TREATMENT OF THE P815 MURINE MASTOCYTOMA WITH CISPLATIN OR ETOPOSIDE UP-REGULATES CELL-SURFACE FAS (CD95) EXPRESSION AND INCREASES SENSITIVITY TO ANTI-FAS ANTIBODY-MEDIATED CYTOTOXICITY AND TO LYSIS BY ANTI-CD3-ACTIVATED KILLER-T CELLS

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We have investigated the effect of pre-treatment with the anti-cancer drugs cisplatin and etoposide on the susceptibility of P815 murine mastocytoma cells to lysis by murine spleen-derived anti-CD3-activated killer-T (AK-T) cells. A 20 hr pre-treatment with cisplatin (0.2–2 μg/ml) or etoposide (0.01–1 μg/ml) rendered P815 cells significantly more sensitive to AK-T cell–mediated lysis in a 4 hr ^{51}Cr-release assay than untreated control tumor cells. At lower concentrations, pre-treatment with cisplatin or etoposide had no direct cytotoxic effects on P815 tumor cells, as measured by the MTT assay. AK-T cell–mediated killing of P815 tumor cells pre-treated with 2 μg/ml cisplatin or 1 μg/ml etoposide was only partially inhibitable by the Ca^{2+} chelator EGTA, suggesting that the Ca^{2+}-independent Fas (CD95)/Fas ligand cytolytic pathway of AK-T cells contributes to cytotoxicity. In comparison to untreated control P815 cells, 2 μg/ml cisplatin– or 1 μg/ml etoposide–treated P815 cells exhibited increased expression of Fas mRNA and cell-surface Fas, which correlated with increased sensitivity to lysis by AK-T cells. In addition, pre-treatment with cisplatin or etoposide caused P815 tumor cells to become sensitive to the cytotoxic effects of anti-Fas antibody in a 4 hr ^{51}Cr-release assay. Taken together, our results demonstrate that short-term exposure to concentrations of cisplatin and etoposide in the low cytotoxic range and below up-regulates Fas expression by P815 tumor cells, thereby facilitating cytotoxicity mediated through the Fas/Fas ligand cytolytic pathway. *Int. J. Cancer* 73:416–423, 1997.
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Conventional treatment of cancer has focused on surgical resection, chemotherapy and radiation therapy. Although surgical procedures are useful in removing large primary tumors, such as carcinomas of the breast, treatment of metastatic disease by surgical resection is not feasible. Anti-cancer drugs or radiotherapy, therefore, are commonly used as adjuncts to the surgical treatment of cancer. However, many tumors eventually become resistant to the cytotoxic effects of drugs or radiation (Kasid *et al.,* 1993; Hickman *et al.,* 1994). This has aroused interest in developing strategies to eliminate cancers by immunotherapy since many human tumor cell lines (*e.g.,* squamous carcinoma, sarcoma, renal cell carcinoma and leukemia) that are resistant to cytotoxic drugs often are sensitive to lysis by activated killer lymphocytes (Harker *et al.,* 1990). Results from clinical trials indicate that adoptive immunotherapy with *ex vivo*–activated killer lymphocytes can be used with some success in the treatment of metastatic melanoma and renal carcinoma, but unfortunately, common cancers, such as adenocarcinomas of the lung, breast and colon, respond poorly to such treatment (reviewed in Rosenberg, 1991). Poor responses of common cancers to adoptive immunotherapy is in large part due to the inherent resistance of many solid tumors to attack by immune effector cells (reviewed in Walker *et al.,* 1997).

Over the past decade, interest has gravitated toward using combinations of chemotherapy and adoptive immunotherapy for the treatment of human cancers (Kedar and Klein, 1992). The immune function of cancer patients often improves shortly after chemotherapy (Head *et al.,* 1993). More importantly, results from clinical trials investigating the effectiveness of combined chemotherapy and immunotherapy regimens indicate improved response rates in patients with a variety of cancers (Mantovani *et al.,* 1994;

Kimura and Yamaguchi, 1995). However, the ability of anti-neoplastic drugs to enhance the effectiveness of immunotherapy is not fully understood. An obvious beneficial effect of prior treatment with chemotherapeutic agents is to lower the tumor burden with which the immune effector cells have to contend. However, this also occurs at low drug concentrations that cause little or no cytotoxicity (Utsugi *et al.,* 1989) and, therefore, need not be linked to reductions in tumor mass. It has been suggested that enhancement of immunotherapy following administration of anti-cancer drugs may be due to elimination of a suppressor cell population (Awwad and North, 1988), though it is uncertain whether suppressor cells impact on the effectiveness of adoptive immunotherapy in cancer patients (Kedar and Klein, 1992). Interestingly, there is considerable evidence that preliminary exposure to chemotherapeutic agents enhances the lysis of tumor cells by activated killer lymphocytes. For instance, cisplatin pre-treatment of human lung squamous carcinoma, leukemia, renal carcinoma, Burkitt's lymphoma and gastric cancer cells increases their susceptibility to subsequent killing by cytotoxic lymphocytes (Kawai *et al.,* 1992; Mizutani *et al.,* 1993; Gold *et al.,* 1995; Ishihata *et al.,* 1996). Other anti-cancer drugs that have been shown to enhance tumor cell sensitivity to killer cell–mediated lysis include doxorubicin, mitomycin C, dacarbazine and alkyl-lysophospholipid compounds (LoRusso *et al.,* 1990; Kawai *et al.,* 1992; Botzler *et al.,* 1996; Ishihata *et al.,* 1996). These findings suggest that chemo-immunotherapy may surmount a fundamental obstacle that has limited the application of immunotherapy to cancer treatment: that tumor cells are often poorly sensitive to attack by cytotoxic effector cells. However, the molecular mechanisms by which anti-cancer drugs increase the susceptibility of tumor cells to lymphocyte-mediated killing are not well understood.

In this study, we have determined the effect of pre-treatment with cisplatin or etoposide, 2 widely used anti-neoplastic agents (Chabner *et al.,* 1996), on murine P815 mastocytoma sensitivity to lysis by murine spleen-derived anti-CD3-activated killer-T (AK-T) cells and investigated the molecular basis of the effect. AK-T cells previously have been shown to kill a range of tumor target cells in a major histocompatibility complex–unrestricted and antigen-non-specific manner (Stankova *et al.,* 1989). Cisplatin is an anti-neoplastic agent which exerts its anti-tumor effect by binding to DNA and forming inter-strand, intra-strand and DNA–protein cross-linkages which disrupt the process of cell replication (Eastman, 1987). Additional cytotoxic effects may result from positively charged platinum binding to and inactivation of thiol-containing enzymes within the cells. Cisplatin is commonly used to treat

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cancers of the bladder, head, neck and endometrium; small cell carcinoma of the lung; carcinoma of the ovary; and advanced testicular cancer (Chabner *et al.,* 1996). Etoposide exerts cytocidal effects on tumor cells by inhibiting the function of topoisomerase II, an enzyme which is essential for DNA replication and repair (Smith, 1990). Clinical studies show etoposide to be active against carcinoma of the breast, non-Hodgkin's lymphomas, acute non-lymphocytic leukemia and Kaposi's sarcoma; in combination with cisplatin, etoposide is used to treat small cell carcinoma of the lung and testicular tumors (Chabner *et al.,* 1996).

MATERIAL AND METHODS

Animals

Male C57BL/6 mice (6–8 weeks old) were purchased from Charles River (Lasalle, Canada) and housed in the Carleton Animal Care Facility of Dalhousie University. Mice were maintained on standard mouse chow and water was supplied *ad libitum.*

Material

RPMI 1640 medium was obtained from ICN (Montreal, Canada) and supplemented with 5% heat-inactivated FCS (at 56°C for 30 min), 2 mM L-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin (all from ICN) and 5 mM HEPES (*N*-2-hydroxyethylpiperazine-H′-2-ethanesulfonic acid) buffer. Hereafter this is referred to as complete medium. HEPES and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). BSA was from Boehringer-Mannheim (Laval, Canada). FITC-conjugated hamster anti-mouse Fas (CD95) monoclonal antibody (MAb) was purchased from PharMingen (San Diego, CA). Hamster anti-mouse MAb against the ε chain of CD3 was used in the form of culture supernatant from a hybridoma (clone 145-2C11) kindly provided by Dr. J. Bluestone (Chicago, IL). P815 mastocytoma cells were from the ATCC (Rockville, MD). Rabbit anti-asialo-GM1 anti-serum was obtained from Wako (Richmond, VA). Low-toxicity rabbit complement was obtained from Cedarlane (Hornby, Canada). Cisplatin (1 mg/ml in saline) was from David Bull Laboratories (Vaudreuil, Canada), and etoposide (20 mg/ml) was from Bristol (Montreal, Canada).

Preparation of AK-T cells

Spleens were removed aseptically from mice killed by cervical dislocation, placed in a 15 ml polystyrene round-bottomed tube and mechanically dissociated in PBS using a sterile loose-fitting tissue homogenizer. Erythrocytes were eliminated from spleen cell preparations by osmotic shock. Following 2 washes in PBS, spleen cells were resuspended in complete RPMI 1640 medium and passaged through a nylon wool (Cellular Products, Buffalo, NY) column to enrich for T lymphocytes, as previously described (Fitzpatrick *et al.,* 1996). Contaminating natural killer cells were removed from nylon wool–non-adherent spleen cell preparations by a 2-step treatment with anti-asialo-GM1 antibody (1:40) and rabbit complement (1:12). The resulting T lymphocyte–enriched cell preparation was typically 90–95% viable by Trypan blue dye exclusion and contained >90% T cells and <0.1% natural killer cells by flow-cytofluorimetric analysis (Fitzpatrick *et al.,* 1996). T cells were adjusted to 4×10^6 cells/ml in complete RPMI 1640 medium containing anti-CD3 MAb (1:20 hybridoma culture supernatant), placed in tissue culture flasks or 24-well plates (Sarstedt, St. Leonard, Canada) and cultured for 48 hr at 37°C in a humidified atmosphere containing 5% CO_2. The resulting AK-T cells were washed extensively with PBS prior to use in order to remove residual anti-CD3 MAb and resuspended in complete RPMI 1640 medium.

Pre-treatment of P815 tumor cells with cytotoxic drugs

Equal numbers of P815 mastocytoma cells were cultured in the presence or absence of cisplatin or etoposide for 20 hr at 37°C in a humidified atmosphere containing 5% CO_2. Cisplatin was used at concentrations of 0.02, 0.2, 2 and 20 µg/ml and etoposide at concentrations of 0.01, 0.1, 1 and 10 µg/ml. P815 cells were washed extensively prior to use in order to remove all traces of the drug from the cell preparation.

MTT cell viability assay

The effect of cytotoxic drug pre-treatment on P815 cell viability was determined using the MTT assay, which measures changes in the function of mitochondrial dehydrogenases (Mosmann, 1983). Following pre-treatment of P815 mastocytoma cells with cytotoxic drugs for 20 hr, 10^5 P815 tumor cells were placed into wells of a 96-well U-bottomed microtiter plate and incubated in the presence of MTT (500 µg/ml) for 2.5 hr at 37°C in a humidified atmosphere containing 5% CO_2. Following centrifugation, cell pellets were solubilized in 100 µl of DMSO and transferred to a 96-well flat-bottomed microtiter plate. Spectrometric absorbance was measured at 492 nm using a Titertek plate reader. Data are expressed as percentages of control MTT readings (±SD) obtained with P815 cells incubated in complete RPMI 1640 medium in the absence of cytotoxic drugs.

Cytolytic ^{51}Cr-release assay

P815 mastocytoma cells were incubated with 100 µCi (0.1 ml) of Na_2 $^{51}CrO_4$ (specific activity 250–500 mCi/mg Cr; ICN) for 1 hr at 37°C, washed 3 times and resuspended in complete RPMI 1640 medium at a concentration of 5×10^4 cells/ml. Various numbers of AK-T cells and 5×10^3 radiolabeled P815 cells were added sequentially to wells of a 96-well V-bottomed microtiter plate (0.2 ml final vol.) to achieve the desired effector:target (E:T) cell ratios. In some experiments, anti-Fas MAb, at concentrations ranging from 0.01 to 1.00 µg/ml, was added to wells instead of AK-T cells. Plates were incubated for 4 hr at 37°C in a humidified atmosphere containing 5% CO_2 and centrifuged at 400 *g* for 5 min, and 100 µl of supernatant were collected from each well. The amount of ^{51}Cr present in supernatants was determined using a Beckman (Fullerton, CA) Gamma 8000 counter. Percent lysis was calculated using the formula:

$$\% \text{ lysis} = (E - S)/(M - S) \times 100$$

where E is the ^{51}Cr-release from experimental samples, S is the spontaneous release in the presence of complete RPMI 1640 medium and M is the maximum release upon cell lysis with 10% SDS. Data are presented as the mean percent lysis of triplicate samples (±SD) from a representative experiment.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

P815 cells were cultured in the presence or absence of various concentrations of cisplatin or etoposide for 20 hr, and total cellular RNA was isolated using Trizol reagent (GIBCO BRL, Burlington, Canada) according to the supplier's instructions. Single-strand cDNA was synthesized from 0.5 µg RNA with 200 U of Moloney murine leukemia virus–derived reverse transcriptase (GIBCO BRL) in the presence of 5 mCi ^{32}P-dCTP (Du Pont, Mississauga, Canada). Amplification of equal amounts of cDNA was insured by monitoring ^{32}P-dCTP incorporation during reverse transcription. PCR were carried out in an automatic DNA thermal cycler (MJ Research, Watertown, MA). Each reaction used 5×10^4 cpm of cDNA, 2.5 U Taq polymerase (GIBCO BRL), 200 µM deoxynucleotide triphosphates (GIBCO BRL) and 50 nM β-actin or Fas gene-specific 5′ and 3′ primers in a final vol. of 50 µl. Gene-specific primers for Fas (5′ primer: TTGCTGTCAACCATGC-CAACC and 3′ primer: CAACCATAGGCGATTTCTGGG) were obtained from GIBCO BRL, while the β-actin primers (5′ primer: CTGGAGAAGAGCTATGAGC and 3′ primer: TTCTGCATCCT-GTCAGCAATG) were a generous gift from Dr. A. Stadnyk (Department of Pediatrics, Dalhousie University). The specificity of the individual primers was verified by Southern blot analysis using ^{32}P-labeled cDNA probes for Fas (kindly provided by S. Nagata, Osaka, Japan) and β-actin. PCR conditions for Fas and β-actin amplifications were 92°C for 30 sec, 57°C for 30 sec and 72°C for 1 min for a total of 35 and 22 cycles, respectively. The

number of PCR cycles chosen for Fas and β-actin amplifications were determined previously to generate PCR product during the exponential phase of amplification. RT-PCR performed under these conditions has been shown to yield reasonably accurate data about relative changes in mRNA levels, providing reliable detection of 2-fold or greater differences even without specially prepared internal standards (Singer-Sam *et al.,* 1990). PCR products were resolved on a 1.7% agarose gel containing 0.01% (v/v) ethidium bromide and visualized by UV light illumination. The detected cDNA was compared to a 100 bp ladder (Promega, Madison, WI) and quantified by densitometric scanning with a Macintosh Color OneScanner and NIH Image Software (version 1.6). PCR product levels, as determined by densitometric scans, were normalized relative to steady-state expression of β-actin.

Flow-cytofluorimetric analysis

P815 mastocytoma cells were washed with PBS and exposed to FITC-conjugated anti-Fas MAb (10 µg/ml) in PBS containing 2.5% BSA and 0.2% sodium azide. Following incubation at 4°C for 45 min, P815 cells were washed once with PBS containing 1% BSA and 0.2% sodium azide and twice with PBS containing 1% BSA. Finally, P815 cells were resuspended in 1% paraformaldehyde in a 10 mM NaCl solution. Samples were stored in the dark overnight at 4°C. Fas expression on P815 cells was determined by analysis of 10^4 cells with a FacScan (Becton Dickinson, Mississauga, Canada).

Statistical analysis

Data analysis was performed using the Instat statistics program (GraphPad Software, San Diego, CA). Statistical significance of data was tested using the *t* statistic evaluation and Student's *t* distribution. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Dose-dependent toxicity of cisplatin and etoposide treatments to P815 cells

The *in vitro* sensitivity of P815 murine mastocytoma to cisplatin and etoposide treatments was determined by incubation for 20 hr in the presence of different concentrations of cisplatin (0.02–20 µg/ml) or etoposide (0.01–10 µg/ml). We chose 20 hr as a pre-incubation time on the basis of previous unpublished work (data not shown) on the schedule-dependent interactions of cisplatin with topoisomerase inhibitors, which suggested that increases in tumor cell susceptibility to killing by cytotoxic drugs were best revealed within this time frame. Following the incubation period, the effect on P815 viability was determined by an MTT assay. At lower concentrations, neither cisplatin (Fig. 1*a;* 0.02–0.2 µg/ml) nor etoposide (Fig. 1*b;* 0.01–0.1 µg/ml) had a negative effect on the viability of P815 cells. Exposure to higher concentrations of cisplatin (Fig. 1*a;* 2–20 µg/ml) or etoposide (Fig. 1*b;* 1–10 µg/ml) led to a dose-dependent decrease in tumor cell viability.

Pre-treatment with cisplatin or etoposide sensitizes P815 tumor cells to lysis by AK-T cells

We next investigated the effect of pre-treatment with low (non-cytotoxic) to moderate (approximating the EC_{50} concentration for cytotoxicity) doses of cisplatin (0.02–2 µg/ml) or etoposide (0.01–1 µg/ml) on the susceptibility of P815 tumor cells to killing by C57BL/6 murine spleen–derived AK-T cells. Cytolysis mediated by AK-T cells was significantly potentiated at all E:T ratios following exposure of P815 tumor cells to 0.2 or 2 µg/ml cisplatin (Fig. 2*a; p < 0.02* or better). However, prior exposure of P815 tumor cells to 0.02 µg/ml cisplatin failed to enhance subsequent AK-T cell–mediated lysis of these tumor cells (Table I). Pre-treatment of P815 tumor cells with all concentrations of etoposide tested (0.01–1 µg/ml) rendered P815 tumor cells significantly more susceptible to killing by AK-T cells at all E:T ratios than untreated controls (Fig. 2*b; p < 0.04* or better).
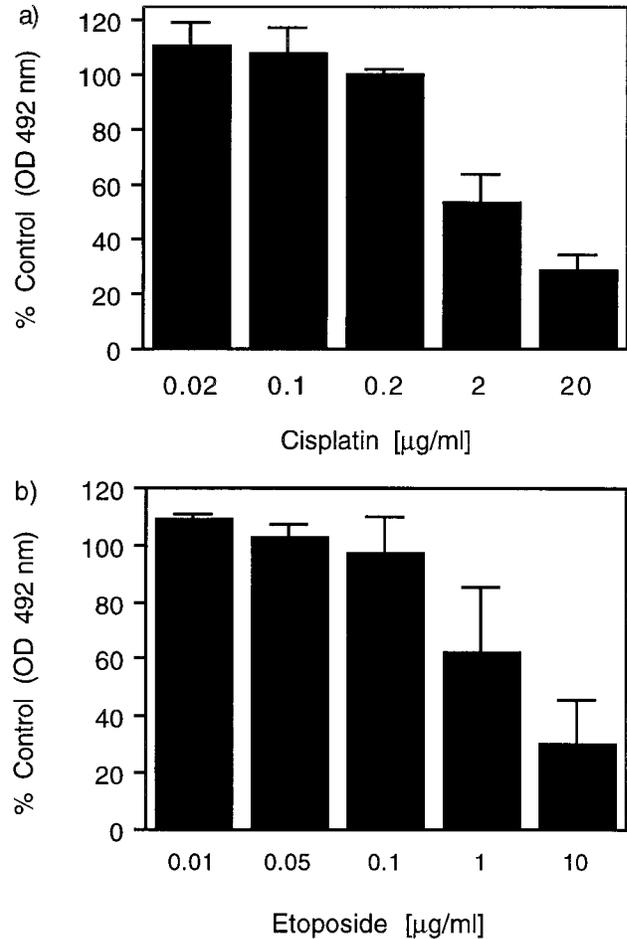

FIGURE 1 – Effect of pre-treatment with cisplatin or etoposide on the viability of P815 tumor cells. P815 cells incubated for 20 hr in the absence or presence of the indicated concentrations of *(a)* cisplatin or *(b)* etoposide were washed and viability was measured by MTT assay, as described in "Material and Methods". Results from 3 independent experiments were averaged and expressed as % control (±SD).

Cytotoxic T lymphocytes mediate target cell destruction through 2 distinct cytolytic pathways (reviewed in Berke, 1995). One pathway is dependent on extracellular Ca^{2+} and involves exocytosis by effector cells of cytotoxic granule contents, including perforin and various granzyme molecules. The other pathway, which functions independently of extracellular Ca^{2+}, involves the interaction of Fas ligand on activated cytotoxic T lymphocytes with Fas receptor, which is transcriptionally activated by p53 in target cells. The result is transduction of an apoptosis-inducing signal to the target cell nucleus. To further characterize the mechanism of cisplatin- and etoposide-induced sensitization of P815 tumor cells to killing by AK-T cells, granule-dependent cytolysis was blocked by adding EGTA (to chelate extracellular Ca^{2+} cations, which are required for granule-mediated killing) to cytotoxicity assays. Representative results obtained at an E:T ratio of 12:1 are shown in Table I. Similar results were obtained at other E:T ratios (data not shown). The data indicate that AK-T cells employ the Ca^{2+}-dependent exocytotic pathway of cytolysis to kill untreated P815 tumor cells since killing was abrogated in the presence of EGTA. P815 tumor cells that were pre-treated with lower (non-cytotoxic) concentrations of cisplatin (0.2 µg/ml) or etoposide (0.01 or 0.1 µg/ml) were rendered more sensitive to cytolysis than untreated P815 tumor cells. This effect was mediated through Ca^{2+}-dependent cytolytic mechanisms since killing was reduced to
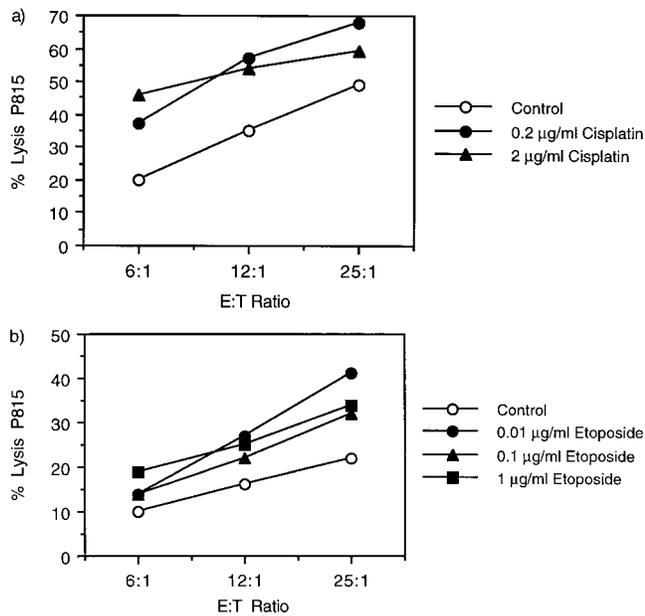
FIGURE 2 – Pre-treatment with cisplatin or etoposide enhances P815 susceptibility to AK-T cell–mediated lysis. P815 cells incubated for 20 hr in the absence or presence of the indicated concentrations of *(a)* cisplatin or *(b)* etoposide were washed, labeled with ^{51}Cr and added to AK-T cells at the indicated E:T ratios. Four hours later, ^{51}Cr-release was measured by gamma counting. Values represent mean cytotoxicity in triplicate wells. Standard deviations were less than 10% of the mean. Percent spontaneous releases of ^{51}Cr from P815 cells pre-treated with 0.2 or 2 µg/ml cisplatin or medium alone were 12%, 36% and 14%, respectively. Percent spontaneous releases of ^{51}Cr from P815 cells pre-treated with 0.01, 0.1 or 1 µg/ml etoposide or medium alone were 11%, 11%, 22% and 8%, respectively. Results from one experiment representative of at least 3 independent experiments are shown.

TABLE I – EFFECT OF EGTA ON AK-T CELL–MEDIATED CYTOLYSIS OF P815 TUMOR CELLS PRE-TREATED WITH CISPLATIN OR ETOPOSIDE[1]

Experiment	P815 pre-treatment	EGTA added to assay	% Lysis	Significance (*p*)
1	Medium	−	22 ± 2	—
		+	3 ± 1	—
	0.02 µg/ml cisplatin	−	23 ± 2	NS
		+	1 ± 1	NS
	0.2 µg/ml cisplatin	−	31 ± 1	0.0022
		+	5 ± 1	NS
	2 µg/ml cisplatin	−	40 ± 8	0.0194
		+	27 ± 1	<0.0001
2	Medium	−	16 ± 1	—
		+	2 ± 1	—
	0.01 µg/ml etoposide	−	27 ± 2	0.0010
		+	4 ± 1	NS
	0.1 µg/ml etoposide	−	22 ± 1	0.0018
		+	4 ± 1	NS
	1 µg/ml etoposide	−	25 ± 2	0.0022
		+	15 ± 3	0.0021

[1]P815 cells incubated for 20 hr in the presence or absence of the indicated concentrations of cisplatin or etoposide were washed, labeled with ^{51}Cr and added to AK-T cells at an E:T ratio of 12:1 in the absence or presence of 4 mM EGTA and 3 mM $MgCl_2$. Four hours later, ^{51}Cr-release was measured by gamma counting. Values represent mean cytotoxicity in triplicate wells (±SD). Percent spontaneous releases of ^{51}Cr from P815 cells pre-treated with 0.02, 0.2 or 2 µg/ml cisplatin or medium alone were 12%, 11%, 25% and 12%, respectively. Percent spontaneous releases of ^{51}Cr from P815 cells pre-treated with 0.01, 0.1 or 1 µg/ml etoposide or medium alone were 11%, 11%, 22% and 8%, respectively. Statistical significance of data relative to the appropriate medium controls was determined by Student's *t*-test; NS = not significant. Results from 1 experiment representative of 3 independent experiments are shown.
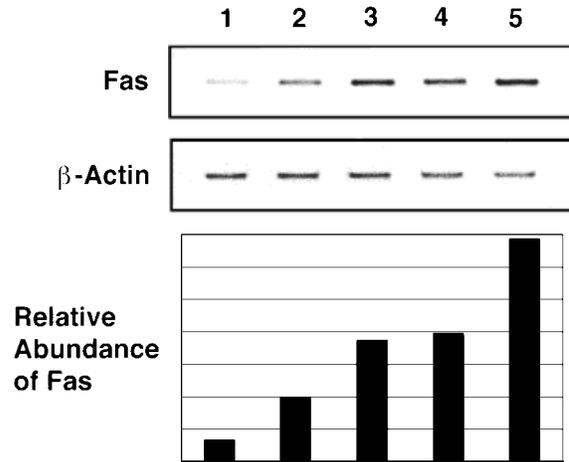

FIGURE 3 – Increased Fas mRNA expression by P815 tumor cells pre-treated with cisplatin or etoposide. P815 cells incubated for 20 hr in the presence of complete medium alone (lane 1), 0.2 µg/ml cisplatin (lane 2), 2 µg/ml cisplatin (lane 3), 0.1 µg/ml etoposide (lane 4) or 1 µg/ml etoposide (lane 5) were washed and mRNA was isolated and reverse-transcribed. A semi-quantitative PCR with primers specific for Fas or β-actin was performed, and ethidium bromide–stained cDNA was visualized under UV light. Detected cDNA was compared to the m.w. standard (not shown) to confirm PCR product size. PCR products were quantified by densitometric scanning, and Fas mRNA expression was normalized relative to the steady-state expression of mRNA coding for β-actin.

background levels when EGTA was added to the cytotoxicity assay. However, P815 cells that were previously exposed to 2 µg/ml cisplatin or 1 µg/ml etoposide (around the EC_{50} concentrations for cytotoxicity) exhibited a marked increase in susceptibility to killing by AK-T cells in the absence of Ca^{2+}, suggesting that pre-treatment with these particular concentrations of cisplatin or etoposide renders P815 tumor cells susceptible to killing by the Fas ligand/ Fas pathway of cytolysis.

Fas expression by P815 tumor cells increases upon exposure to cisplatin or etoposide

We therefore used semi-quantitative RT-PCR to determine whether P815 tumor cells that were pre-treated with higher concentrations of cisplatin (0.2, 2 µg/ml) or etoposide (0.1, 1 µg/ml) up-regulated expression of Fas mRNA. As shown in Figure 3, untreated P815 cells express only low levels of Fas mRNA. P815 expression of Fas mRNA was up-regulated most markedly following a 20 hr incubation in the presence of 1 µg/ml etoposide, though a modest increase in Fas gene expression was observed following exposure to cisplatin (0.2, 2 µg/ml) or to a lower concentration of etoposide (0.1 µg/ml). We next used flow cytofluorimetry with FITC-conjugated anti-Fas MAb to confirm these results. Only low levels of Fas were detected on the surface of untreated P815 tumor cells (Fig. 4). Treatment of P815 tumor cells with cisplatin led to a dose-dependent increase in cell-surface Fas expression that was maximal on P815 cells exposed to 2 µg/ml cisplatin (Fig. 4*a*). P815 tumor cells that were incubated in the presence of etoposide also up-regulated cell-surface expression of Fas in a dose-dependent fashion (Fig. 4*b*). Cell-surface Fas expression was greatest following exposure of P815 cells to 0.1–1.0 µg/ml etoposide.

Sensitization of P815 tumor cells by cisplatin or etoposide to anti-Fas antibody–mediated cytotoxicity

We also tested whether P815 tumor cells which were pre-treated with cisplatin or etoposide were susceptible to killing mediated by anti-Fas MAb in the absence of complement. It is well established
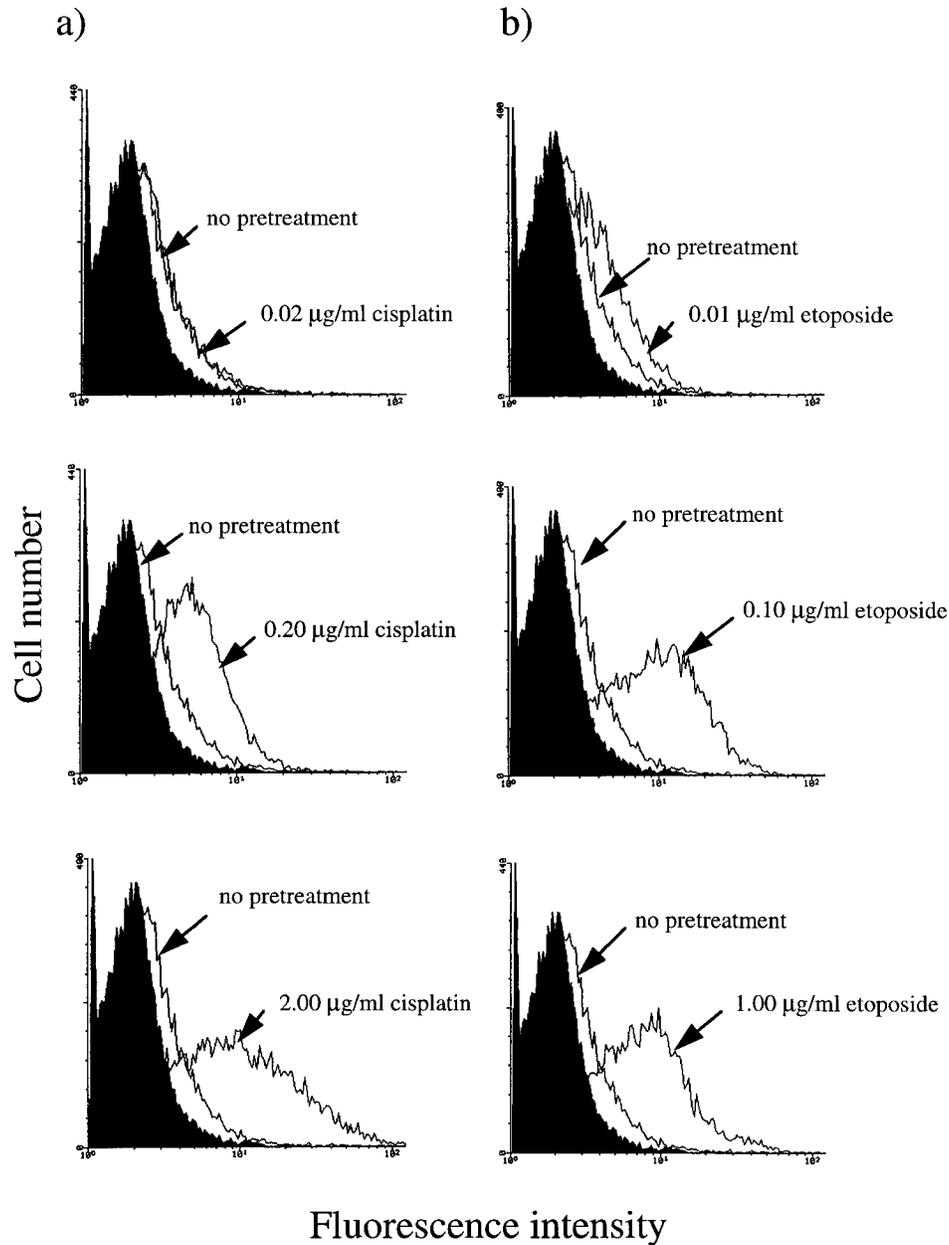
a)   b)

Figure 4 – Flow-cytofluorimetric analysis of cell surface Fas (CD95) expression by cisplatin- or etoposide-treated P815 tumor cells. P815 cells were incubated for 20 hr in the absence or presence of the indicated concentrations of *(a)* cisplatin or *(b)* etoposide, washed and stained for cell-surface Fas expression with a FITC-conjugated anti-Fas MAb (10 μg/ml), as described in "Material and Methods". Cytofluorimetric profiles for unstained P815 cells (filled peaks) and for P815 cells stained with Fas-specific MAb (open peaks) are shown.

that anti-Fas MAbs cause cytotoxicity and apoptosis in cultures of Fas-bearing cells as a result of cross-linking cell-surface Fas and subsequent induction of programmed cell death (Trauth *et al.,* 1989). P815 cells were pre-treated with cisplatin (0.2, 2 μg/ml) or etoposide (0.01, 0.1 μg/ml) for 20 hr, radiolabeled and incubated for 4 hr in the presence of anti-Fas MAb at concentrations ranging from 0.01 to 1 μg/ml. As shown in Figure 5, anti-Fas MAb had no discernable cytotoxic effect on untreated P815 tumor cells. In contrast, P815 cells which were pre-treated with cisplatin became sensitive to anti-Fas MAb–mediated cytotoxicity (Fig. 5*a*). For example, pre-treatment of P815 cells with 2 μg/ml of cisplatin followed by exposure to 1 μg/ml of anti-Fas MAb resulted in 20% lysis over the 4 hr cytotoxicity assay. Pre-treatment with etoposide also sensitized P815 cells to anti-Fas MAb–mediated cytotoxicity

(Fig. 5*b*). These data indicate that cisplatin- and etoposide-induced Fas is functionally active in the P815 mastocytoma.

DISCUSSION

Cisplatin and etoposide are 2 anti-neoplastic drugs widely used in the treatment of human cancers (Chabner *et al.,* 1996). In our study, cisplatin and etoposide were used at concentrations within and below the plasma therapeutic range. In patients receiving well-tolerated doses of i.v. cisplatin, plasma levels of the drug typically peak at 2.8 μg/ml (Reese *et al.,* 1989), while plasma levels of etoposide reach 30 μg/ml following i.v. injection of a typical therapeutic dose of the drug (Chabner *et al.,* 1996). Pre-treatment of P815 mastocytoma cells with low doses of cisplatin or etoposide
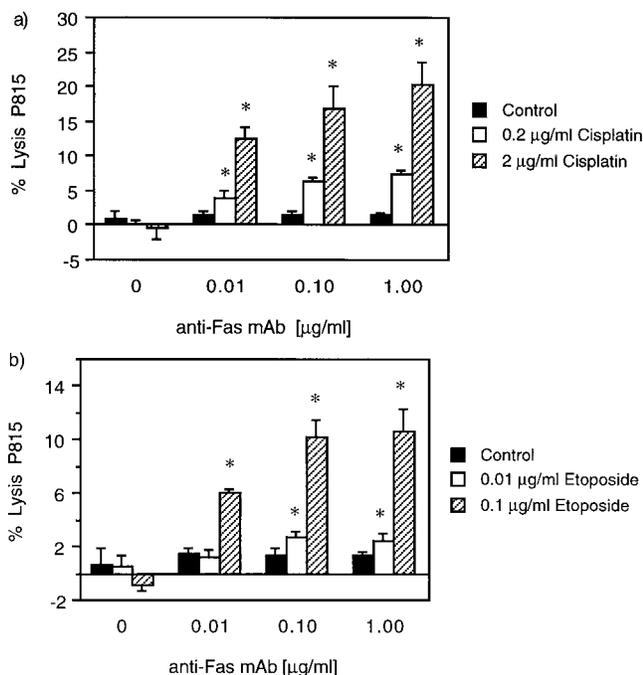
FIGURE 5 – Sensitivity of P815 tumor cells to anti-Fas antibody–mediated cytotoxicity increases following exposure to cisplatin or etoposide. P815 cells incubated for 20 hr in the absence or presence of the indicated concentrations of *(a)* cisplatin or *(b)* etoposide were washed, labeled with ^{51}Cr and added to the indicated concentrations of anti-Fas MAb. Four hours later, ^{51}Cr-release was measured by gamma counting. Values represent mean cytotoxicity ($\pm$SD) in triplicate wells. Asterisk indicates statistical significance compared to control values, as determined by Student's *t*-test. Percent spontaneous releases of ^{51}Cr from P815 cells pre-treated with 0.2 or 2 µg/ml cisplatin or medium alone were 14%, 35% and 9%, respectively. Percent spontaneous releases of ^{51}Cr from P815 cells pre-treated with 0.01 or 0.1 µg/ml etoposide or medium alone were 9%, 16% and 9%, respectively. Results from one experiment representative of 3 independent experiments are shown.

for 20 hr had no significant direct cytotoxic effect on P815 cells, as determined by MTT assay (Fig. 1). Exposure of P815 tumor cells to higher doses of cisplatin or etoposide revealed that the EC_{50} values for cisplatin and etoposide are approx. 2 µg/ml and 1 µg/ml, respectively. Pre-treatment with concentrations of cisplatin that lie on the threshold of, or on the dose-response curve for, cytotoxicity sensitized the P815 mastocytoma cells to subsequent lysis by AK-T cells (Fig. 2). This finding is in agreement with a previous report that *in vitro* exposure of human K562 erythroleukemia cells to 10 µg/ml cisplatin renders the tumor cells more susceptible to attack by natural killer cells and lymphokine-activated killer cells (Mizutani *et al.,* 1993). In comparison to cisplatin, etoposide appeared to be the more potent sensitizing agent since pre-treatment with a 0.01 µg/ml concentration of etoposide, which is well below the threshold for toxicity, potentiated tumor cell lysis by AK-T cells at least as well as exposure to concentrations of etoposide at the threshold of, or on the dose-response curve for, toxicity. Our data are consistent with an earlier report that murine natural cell–mediated cytotoxicity against L929 and WEHI-164 fibrosarcoma cells is enhanced following treatment of the tumor cells with 5–50 µM etoposide (Utsugi *et al.,* 1989).

To date, few studies have addressed the mechanisms by which cytotoxic anti-neoplastic drugs render tumor cells more susceptible to destruction by immune effector cells. Cisplatin enhances lymphokine-activated killer cell–mediated lysis of Daudi lymphoma cells and KATO-III gastric cancer cells by up-regulating tumor cell

expression of ICAM-1 and LFA-3, adhesion molecules which play important roles in effector cell adhesion to tumor targets (Ishihata *et al.,* 1996). One study suggests that ET-18-OCH3 is able to sensitize K562 erythroleukemia cells to *in vitro* lysis by lymphokine-activated killer cells by inducing expression of HSP72 heat shock protein on the surface of tumor cells (Botzler *et al.,* 1996). In the present study, we have demonstrated that pre-treatment with sub-cytotoxic concentrations of cisplatin or etoposide can enhance the sensitivity of P815 mastocytoma cells to Ca^{2+}-dependent, AK-T cell–mediated cytolysis (Table I). Furthermore, when P815 mastocytoma cells were exposed to concentrations of cisplatin or etoposide in the low cytotoxic range, we observed increased susceptibility to killing by AK-T cells in the presence of the Ca^{2+} chelator EGTA, indicating the involvement of a Ca^{2+}-independent cytolytic pathway (Table I). Interestingly, increased sensitivity of P815 tumor cells pre-treated with low cytotoxic doses of cisplatin or etoposide to killing by AK-T cells in the absence of Ca^{2+} correlated well with a marked increase in Fas mRNA expression by the tumor cells (Fig. 3). Human ovarian tumor cells also have been reported to up-regulate Fas gene expression following treatment with sub-cytotoxic concentrations of cisplatin (Uslu *et al.,* 1996). We report here a similar effect mediated by etoposide. We also observed a dose-dependent increase in Fas molecules present on the surface of P815 mastocytoma cells following treatment with cisplatin or etoposide (Fig. 4).

Cross-linking of Fas molecules on the surface of target cells by Fas ligand expressed by activated T cells leads to signal-transduction events that cause the target cell to undergo apoptosis (Hanabuchi *et al.,* 1994). Since anti-CD3-activated T lymphocytes express abundant Fas ligand (Hoskin *et al.,* 1996), it is likely that AK-T cells employ the Fas/Fas ligand cytolytic pathway to kill P815 tumor cells which have up-regulated cell-surface Fas expression beyond some critical threshold level as a result of prior exposure to concentrations of cisplatin or etoposide which approximate the EC_{50} values for these drugs. Although we have not determined the phenotype of AK-T cells which kill by the Fas/Fas ligand cytolytic pathway, both $CD4^{+}$- and $CD8^{+}$-activated T lymphocytes have been shown to express Fas ligand and to kill target cells in a Ca^{2+}-independent manner (Berke, 1995). Because levels of Fas expression on tumor cell lines do not always correlate with biological responsiveness to engagement of the Fas protein (Owen-Schaub *et al.,* 1994), we also examined the effect of anti-Fas MAb on cisplatin- or etoposide-treated P815 tumor cells. We observed that P815 tumor cells which had been pre-treated with the anti-neoplastic agents exhibited a dose-dependent increase in susceptibility to anti-Fas MAb–mediated cytotoxicity, whereas untreated P815 tumor cells were completely resistant to the cytotoxic effect of anti-Fas MAb (Fig. 5). The finding that the Fas pathway is functional in cisplatin- or etoposide-treated P815 mastocytoma cells is consistent with the involvement of the Fas/Fas ligand cytolytic pathway in AK-T cell–mediated lysis of these tumor cells following treatment with low cytotoxic concentrations of cisplatin or etoposide. However, it was not possible to block AK-T cell–mediated lysis of Fas-bearing P815 mastocytoma cells with anti-Fas MAb because the anti-Fas MAb alone triggered apoptotic death of P815 tumor cells over the course of a 4-hr ^{51}Cr-release assay.

The mechanism by which cisplatin or etoposide induces Fas expression by P815 and other tumor cells is not yet completely understood. It is known that the tumor-suppressor protein p53 is involved in the transcriptional activation of the *Fas* gene in human tumor cell lines (Owen-Schaub *et al.,* 1995). In response to DNA damage, p53 protein levels in cells increase, resulting in the delay of cell-cycle progression from G_1 into S phase in order to allow DNA repair to occur (Kastan *et al.,* 1991). Failure to repair DNA leads the cell to undergo programmed cell death. Human testicular tumor and bladder cancer cells which have been exposed to a threshold cytotoxic dose of etoposide exhibit increased expression

of the p53-induced gene *waf-1* (Chresta *et al.*, 1996), suggesting the expression of functional p53 by these tumor cells. However, certain cells modulate the expression of *waf-1* independently of p53 (Munker *et al.*, 1996). Alkylating agents also are known to induce p53 expression (Artuso *et al.*, 1995). Exposure to 1 μM cisplatin induces the synthesis of p53 by malignant glioma cells (Roth *et al.*, 1997). Moreover, bleomycin-induced up-regulation of cell-surface Fas expression in human hepatoma cells depends on the transient accumulation of nuclear wild-type p53 (Müller *et al.*, 1997). It is, therefore, reasonable to speculate that the up-regulation of Fas expression by P815 tumor cells following exposure to cisplatin or etoposide depends on p53 induction in response to drug-mediated damage to tumor cell DNA. Interestingly, restoration of wild-type p53 expression in a p53-defective colorectal cancer cell line potentiates the *in vitro* cytotoxicity of the topoisomerase I inhibitor topotecan (Yang *et al.*, 1996), suggesting that engagement of topotecan-induced Fas by Fas ligand expressed by many colon carcinoma cells (Walker *et al.*, 1997) may contribute to the anti-neoplastic activity of topotecan.

In conclusion, we have shown that cisplatin and etoposide, in the low cytotoxic dose range and below, sensitize P815 tumor cells to killing by major histocompatibility complex–unrestricted cytotoxic T lymphocytes. The potentiating effect at low cytotoxic drug concentrations is mediated in part by effector cell Fas ligand engagement of tumor cell-surface Fas molecules induced in response to cisplatin or etoposide treatment. It is well known that anti-neoplastic agents frequently have devastating toxic side effects when administered to cancer patients at the maximal tolerated dosage. In addition, endogenous immune responses often are compromised by aggressive chemotherapy. However, our data indicate that it may be possible to employ low-dose chemotherapy with anti-cancer drugs such as cisplatin and etoposide in combination with the adoptive transfer of activated Fas ligand-positive killer T lymphocytes for the treatment of certain cancers since relatively low doses of these anti-neoplastic agents can facilitate T cell–mediated oncolysis mediated through granule- and Fas/Fas ligand-dependent cytolytic pathways. Although the systemic administration of agonistic anti-Fas antibodies *via* the i.v. route is associated with hepatocyte apoptosis and acute liver failure in mice (Ogasawara *et al.*, 1993), i.p. injection of soluble Fas ligand has been shown to eradicate Fas-bearing lymphoma cells growing within the peritoneal cavity without causing liver failure (Rensing-Ehl *et al.*, 1995). Therefore, it may be possible to combine Fas-inducing low-dose chemotherapy with loco-regional administration of soluble Fas ligand or agonistic anti-Fas antibodies to treat localized neoplasms.

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