Doxorubicin Sensitizes Human Bladder Carcinoma Cells to Fas-Mediated Cytotoxicity

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BACKGROUND. The resistance of bladder carcinoma to anticancer chemotherapeutic agents remains a major problem. Hence, several immunotherapeutic approaches have been developed to treat the drug-resistant cancer cells. Fas antigen (Fas) and Fas ligand participate in cytotoxicity mediated by T lymphocytes and natural killer cells. Like Fas ligand, anti-Fas monoclonal antibody (MoAb) induces apoptosis of the cells expressing Fas. This study examined whether bladder carcinoma cells are sensitive to cytotoxicity mediated by anti-Fas MoAb and whether anticancer agents synergize with anti-Fas MoAb in cytotoxicity.

METHODS. Cytotoxicity was determined by a 1-day microculture tetrazolium dye assay. Synergy was assessed by isobolographic analysis.

RESULTS. The T24 human bladder carcinoma cell line constitutively expressed the Fas on the cell surface; however, T24 line was resistant to anti-Fas MoAb. Treatment of T24 cells with anti-Fas MoAb in combination with mitomycin C, methotrexate, or 5-fluorouracil did not overcome their resistance to these agents. However, treatment of T24 cells with a combination of anti-Fas MoAb and doxorubicin resulted in a synergistic cytotoxic effect. In addition, the doxorubicin-resistant T24 cells were sensitive to treatment with a combination of anti-Fas MoAb and doxorubicin. Synergy was also achieved in three other bladder carcinoma cell lines and four freshly derived human bladder carcinoma cells. Treatment with anti-Fas MoAb in combination with epirubicin or pirarubicin also resulted in a synergistic cytotoxic effect on T24 cells. The mechanisms of synergy were examined. Anti-Fas MoAb did not affect the intracellular accumulation of doxorubicin, the expression of P-glycoprotein, or the expression of the antioxidant glutathione S-transferase-π mRNA. However, treatment with doxorubicin enhanced the expression of Fas on T24 cells.

CONCLUSIONS. This study demonstrated that treatment of bladder carcinoma cells with doxorubicin sensitized the cells to lysis by anti-Fas MoAb. The synergistic effect obtained with established doxorubicin-resistant bladder carcinoma cells and freshly isolated bladder carcinoma cells suggests that drug-resistant bladder carcinoma cells can be sensitized by doxorubicin to Fas- and Fas ligant-mediated cytotoxicity by lymphocytes. Furthermore, the sensitization required low concentrations of doxorubicin, thus supporting the in vivo application of a combination of chemotherapy and immunotherapy in the treatment of drug-resistant and/or immunotherapy-resistant bladder carcinoma. ***Cancer* 1997;79:1180–9.**

KEYWORDS: Fas, doxorubicin, bladder carcinoma, synergy, apoptosis.

Previous studies have demonstrated that cancer cells have different degrees of sensitivity and resistance to various kinds of anticancer cytotoxic agents.[1,2] When anticancer chemotherapeutic agents are administered, only the drug-sensitive cancer cells are eliminated, and cancer cells with acquired resistance develop.[3] Accordingly, inherent

and acquired drug resistance are the major causes of failure in cancer chemotherapy. Therefore, new therapeutic modalities have been designed to treat drug-resistant cancers. Combination treatment with anticancer agents and biologic response modifiers have been considered as a possible means of reversing drug resistance. For example, recent studies from our laboratories demonstrated that treatment with tumor necrosis factor-α (TNF-α) in combination with anticancer drugs resulted in significant potentiation of cytotoxicity and synergy against a variety of sensitive and resistant human cancer cells.[4,5]

The interaction of Fas antigen (Fas) and Fas ligand plays an important role in cytotoxic T cell–mediated and natural killer (NK) cell–mediated cytotoxicity and apoptosis against cancer cells.[6,7] Activation of cytotoxic T cells and NK cells up-regulates Fas ligand on their surfaces and thus induces apoptosis in target cells expressing Fas. Fas is a member of the TNF-receptor family of cell surface proteins, which include two TNF receptors, the low-affinity nerve growth factor receptor, CD40, CD27, CD30, and OX40.[8,9] Members of this family are homologous in amino acid sequences in the extracellular regions. A high level of Fas mRNA expression has been detected in various tissues, such as thymus, liver, lung, heart, and ovary tissues, as well as cancer cells.[10,11] The Fas ligand is a type II membrane protein homologous to members of the TNF family, which include TNF-α, TNF-β, lymphotoxin-β, and the ligands for CD40, CD27, and CD30.[12,13]

Some monoclonal antibodies (MoAb) against Fas work as agonists of Fas ligand and induce apoptosis of the cell expressing Fas.[14,15] Like anti-Fas MoAb–mediated or Fas ligand–mediated apoptosis, several cytotoxic drugs also mediate apoptosis and may share common intracellular pathways leading to cell killing. We reasoned that combination treatment of drug-resistant cells with anti-Fas MoAb and drugs might overcome their resistance. This study investigated whether the resistance of bladder carcinoma cells to anti-Fas MoAb or commonly used anticancer chemotherapeutic agents could be overcome by combination treatment with anti-Fas MoAb and anticancer agents such as doxorubicin, mitomycin C (MMC), 5-fluorouracil (5-FU), and methotrexate (MTX). Further, this study explored a possible underlying mechanism involved in the reversal of drug resistance.

MATERIALS AND METHODS

Tumor Cells

The T24, J82, HT1197, and KK47 human bladder carcinoma cell lines were maintained in monolayers on plastic dishes in RPMI-1640 medium (Gibco, Bio-cult, Glasgow, Scotland, U.K.) supplemented with 25 mM N-[2-Hydroxyethyl]piperazine - N′ - [2 - ethanesulfonic acid] HEPES (Gibco), 2 mM L-glutamine (Gibco), 1% nonessential amino acid (Gibco), 100 units/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco), hereafter referred to as complete medium.[16,17] The T24/doxorubicin line is a doxorubicin-resistant subline of the T24 cell line. The T24/doxorubicin line was established by the continuous exposure of T24 line to complete medium containing progressively increasing concentrations of doxorubicin at 0.001–0.1 μg/mL. The line was then cloned in the presence of doxorubicin after it had been maintained for 1 year. Continuous maintenance of the line in a drug-free medium for at least 3 months did not change its doxorubicin resistance. Both T24 cells and T24/doxorubicin cells constitutively express P-glycoprotein. The percentages of fluorescent cells in T24 and T24/doxorubicin lines were 21.0% and 23.3%, respectively.

Fresh bladder carcinoma cells from four patients were separated from surgical specimens as previously described.[18,19] The histologic diagnosis revealed that all patients had transitional cell carcinoma of the bladder. Their histologic classification and grading according to the TNM staging system were as follows: Patient 1: $T_3N_0M_0$, Grade 3; Patient 2: $T_aN_0M_0$, Grade 2; Patient 3: $T_1N_1M_0$, Grade 3; and Patient No. 4: $T_aN_0M_0$, Grade 1. Briefly, cell suspensions were prepared by treating finely minced tumor tissues with collagenase (3 mg/mL, Sigma Chemical Co., St. Louis, MO). After washing 3 times in RPMI-1640 medium, the cell suspensions were layered on discontinuous gradients consisting of 2 mL of 100%, 2 mL of 80%, and 2 mL of 50% Ficoll-Hypaque in 15-mL plastic tubes and were centrifuged at 400 $\times$ g for 30 minutes. Lymphocyte-rich mononuclear cells were collected from the 100% interface, and tumor cells and mesothelial cells from the 80% interface. Cell suspensions enriched with tumor cells were sometimes contaminated by monocyte-macrophages, mesothelial cells, or lymphocytes. To avoid further contamination of host cells, we layered the cell suspensions on a discontinuous gradient containing 3 mL each of 25%, 15%, and 10% Percoll in complete medium in 15-mL plastic tubes and centrifuged them for 7 minutes at 25 $\times$ g at room temperature. Tumor cells depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. To avoid further contamination of mesothelial cells and monocyte-macrophages, we incubated the cell suspension in plastic dishes for 30–60 minutes at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, nonadherent cells were recovered, washed, and suspended in complete medium. Usually, the nonadherent cells mainly contained tumor cells with less than 5% contaminating

nonmalignant cells, as judged by morphologic examination of smears stained with Wright–Giemsa stain, and were more than 93% viable according to the trypan blue dye exclusion test. Cells having less than 5% contamination with nonmalignant cells were accepted for use as tumor cells.

Reagents

Antihuman Fas MoAb and isotype matched control antibody were purchased from MBL, Nagoya, Japan. The isotype of the antibodies is immunoglobulin (Ig)M. Doxorubicin (Adriamycin, Lot. No. 705ACB), Epirubicin (EPI) (Lot. No. 3015AG), MMC (Lot. No. 624A11), and 5-FU (Lot. No. 469ABG) were kindly supplied by Kyowa Hakkou Co. Ltd., Tokyo, Japan. Pirarubicin (THP) (Lot. No. THPMR100) was obtained from Meiji Pharmaceutical Co. Ltd., Tokyo, Japan. MTX (Lot. No. 119A) was supplied by Takeda Pharmaceutical Co. Ltd., Tokyo, Japan. Glutathione *S*-transferase-π (GST-π) cDNA used in making probes for Northern blot analysis was a gift from Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan.

Cytotoxicity Assay

The microculture tetrazolium dye (MTT) assay was used to determine tumor cell lysis as previously described.[20,21] Briefly, one hundred μL of target cell suspension (2×10^4 cells) were added to each well of 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, NY), and each plate was incubated for 24 hours at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, 100 μL of drug solution or complete medium for control were distributed in the 96-well plates, and each plate was incubated for 24 hours at 37 °C. Following incubation, 20 μL of MTT working solution (5 mg/mL, Sigma Chemical Co., St. Louis, MO) was added to each culture well, and the cultures were incubated for 4 hours at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was removed from the wells and replaced with 100 μL of isopropanol (Sigma) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Immunoreader, Japan Intermed Co. Ltd., Tokyo, Japan) at 540 nm. The percentage of cytotoxicity was calculated by the following formula: Percentage of cytotoxicity = [1 − (absorbance of experimental wells/absorbance of control wells)] $\times$ 100.

Flow Cytometric Examination

The expression of Fas on T24 cells and J82 cells was quantitated by flow cytometry. Immunofluorescent staining was performed in 96-well U-bottom plates. Dilutions and washing were performed throughout in 1$\times$ phosphate-buffered saline (PBS) containing 2% heat-inactivated FBS and 0.1% sodium azide. Before staining, the cells (2×10^5 cells) were pretreated with human AB serum for 1 hour, washed twice, and resuspended in 50 μL of 1$\times$ PBS. Thereafter, the cells were incubated with 10 μg/mL of anti-Fas MoAb or 10 μg/mL of control IgM. The cells were washed twice again and resuspended in 50 μL of 1$\times$ PBS containing goat antimouse fluorescein isothiocyanate–conjugated IgM antibody for 1 additional hour. The cells were washed twice and fixed in 2% paraformaldehyde solution. Flow cytometry was performed using an Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Acridine-Orange Staining

Apoptosis was observed by acridine-orange staining as previously described.[22] T24 cells in a chamber/slide (Miles Scientific, Naperville, IL) were incubated with anti-Fas MoAb at 1 μg/mL in the absence or presence of doxorubicin at 1 μg/mL for 12 hours at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, the supernatant was discarded and acridine-orange (20 μg/mL: Lot. No. CAE 2276, Wako Pure Chemical Industry Ltd., Osaka, Japan) was added on a slide glass. The specimens were examined under a Nikon fluorescence microscope with B2-type filter (magnification $\times$200). When apoptotic bodies or chromatin condensation were observed, the observation was defined as apoptosis.

Doxorubicin Determination

The doxorubicin content in T24 cells or T24/doxorubicin cells was determined by high-performance liquid chromatography using a Hitachi Model 635A (Hitachi Co. Ltd., Tokyo, Japan) as described in detail elsewhere.[23]

Northern Blot Analysis

Cytoplasmic RNA from tumor cells was prepared as described in detail elsewhere.[24,25] Briefly, 10 μg/lane of tumor cell RNA was electrophoresed in 1.2% agarose-2.2 M formaldehyde gels in 1$\times$ 3-morpholinopropanesulfonic acid (MOPS) buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM sodium ethylenediamine tetraacetic acid). The RNA was transferred to Biodyne A membranes (East Hills, NY) in 20$\times$ standard saline citrate (3 M NaCl, 0.3 M sodium citrate, pH 7.0). cDNA probe (50–100 ng) was labeled with [α^{32}P] dCTP (NEN, MA) by random oligo-primer extension. The nylon membranes were ultraviolet-crosslinked and hybridized.

Statistical Analysis

All determinations were made in triplicate, and the results were expressed as the mean $\pm$ standard devia-

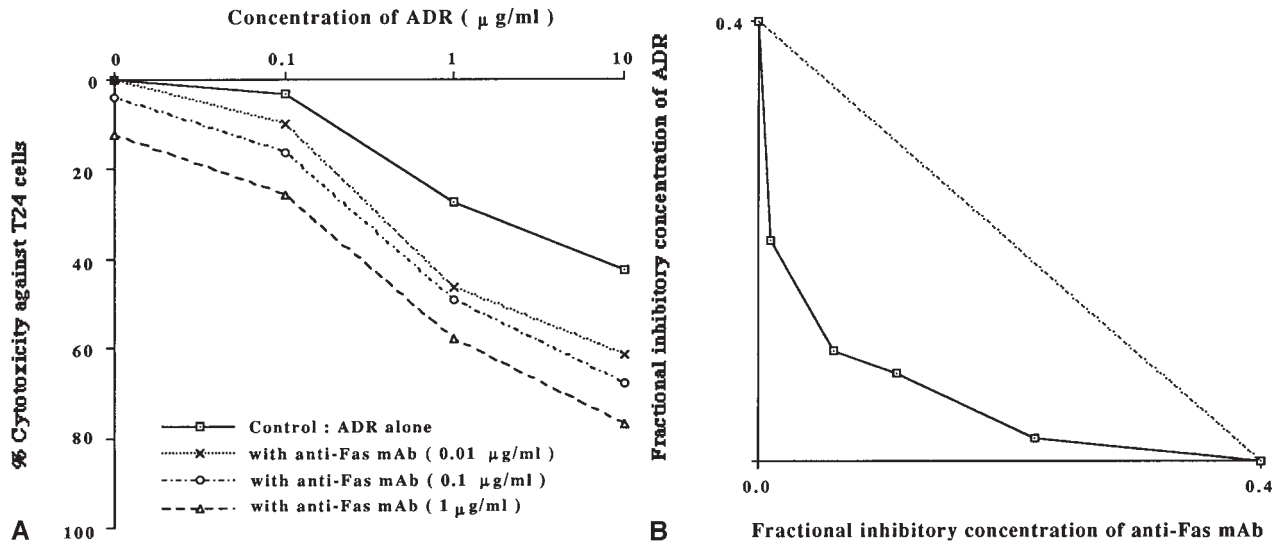

FIGURE 1. The synergistic cytotoxic effect of anti-Fas monoclonal antibody (MoAb) and doxorubicin (ADR), used in combination against T24 cells, is shown. The cytotoxic effect of anti-Fas MoAb and doxorubicin was assessed in a 1-day microculture tetrazolium dye assay (A) and was estimated by isobolographic analysis (B). The results were derived from three different experiments.

tion (SD). Statistical significance was determined by Student's *t* test. A *P* value of 0.05 or less was considered significant.

Calculations of synergistic cytotoxicity were determined by isobolographic analysis as described by Berenbaum.[21,26,27] Whether any particular dose combination is additive, synergistic, or antagonistic is shown by whether the point representing that combination lies on, below, or above the straight line joining the doses of the two drugs that, when given alone, produce the same effect as that combination in isobolographic analysis.

RESULTS

Synergistic Antitumor Cytotoxicity against Bladder Carcinoma Cells by Anti-Fas MoAb and Doxorubicin Used in Combination

We examined whether treatment of T24 bladder carcinoma cell line with anti-Fas MoAb in combination with doxorubicin, MMC, 5-FU, or MTX could overcome the drug resistance. Cytotoxicity was determined in a 1-day MTT assay, and synergy was assessed by isobolographic analysis as described in ''Materials and Methods.'' T24 cells were relatively resistant to anti-Fas MoAb. There was no synergistic cytotoxic effect of anti-Fas MoAb in combination with MMC, 5-FU, or MTX (data not shown). However, when T24 cells were treated with a combination of anti-Fas MoAb and doxorubicin, significant potentiation of cytotoxicity and synergy were achieved (Fig. 1). We concluded that the synergy was specific for the anti-Fas MoAb because a control IgM in combination with doxorubicin had no effect (data not shown). We then examined the sensitivity of the T24 doxorubicin-resistant variant, T24/doxorubicin, after treatment with anti-Fas MoAb and doxorubicin. The T24/doxorubicin line was resistant to both doxorubicin and anti-Fas MoAb, as compared with the T24 parental line. The doxorubicin concentrations required for 20% lysis of the T24 line and the T24/doxorubicin line were 0.6 μg/mL and 4.0 μg/mL, respectively. Clearly, significant synergy was also achieved by combination treatment with anti-Fas MoAb and doxorubicin (Fig. 2). We concluded that the findings obtained with both T24 and T24/doxorubicin lines were not selective for these lines because three other bladder cancer lines, J82, HT1197 and KK47 lines, were also sensitized to anti-Fas MoAb and doxorubicin cytotoxicity (data not shown). To determine if the synergy observed was a reflection of the properties of established tumor lines, we tested for synergy on four freshly isolated bladder carcinoma cells. In all cases, a significant synergy was achieved irrespective of the sensitivity of the cancer cells to either doxorubicin or anti-Fas MoAb used alone (data not shown). All together, these findings clearly demonstrate that treatment of bladder carcinoma cell lines or freshly derived cancer cells with a combination of anti-Fas MoAb and doxorubicin resulted in potentiation of cytotoxicity. Furthermore, the synergy was observed with both sensitive and resistant cancer cells. In all of the above findings, synergy was achieved with subtoxic concentrations of doxorubicin. The synergy ob-

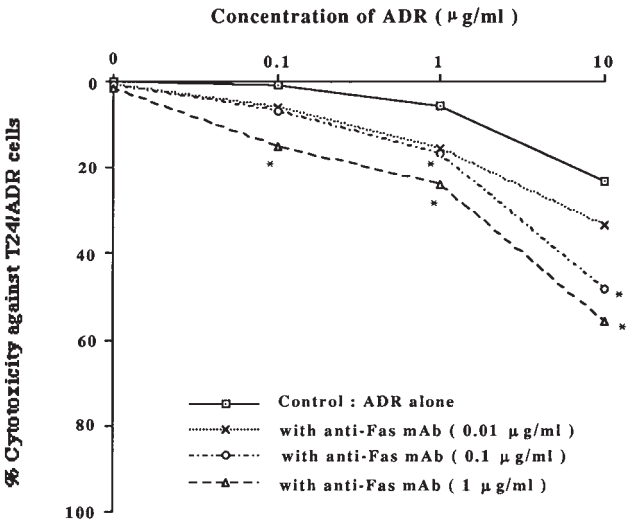

FIGURE 2. The synergistic cytotoxic effect of anti-Fas monoclonal antibody (MoAb) and doxorubicin (ADR), used in combination against T24/doxorubicin (T24/ADR) cells, is shown. The cytotoxic effect of anti-Fas MoAb and doxorubicin was assessed in a 1-day microculture tetrazolium dye assay. The results were derived from three different experiments. Anti-Fas MoAb at 0.01–1 μg/mL had almost no cytotoxic activity against T24/doxorubicin cells. *Values in the combination treatment are significantly higher than those achieved by treatment with doxorubicin alone ($P < 0.05$).

tained with low concentrations of the agents is of clinical relevance because high concentrations of drugs are toxic in vivo.

Effect of the Sequence of Treatment with Anti-Fas MoAb and Doxorubicin on Synergy

The findings just described demonstrate that simultaneous treatment of T24 cells with the anti-Fas MoAb and doxorubicin resulted in synergy. The effect of sequential treatment with anti-Fas MoAb and doxorubicin was examined and compared with treatment consisting of both agents added together. The T24 bladder carcinoma cells were treated for 6 hours with one agent, and the medium was removed; the second agent was subsequently added for 18 hours, and the cells were tested for viability. The results show that enhanced cytotoxicity was obtained irrespective of the sequence of treatment (Table 1). These findings demonstrate that the sequence of treatment with anti-Fas MoAb and doxorubicin may not be critical for obtaining synergy.

Effect of the Doxorubicin-Related Cytotoxic Drugs, EPI and THP, on Synergy with Anti-Fas MoAb

Two closely related compounds of doxorubicin, namely, EPI and THP, are currently used in a clinical setting. The cytotoxic effects of these two drugs when each was used in combination with anti-Fas MoAb were tested against T24 cells. Clearly, synergy was achieved with both EPI (Fig. 3) and THP (Fig. 4) in combination with anti-Fas MoAb.

TABLE 1
Effect of Sequence of Treatment with Anti-Fas MoAb and Doxorubicin on Their Cytotoxic Activity against T24 Cells

First treatment[a] (6 hrs)	Second treatment[a] (18 hrs)	% Cytotoxicity (mean ± SD)[b]
Medium	Anti-Fas MoAb	11.0 ± 0.6
Medium	Doxorubicin	27.9 ± 3.5
Medium	Anti-Fas MoAb plus doxorubicin	56.7 ± 2.9[c]
Anti-Fas MoAb	Doxorubicin	45.2 ± 6.6[c]
Doxorubicin	Anti-Fas MoAb	51.3 ± 2.9[c]

MoAb: monoclonal antibody; SD: standard deviation.

[a] T24 cells were pretreated with medium only, anti-Fas MoAb (1 μg/mL), or doxorubicin (1 μg/mL) for 6 hrs (first treatment). The medium was aspirated and T24 cells were washed twice with RPMI-1640 medium. The cells were then incubated with anti-Fas MoAb (1 μg/mL) and/or doxorubicin (1 μg/mL) for 18 hrs (second treatment). Cytotoxicity was assessed in a 1-day microculture tetrazolium dye assay.

[b] The results are expressed as the mean ± SD of three separate experiments.

[c] Values in the combination treatment are significantly higher than those achieved by treatment with anti-Fas MoAb alone or those achieved by treatment with doxorubicin alone ($P < 0.05$).

Mechanism of Synergy Achieved by Anti-Fas MoAb and Doxorubicin

Expression of Fas on the cell surface

We examined whether treatment of bladder carcinoma cells with doxorubicin up-regulates surface Fas expression, thus enhancing cytotoxicity by anti-Fas MoAb. There was up-regulation of Fas expression in both T24 and J82 cells after treatment with doxorubicin (data not shown). The up-regulation of Fas was dependent on the concentration of doxorubicin used.

Induction of apoptosis

Because both anti-Fas MoAb and doxorubicin can mediate apoptosis, we examined, by acridine-orange staining, whether the synergy achieved in cytotoxicity with anti-Fas MoAb and doxorubicin was also achieved in apoptosis. When apoptotic bodies or chromatin condensation were observed, we defined the observation as apoptosis. Doxorubicin, at a concentration of 1 μg/mL, was cytotoxic against T24 cells and mediated modest apoptosis (Fig. 5B). Approximately 2% of T24 cells treated with doxorubicin were considered to be apoptotic. Likewise, anti-Fas MoAb at a concentration of 1 μg/mL was slightly cytotoxic against T24 cells and also mediated some apoptosis (Fig. 5C). Approximately half of the cells were considered to be apoptotic. When anti-Fas MoAb and doxorubicin were used in combination, we consid-

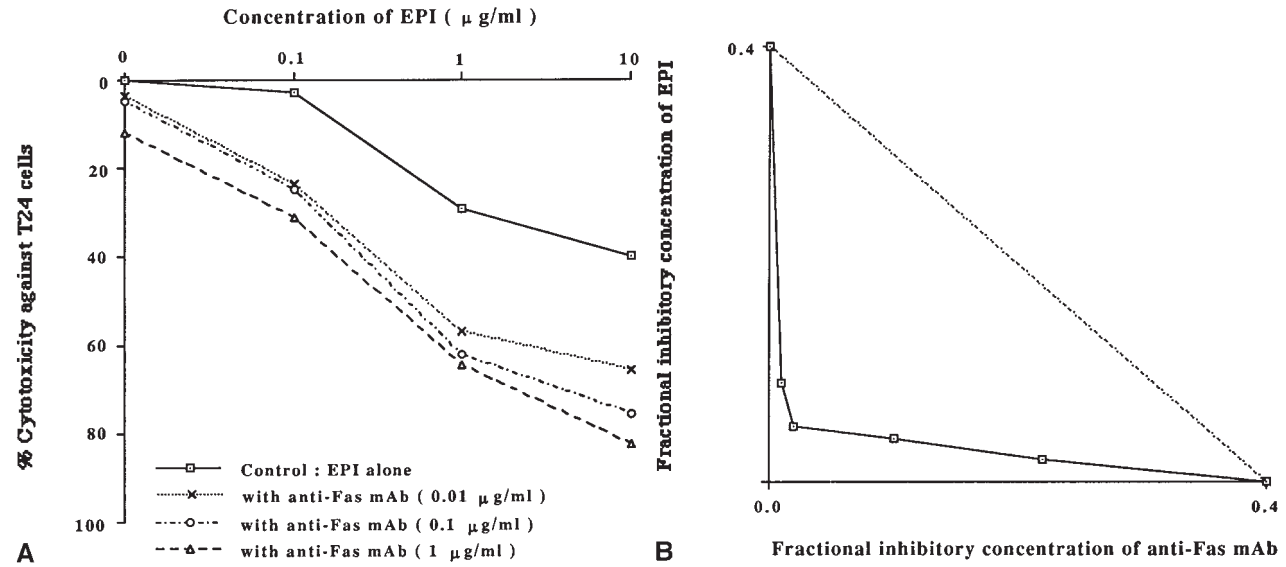

FIGURE 3. The synergistic cytotoxic effect of anti-Fas monoclonal antibody (MoAb) and epirubicin (EPI), used in combination against T24 cells, is shown. The cytotoxic effect of anti-Fas MoAb and EPI was assessed in a 1-day microculture tetrazolium dye assay (A) and was estimated by isobolographic analysis (B). The results were derived from three different experiments.

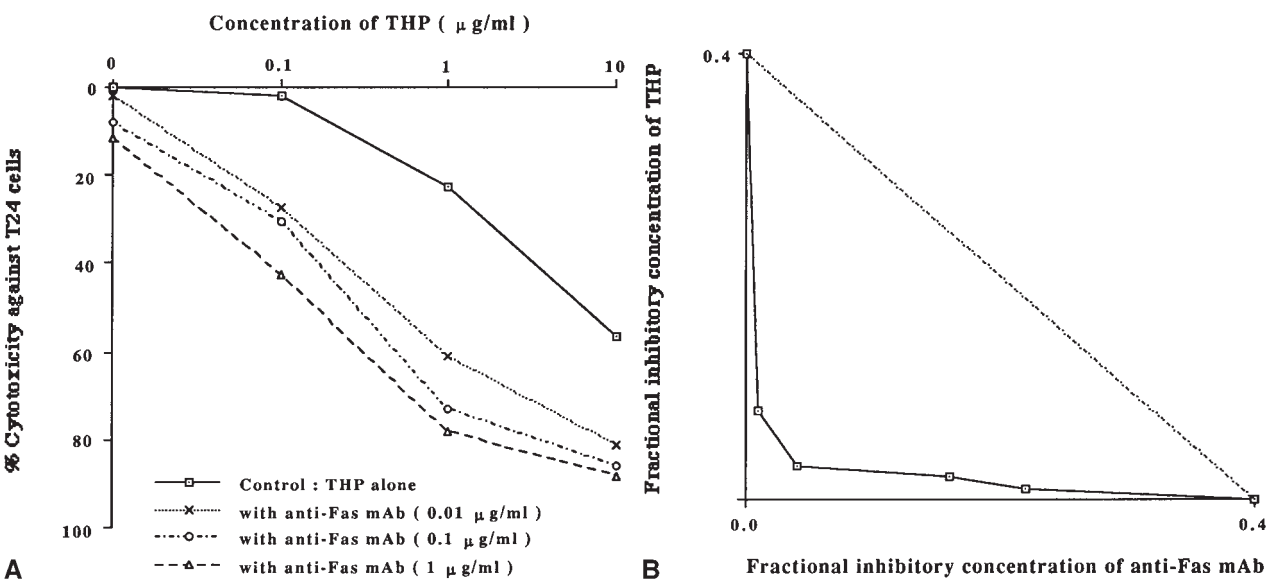

FIGURE 4. The synergistic cytotoxic effect of anti-Fas monoclonal antibody (MoAb) and pirarubicin (THP), used in combination against T24 cells, is shown. The cytotoxic effect of anti-Fas MoAb and THP used in combination on T24 cells was assessed in a 1-day microculture tetrazolium dye assay (A) and was estimated by isobolographic analysis (B). The results were derived from three different experiments.

ered all T24 cells apoptotic, and synergy in apoptosis was observed (Fig. 5D). Also, treatment of bladder carcinoma cells from Patient 1 with anti-Fas MoAb and doxorubicin resulted in synergy in apoptosis (data not shown). These results indicate that there was a good correlation between cytotoxicity and apoptosis after treatment of bladder carcinoma cells with a combination of anti-Fas MoAb and doxorubicin.

ADR intracellular accumulation

A possible mechanism of the augmentation of cytotoxicity may be the result of increased accumulation of

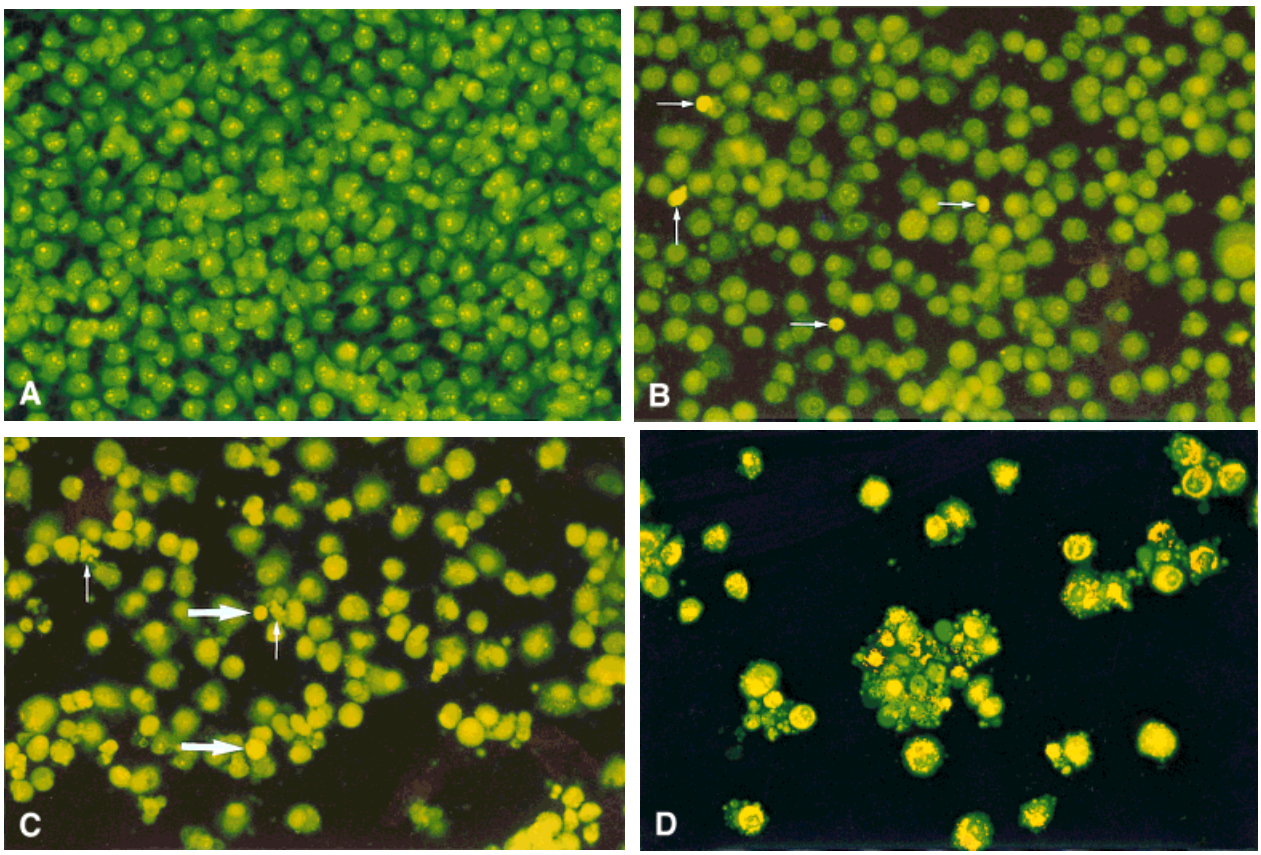

FIGURE 5. Apoptosis in T24 cells treated with anti-Fas monoclonal antibody (MoAb) and/or doxorubicin is shown. Apoptosis was determined by acridine-orange staining as described in "Materials and Methods." Each figure is a fluorescence microscopic view of acridine-orange staining (×200) after the following treatment: (A) medium only, (B) doxorubicin at 1 μg/mL, (C) anti-Fas MoAb at 1 μg/mL, and (D) doxorubicin at 1 μg/mL and anti-Fas MoAb at 1 μg/mL. A: No apoptotic cells were observed. B: Small arrow: apoptotic cells (condensation of chromatin and cytoplasm was observed). C: Small arrow: typical apoptotic bodies; large arrow: typical apoptotic cells (condensation of chromatin and cytoplasm was observed). D: All cells were considered to be apoptotic. Nuclear fragmentation and condensation of chromatin were apparent.

doxorubicin in the cells treated with anti-Fas MoAb. When T24 cells or T24/doxorubicin cells were treated with combination of doxorubicin and anti-Fas MoAb, doxorubicin accumulation inside the cells did not change significantly (data not shown). In addition, a preliminary experiment showed that T24 cells constitutively expressed P-glycoprotein, and treatment of T24 cells with anti-Fas MoAb did not affect the expression of P-glycoprotein on their surface.

Expression of GST-π mRNA

Previous findings indicated that doxorubicin resistance may be in part due to up-regulation of the expression of the antioxidant enzyme GST-π.[28,29] However, treatment of T24 cells with anti-Fas MoAb did not affect the level of constitutive GST-π mRNA expression (data not shown).

Altogether, these findings suggest that the synergy achieved with anti-Fas MoAb and doxorubicin is in part due to up-regulation of surface Fas expression.

DISCUSSION

The current study shows that the combined treatment of anti-Fas MoAb and doxorubicin resulted in a synergistic cytotoxic activity against bladder carcinoma cells and reversed their resistance. Synergy was achieved in the parental T24 bladder carcinoma cell line, its doxorubicin-resistant T24/doxorubicin line, three other bladder carcinoma cell lines, and four freshly isolated bladder carcinoma cells. The mechanism of overcoming the resistance by anti-Fas MoAb and doxorubicin was examined. Treatment of T24 cells with doxorubicin up-regulated the surface expression of Fas, suggesting a possible role of this up-regulation in overcoming the resistance. Synergy was achieved with low concentrations of each agent,

thus minimizing their toxicity in vivo and maximizing their therapeutic application in vivo.

The mechanisms responsible for cellular resistance to doxorubicin are believed to be multifactorial and include alterations in the transmembrane transport of doxorubicin, decreased formation of DNA single- and double-strand breaks, earlier onset of DNA repair, the cytosolic quenching of doxorubicin due to increased levels of glutathione and its relate enzymes, and the decreased cellular level of DNA topoisomerase II (Topo II). Alterations in the transmembrane transport of doxorubicin in cancer cells by P-glycoprotein or multidrug resistance–associated protein result in reduced intracellular accumulation of doxorubicin and resistance to the drug.[30,31] The resistance to doxorubicin has been attributed to reduced levels of DNA single- and double-strand breaks induced by the drug in doxorubicin-resistant P388 leukemia cells.[32,33] The doxorubicin-resistant P388 cells appeared to display an earlier onset of DNA repair as compared with drug-sensitive cells.[33] Some doxorubicin-resistant cells have higher levels of intracellular glutathione or its relate enzymes.[28,29] Because doxorubicin inhibits DNA Topo II, the reduced cellular level of DNA Topo II has been proposed as a possible mechanism of tumor cell resistance to doxorubicin.[34,35]

Several possible mechanisms of resistance to cell killing by anti-Fas MoAb in cancer cells have been reported, such as lack of Fas expression, synthesis of protective proteins, alterations on Fas intracellular signaling pathways, and a soluble form of Fas.[36,37] Protection against apoptosis via synthesis of an intracellular protein is a well-established paradigm. The protein product of *bcl*-2 has been shown to inhibit DNA fragmentation induced by a variety of stimuli, including anti-Fas MoAb.[38,39] Fas-associated phosphatase-1 has been reported to have an inhibitory effect on Fas signal transduction.[40,41] Further studies are required to elucidate the mechanism responsible for the acquisition of resistance of bladder carcinoma cells to Fas-mediated cytotoxicity.

Treatment of T24 cells with doxorubicin resulted in up-regulation of Fas expression by flow cytometry, suggesting a possible role of this up-regulation in overcoming their resistance. It is unclear whether this up-regulation is secondary to increases in mRNA for Fas or by posttranslational stabilization of the protein. Further studies are needed to corroborate these possibilities.

Although the up-regulation of Fas expression by doxorubicin is suggestive of synergistic cytotoxicity of anti-Fas MoAb and doxorubicin, the precise mechanism of the synergy by combination treatment with anti-Fas MoAb and doxorubicin is not fully understood. Doxorubicin and anti-Fas MoAb may share a common lytic mechanism. Anti-Fas MoAb induces apoptosis of cancer cells, and doxorubicin also exerts its cytotoxic activity partly through the induction of apoptosis.[42,43] Another possibility is that doxorubicin inhibits DNA synthesis by blocking DNA polymerase action and anti-Fas MoAb also inhibits DNA synthesis. Thus, under such conditions, anti-Fas MoAb and doxorubicin might act synergistically. This study shows that treatment of T24 cells with anti-Fas MoAb had no effect on their expression of GST-π mRNA. However, the expression of GST-π protein might have been increased by posttranslational stabilization of the protein, or the activity of GST-π itself might have been increased. The mechanisms responsible for synergistic cytotoxicity by combination treatment with anti-Fas MoAb and doxorubicin await further investigation.

The interaction of Fas and Fas ligand plays an important role in cytotoxic T cell–mediated and NK-mediated apoptosis in cancer cells.[6,7] Preliminary experiments showed that treatment of freshly isolated bladder cancer cells with doxorubicin augmented their susceptibility to lysis by autologous peripheral blood lymphocytes (data not shown). Since doxorubicin enhanced Fas expression on bladder carcinoma cells, the enhanced Fas expression might be one of the mechanisms responsible for the augmented susceptibility of doxorubicin-treated bladder carcinoma cells to lysis by autologous lymphocytes.

Because a 1-day MTT assay was used to determine cytotoxicity, the assay may indicate inhibition of cell proliferation, which could be due to either cytotoxicity or cell stasis. Therefore, it may not be specific for cytotoxicity, but rather indicative of the inhibition of cell proliferation.

The overall response rate of patients with bladder carcinoma to anticancer chemotherapeutic agents involving doxorubicin has improved. However, drug resistance and recurrence of cancer remain major problems, and a more effective therapy is necessary for these patients. The current study shows that combination treatment with anti-Fas MoAb and doxorubicin results in a synergistic cytotoxicity against both acquired and natural doxorubicin-resistant bladder carcinoma cells, and the synergistic effect is not restricted to established cell lines but is also observed in freshly derived cancers. These findings suggest that the therapeutic use of doxorubicin in combination with Fas-mediated immunotherapy might be useful for patients with doxorubicin-resistant or immunotherapy-resistant bladder carcinoma.

REFERENCES

1. Safrit JT, Bonavida B. Hierarchy of sensitivity and resistance of tumor cells to cytotoxic effector cells, cytokines, drugs, and toxins. *Cancer Immunol Immunother* 1992;34:321–8.
2. Bonavida B, Tsuchitani T, Safrit JT, Zighelboim J. Hierarchy of tumor cell sensitivity and resistance to cytolysis by TNF, cytotoxic cells, bacterial toxins, and cytotoxic drugs. In: Bonavida B, Granger G. Tumor necrosis factor: structure, mechanism of action, role in disease and therapy. Basel: Karger, 1990:125–32.
3. Birkhead BG. Evaluating and designing cancer chemotherapy treatment using mathematical models. *Eur J Cancer Clin Oncol* 1986;22:3–8.
4. Mizutani Y, Bonavida B. Overcoming CDDP resistance of human ovarian tumor cells by combination treatment with CDDP and TNF-α. *Cancer* 1993;72:809–18.
5. Safrit JT, Bonavida B. Sensitivity of resistant human tumor cell lines to tumor necrosis factor and Adriamycin used in combination: correlation between down-regulation of tumor necrosis factor-messenger RNA induction and overcoming resistance. *Cancer Res* 1992;52:6630–7.
6. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, et al. Fas and perforin pathways as major mechanisms of T cell–mediated cytotoxicity. *Science* 1994; 265:528–30.
7. Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med* 1995;181:1235–8.
8. Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, et al. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily: sequence identity with the Fas antigen. *J Biol Chem* 1992;267:10709–15.
9. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima SI, Sameshima M, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 1991;66:233–43.
10. Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA, et al. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J Immunol* 1992;148:1274–9.
11. Nagata S. Fas and Fas ligand: a death factor and its receptor. *Adv Immunol* 1994;57:129–44.
12. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 1993;75:1169–78.
13. Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 1994;76:959–62.
14. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al. Lethal effect of the anti-Fas antibody in mice. *Nature* 1993;364:806–9.
15. Trauth BC, Klas C, Peters AMJ, Matzuku S, Moller P, Falk W, et al. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 1989;245:301–5.
16. Mizutani Y, Nio Y, Fukumoto M, Yoshida O. Effects of bacillus Calmette-Guerin on cytotoxic activities of peripheral blood lymphocytes against human T24 lined and freshly isolated autologous urinary bladder transitional carcinoma cells in patients with urinary bladder cancer. *Cancer* 1992; 69:537–45.
17. Mizutani Y, Okada Y, Terachi T, Kakehi Y, Yoshida O. Serum granulocyte colony-stimulating factor levels in patients with urinary bladder tumour and various urological malignancies. *Br J Urol* 1995;76:580–6.
18. Mizutani Y, Okada Y, Terachi T, Yoshida O. Prognostic significance of circulating cytotoxic lymphocytes against autologous tumors in patients with urinary bladder cancer. *J Urol* 1996;155:888–94.
19. Mizutani Y, Nio Y, Yoshida O. The streptococcal preparation OK-432 specifically augments the susceptibility of human urinary bladder tumor cells to the autologous peripheral blood lymphocytes. *Cancer* 1992;69:2999–3007.
20. Mizutani Y, Yoshida O. Overcoming TNF-α resistance of human renal and ovarian carcinoma cells by combination treatment with buthionine sulfoximine and TNF-α. Role of TNF-α mRNA downregulation in tumor cell sensitization. *Cancer* 1994;73:730–7.
21. Mizutani Y, Fukumoto M, Bonavida B, Yoshida O. Enhancement of sensitivity of urinary bladder tumor cells to cisplatin by c-*myc* antisense oligonucleotide. *Cancer* 1994;74:1546–54.
22. Murai N, Ueda T, Takahashi J, Yang H, Kikuchi H, Hatanaka M, et al. Apoptosis of human glioma cells in vitro and in vivo induced by a neutralizing antibody against human basic fibroblast growth factor. *J Neurosurg.* 1996;85:1072–7.
23. Shinozawa S, Mikami Y, Araki Y. Determination of the concentration of adriamycin and its metabolites in the serum and tissues of Ehrlich carcinoma-bearing mice by high-performance liquid chromatography. *J Chromatogr* 1980; 196:463–9.
24. Mizutani Y, Bonavida B, Koishihara Y, Akamatsu K, Ohsugi Y, Yoshida O. Sensitization of human renal cell carcinoma cells to cis-diamminedichloroplatinum (II) by anti-interleukin-6 monoclonal antibody or anti-interleukin-6-receptor monoclonal antibody. *Cancer Res* 1995;55:590–6.
25. Mizutani Y, Bonavida B, Nio Y, Yoshida O. Overcoming TNF-α and drug resistance of human renal cell carcinoma cells by treatment with pentoxifylline in combination with TNF-α or drugs. *J Urol* 1994;151:1697–702.
26. Berenbaum MC. Synergy, additivism and antagonism in immunosuppression. *Clin Exp Immunol* 1977;28:1–18.
27. Berenbaum MC. A method for testing for synergy with any number of agents. *J Infect Dis* 1978;137:122–130.
28. Volm M, Mattern J, Samsel B. Relationship of inherent resistance to doxorubicin, proliferative activity and expression of P-glycoprotein 170, and glutathione *S*-transferase-π in human lung tumors. *Cancer* 1992;70:764–9.
29. Ahn H, Lee E, Kim K, Lee C. Effect of glutathione and its related enzymes on chemosensitivity of renal cell carcinoma and bladder carcinoma cell lines. *J Urol* 1994; 151:263–7.
30. Breuninger LM, Paul S, Gaughan K, Miki T, Chan A, Aaronson SA, et al. Expression of multidrug resistance–associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. *Cancer Res* 1995;55:5342–7.
31. Nooter K, Herweijer H. Multidrug resistance (MDR) genes in human cancer. *Br J Cancer* 1991;63:663–9.
32. Goldenberg GJ, Wang H, Blair GW. Resistance to Adriamycin: relationship of cytotoxicity to drug uptake and DNA single- and double-strand breakage in cloned cell lines of Adriamycin-sensitive and -resistant P388 leukemia. *Cancer Res* 1986;46:2978–83.
33. Deffie AM, Alam T, Senevirante C, Beenken SW, Batra JK, Shea TC, et al. Multifactorial resistance to Adriamycin: relationship of DNA repair, glutathione transferase activity, drug efflux, and P-glycoprotein in cloned cell lines of Adriamycin-sensitive and -resistant P388 leukemia. *Cancer Res* 1988; 48:3595–602.

34. De Jong S, Zijlstra JG, De Vries EGE, Mulder NH. Reduced DNA topoisomerase II activity and drug-induced cleavage activity in an Adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Res* 1990;50:304–9.
35. Deffie AM, Batra JK, Goldenberg GJ. Direct correlation between DNA topoisomerase II activity and cytotoxicity in Adriamycin-sensitive and resistant P388 leukemia cell lines. *Cancer Res* 1989;49:58–62.
36. Owen-Schaub LB, Radinsky R, Kruzel E, Berry K, Yonehara S. Anti-Fas on nonhematopoietic tumors: levels of Fas/APO-1 and *bcl*-2 are not predictive of biological responsiveness. *Cancer Res* 1994;54:1580–6.
37. Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, et al. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science* 1994;263:1759–62.
38. Itoh N, Tsujimoto Y, Nagata S. Effect of *bcl*-2 on Fas antigen-mediated cell death. *J Immunol* 1993;151:621–7.
39. Sentman CL, Shutter JR, Hockenberry D, Kanagawa O, Korsmeyer SJ. *bcl*-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* 1991;67:879–88.
40. Sato T, Irie S, Kitada S, Reed JC. FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science* 1995;268:411–5.
41. Mori S, Mori K, Jewett A, Nakamura S, Bonavida B. Resistance of AIDS-associated Kaposi's sarcoma cells to Fas-mediated apoptosis. *Cancer Res* 1996;56:1874–9.
42. Kerr JFR, Winterford CM, Harmon BV. Apoptosis. *Cancer* 1994;73:2013–26.
43. Ohmori T, Podack ER, Nishio K, Takahashi M, Miyahara Y, Takeda Y, et al. Apoptosis of lung cancer cells caused by some anticancer agents (MMC, CPT-11, ADM) is inhibited by *bcl*-2. *Biochem Biophys Res Comm* 1993;192:30–6.
44. Shima Y, Nishio N, Ogata A, Fujii Y, Yoshizaki K, Kishimoto T. Myeloma cells express Fas antigen/APO-1 (CD95) but only some are sensitive to anti-Fas antibody resulting in apoptosis. *Blood* 1995;85:757–64.