti-cancer agents. Although similar apoptotic changes were produced in SBC-3 using all of these drugs, the *bcl-2* transfectant cell line showed resistance to only 3 of the drugs tested. Moreover the overexpression of *bcl-2* did not inhibit DNA fragmentation induced by the non-cross-resistant drug, CDDP. Therefore, we speculate that some apoptotic pathways are *bcl-2*-sensitive and others *bcl-2*-independent. Previously, Miyashita et al. reported that *bcl-2* gene-transfected murine lymphoid cell lines showed

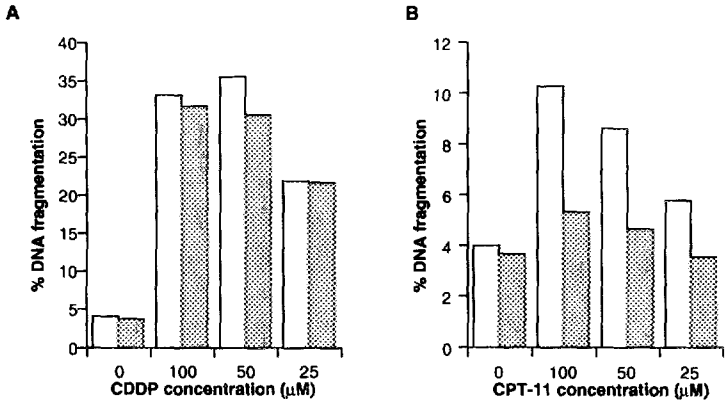

Fig. 3. Percentage DNA fragmentation caused by CDDP (A) and CPT-11 (B) in SBC-3 and SBC-3/Bcl2. SBC-3 and SBC-3/Bcl2 cells were prelabeled with [^{14}C]thymidine overnight and then cultured for 3 days in the continuous presence of the indicated concentrations of drugs. Percentage DNA fragmentation was calculated as described in Materials and Methods. White bar represents SBC-3, and grey bar represents SBC-3/Bcl2. All data points represent the mean of triplicate cultures.

resistance to the cytotoxic effect of dexamethasone. In contrast, these cell lines were not resistant to H_2O_2-induced apoptosis. It was also demonstrated that Bcl-2 did not protect the cells from all types of anticancer drug-induced DNA fragmentation (18). Our results extent these observation to human small cell lung cancer and MMC.

It has been reported that *c-myc*-induced apoptotic cell death was inhibited by *bcl-2*. Fanidi et al. reported that VP-16-induced apoptotic cell death was also inhibited by *bcl-2* in a *c-myc* transfected rat fibroblast cell line (19). They suggested that apoptotic cell death induced by anticancer agents might be due to a change of *c-myc* gene expression. The overexpression of *c-myc* is thought to cause DNA degradated by a pathway that can be counteracted by *bcl-2*. Although, in our experiments, similar *c-myc* expression was detected between the SBC-3 and SBC-3/Bcl2 cell lines by Northern blot analysis, VP-16 caused similar apoptotic changes in both cell lines (data not shown). Furthermore SBC-3/Bcl2 cells did not show resistance to this drug. Accordingly, we speculate the apoptotic cell death caused by VP-16 may not be due to overexpression of *c-myc* in these cell lines, and that this type of apoptotic cell death might result through the bcl-2-independent pathway in SBC-3/Bcl2. The delimitation of the multiple pathways of apoptotic cell death caused by various anti-cancer agents requires further study.

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