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Original Paper

Drug Resistance in Human Neuroblastoma Cell Lines Correlates with Clinical Therapy

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To determine if neuroblastoma acquires a sustained drug-resistant phenotype from patient exposure to therapy, we studied neuroblastoma cell lines established at different points of therapy: at diagnosis prior to therapy, at progressive disease after induction therapy and at relapse after intensive chemoradiotherapy and bone marrow transplantation (post-BMT). Melphalan, cisplatin, carboplatin, doxorubicin, and etoposide cytotoxicities were determined by DIMSCAN assay. Drug resistance progressively increased with therapy and 3/5 post-BMT lines showed high resistance to most drugs. IC 90s 37, 78, 719 and 256 times higher than clinically achievable drug levels were obtained in post-BMT cell lines for melphalan, cisplatin, doxorubicin and etoposide, respectively. Resistance correlated with the therapies patients received: considerable etoposide and doxorubicin resistance (>1000-fold resistance) was seen in cell lines obtained from patients treated with these drugs. These cell lines indicate that neuroblastoma acquires resistance to cytotoxic drugs that is probably due to stable genetic alterations occurring during therapy. © **1997 Published by Elsevier Science Ltd.**

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INTRODUCTION

NEUROBLASTOMA IS a malignant childhood neoplasm of the sympathetic nervous system. While most high-risk tumours show an initial response to chemotherapy, the majority of such patients eventually develop progressive disease that is refractory to chemotherapy [1, 2]. Neuroblastomas which progress on or after chemotherapy could result from altered expression of drug resistance genes [3–5], tumour cells in 'sanctuary' sites of low drug penetration [6], tumour hypoxia [7, 8] tumour cells resting out of cell cycle [9] or all of the above. No current experimental data favours any of the above mechanisms, except that the poor response of relapsed neuroblastoma to further therapy suggests that selection for tumour cells with genetic alterations conferring drug resistance plays a key role.

To develop a model system for understanding the mechanisms by which neuroblastoma cells escape chemotherapy, especially intensive, marrow-ablative chemotherapy, we established a panel of cell lines from patients with neuroblastoma at various points during the course of their disease. In this study we determined sensitivity to cytotoxic drugs of neuroblastoma cell lines established at diagnosis, progressive disease during induction therapy and relapse after intensive chemoradiotherapy and bone marrow transplantation.

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MATERIALS AND METHODS

Cell lines

We used a panel of 17 neuroblastoma cell lines (Table 1) obtained from patients at various points of disease: 6 at diagnosis (DX), 6 at progressive disease (PD-Ind) during induction therapy, and 5 derived at relapse after bone marrow transplantation (post-BMT); 3 after autologous BMT (CHLA-51, CHLA-90, CHLA-134) and 2 after allogeneic BMT (CHLA-8, CHLA-79). Cell lines established at diagnosis were derived from patients before any chemotherapy was given; cell lines established at progressive disease came from patients treated with induction chemotherapy; and cell lines established after BMT were obtained from patients exposed to high-dose, myeloablative chemoradiotherapy.

Table 1. *IC* 90 *values of doxorubicin (Dox, ng/ml), etoposide (ETOP, ng/ml), cisplatin (CDDP, μg/ml), carboplatin (CBDCA, μg/ml), and melphalan (L-PAM, μg/ml) are given for the* 6 *lines established at diagnosis (DX), the* 6 *cell lines at the time of progression during induction therapy (PD-Ind) and* 5 *cell lines at relapse after myeloablative therapy and BMT (post-BMT). The clinically achievable levels are shown in parentheses*

	DOX (60 ng/ml)	ETOP (1000 ng/ml)	CDDP (0.1 μg/ml)	CBDCA (3 μg/ml)	L-PAM (10 μg/ml)
DX					
SMS-KAN	62.7	156	0.31	1.13	4.41
SMS-KCN	0.26	2.7	0.14	0.58	0.08
SK-N-BE(1)	28.6	<0.001	<0.001	<0.04	0.13
CHLA-15	1.14	0.18	0.226	1.05	1.52
SMS-SAN	11.8	0.0017	<0.001	0.064	0.12
CHLA-42	24.26	67.4	0.34	0.52	0.54
PD-Ind					
SMS-KANR	6.22	1.2	0.34	0.7	2.21
SMS-KCNR	0.18	0.21	0.11	1.21	0.97
SK-N-BE(2)	40.33	577	0.054	1.1	3.31
CHLA-20	497.56	691	1.13	3.7	1.31
SMS-LHN	19.89	682	0.097	0.79	0.34
LA-N-6	86.04	24 059	0.93	7.25	3.82
Post-BMT					
CHLA-51	0.0028	24.28	<0.001	2.6	2.22
CHLA-8	8.22	41.77	0.71	2.34	0.3
CHLA-79	43 155	4893	5.13	3.2	4.97
CHLA-90	331.2	51 254	2.54	13.75	375
CHLA-134	768	255 873	7.82	26.3	37.4

SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-BE(1), SK-N-BE(2), SMS-SAN, SMS-LHN and LA-N-6 have been characterised previously [10–12] and were cultured in complete medium made from RPMI-1640 medium (Irvine Scientific, Santa Ana, California, U.S.A.) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco BRL, Grand Island, New York, U.S.A.). CHLA-15, CHLA-20, CHLA-51, CHLA-8, CHLA-79, CHLA-90 and CHLA-134 are newly established neuroblastoma cell lines, and were cultured in complete medium made from Iscove's Modified Dulbecco's Medium (IMDM) (Bio Whittaker, Walkersville, Maryland, U.S.A.) supplemented with 2 mM L-glutamine (Gemini Bioproducts, Inc., Calabasas, California, U.S.A.), insulin and transferrin 16.6 mg/ml each and 16.6 μg/ml of selenous acid (ITS™ Culture Supplement) (Collaborative Biomedical Products, Bedford, Massachusetts, U.S.A.) and 20% heat inactivated FCS. All cell lines used in the study were under passage 25 and were cultured at 37°C in a humidified incubator containing a 95% air/5% CO_2 atmosphere without antibiotics. Cell lines were not selected for drug resistance *in vitro*.

Drugs and chemicals

Melphalan (L-PAM), cisplatin (CDDP), carboplatin (CBDCA) and doxorubicin hydrochloride (DOX) were obtained from the National Institutes of Health, Bethesda, Maryland, U.S.A. Etoposide (ETOP) was obtained from Bristol-Myers Squibb Co., Princeton, New Jersey, U.S.A. Fluorescein diacetate (FDA) was purchased from Eastman Kodak Company, Rochester, New York, U.S.A.

Cytotoxicity assay

Melphalan, cisplatin, carboplatin, doxorubicin and etoposide cytotoxicities were determined with the DIMSCAN assay system. DIMSCAN uses digital imaging microscopy to quantify viable cells stained with fluorescein diacetate (FDA) and is capable of measuring cytotoxicity over a 4 log dynamic range. DIMSCAN measures the total fluorescence per well (which is proportional to viable, clonogenic cells) after eliminating background fluorescence with digital thresholding [13] and eosin Y quenching [14]. Cell lines were seeded at 15 000 cells in 150 μl of complete medium per well into 96-well plates. After overnight incubation, various concentrations of chemotherapeutic drugs in 100 μl of complete medium were added to each well. The concentration ranges used were: cisplatin, etoposide and doxorubicin = 0–10 μg/ml; carboplatin and melphalan = 0–12 μg/ml. Each condition was tested in 12 replicates. After incubation of cell lines with L-PAM for 3 days, DOX and ETOP for 4 days and CDDP and CBDCA for 7 days, 150 μl of medium was removed from each well, FDA in 50 μl of medium (final concentration of FDA was 8 μg/ml) was added, plates were incubated for an additional 30 min at 37°C and 30 μl of 0.5% eosin Y [14] was added to each well. Total fluorescence was then measured using digital imaging microscopy and results were expressed as surviving fractions of treated cells compared to control cells.

Data analysis

IC_{90} values (i.e. the drug concentration that was cytotoxic for 90% of the cell population) were calculated using the software 'Dose–Effect Analysis with Microcomputers' [15]. Fold resistance was defined as a ratio of the IC_{90} of a given cell line to the IC_{90} value of a sensitive cell line for a given drug. Cell lines with IC_{90} values higher than the achievable clinical concentrations (peak plasma level (PPL) for bolus agents or continuous steady-state concentration (CSS) for continuous infusion agents) were considered resistant to that

drug. 60 ng/ml is the reported CSS for doxorubicin [16] and 1 μg/ml is the CSS for etoposide [17]. 3 μg/ml and 0.1 μg/ml are the CSS achieved for carboplatin and cisplatin, respectively, in neuroblastoma patients and 10 μg/ml is the PPL for melphalan in neuroblastoma patients receiving this drug as a part of their conditioning regimen for bone marrow transplantation (Dr J. Villablanca, data not shown).

DNA typing

DNA typing was conducted using the PCR Amplification and Typing Kit AmpliType PM (Perkin Elmer, Foster City, California, U.S.A.) following the manufacturer's instructions. The following genetic loci were studied: low-density lipoprotein receptor (LDLR), haemoglobin G gammaglobin (HBGG), D7S8 and group-specific component (GC). Typing was performed to confirm cell line origin. Allo-BMT cell lines: CHLA-8 and CHLA-79, matched to their corresponding tumour specimen, ABMT cell lines: CHLA-51, CHLA-90 and CHLA-134, matched to their corresponding bone marrow. Paired cell lines matched each other, except for SMS-KAN + SMS-KANR, in which one locus did not match, presumably as a result of chromosomal loss during tumour progression.

RESULTS

Cytotoxicity studies

We determined sensitivity to drugs commonly used for neuroblastoma: doxorubicin, etoposide, cisplatin, carboplatin and melphalan. Results of cytotoxicity assays are expressed as IC_{90} values for each drug tested (Table 1).

All PD-Ind and post-BMT cell lines were derived from patients treated with doxorubicin. As shown in Table 1, IC_{90} values for doxorubicin increased with therapy, with DX cell lines having IC_{90}s of 0.26–62.7 ng/ml, PD-Ind cell lines from 0.17 to 497.56 ng/ml, and post-BMT cell lines from 0.0028 to 43 155 ng/ml. The IC_{90} values for doxorubicin were >60 ng/ml (the CSS for doxorubicin) for two PD-INd and three post-BMT cell lines. CHLA-20 had the highest doxorubicin IC_{90}s value within the PD-Ind group, which was 8 times higher than the clinically obtainable concentration. The IC_{90}s for doxorubicin of the post-BMT cell lines, CHLA-79, CHLA-90 and CHLA-134, were 719, 5.5. and 13 times higher than the reported CSS.

Etoposide or another epipodophilotoxin, teniposide, were given to 2 PD-Ind and to all post-BMT patients. Etoposide IC_{90} values of DX cell lines ranged from <0.001 to 156 ng/ml, those of PD-Ind cell lines range from 0.21 to 24 059 ng/ml and post-BMT cell lines IC_{90}s ranged from 24.28 to 255 873 ng/ml. Resistance to etoposide was high ($>2 \times 10^4$-fold resistance) in cell lines derived from patients treated with etoposide (LA-N-6, CHLA-79, CHLA-90 and CHLA-134). The etoposide IC_{90} for LA-N-6 (a PD-Ind cell line) was 24 times higher than the reported CSS. The post-BMT cell lines CHLA-79, CHLA-90 and CHLA-134 showed IC_{90}s 5, 51, and 256 times higher than CSS. Figure 1 shows representative dose–response curves for cell lines to etoposide.

Carboplatin was more cytotoxic than cisplatin at clinically achievable levels in neuroblastoma cell lines. Carboplatin IC_{90} values for 0/6 DX, 2/6 PD-Ind and 2/5 post-BMT cell lines were higher than clinically achievable CSS. By contrast, cisplatin IC_{90} values for 4/6 DX, 4/6 PD-Ind and 4/5 Post-BMT cell lines were higher than CSS.

Resistance to melphalan (IC_{90} > 10 μg/ml) was seen only in two post-BMT cell lines: CHLA-90 and CHLA-134. Melphalan IC_{90} values for DX cell lines were 0.08–4.41 μg/ml, for PD-lnd cell lines 0.34–3.82 μg/ml and for post-BMT cell lines 0.3–375 μg/ml.

Drug-resistance phenotype in DX + PD-Ind paired cell lines

Within the panel of 17 neuroblastoma cell lines studied, there were four pairs of cell lines derived from the same patients at diagnosis and after progressive disease: SMS-KAN + SMS-KANR; SMS-KCN + SMS-KCNR; SK-N-BE(1) + SK-N-BE(2); and CHLA-15 + CHLA-20. PD-Ind cell lines SMS-KANR and SMS-KCNR were derived from patients treated with cyclophosphamide and doxorubicin. SMS-KANR, SMS-KCNR did not show sustained drug resistance relative to the DX cell lines from the same patients. The IC_{90} values of DX cell line

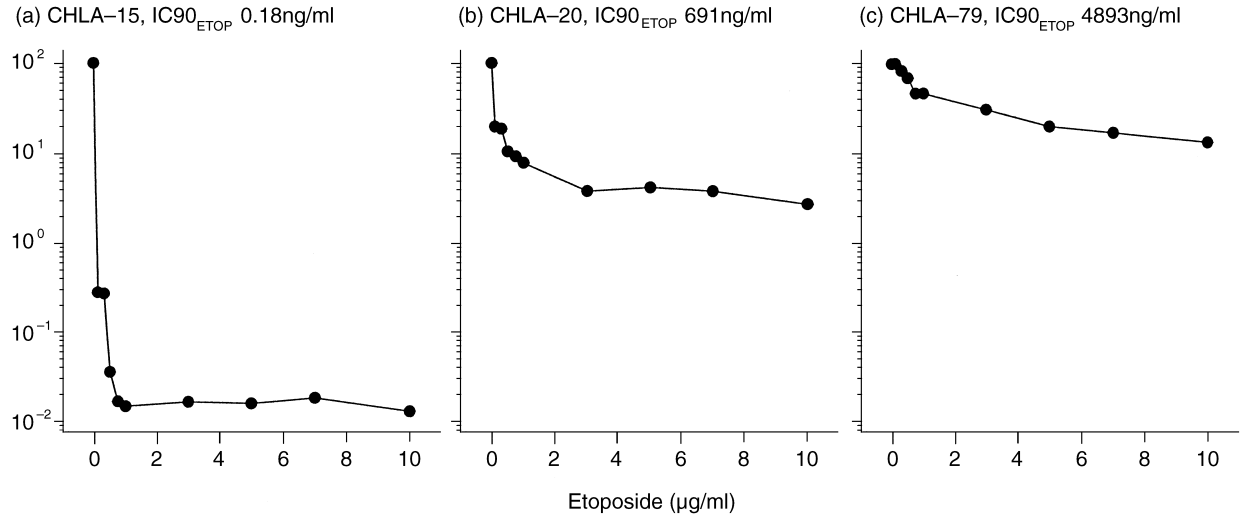

Figure 1. Dose-response curves of representative cell lines to etoposide. Response of cell lines was diminished with therapy. (a) CHLA-15 isolated from the patient at the time of diagnosis prior to any therapy was sensitive to etoposide. (b) CHLA-20 established from the same patient after induction chemotherapy, showed resistance to etoposide. (c) CHLA-79 obtained from a different patient after intensive chemotherapy followed by bone marrow transplantation showed extreme etoposide resistance.

SMS-KAN were higher than those of SMS-KANR for most of the drugs. IC_{90} values of SMS-KCN and SMS-KCNR were within the same range (Table 1).

SK-N-BE(2) was derived from a patient after treatment with doxorubicin, cyclophosphamide and vincristine. IC_{90} values of SK-N-BE(2) for all five drugs were within the clinically achievable ranges, but were considerably higher than those of SK-N-BE(1), the DX cell line from the same patient (Table 1). For example, the IC_{90}s of SK-N-BE(2) were greater than those of SK-N-BE(1) by 5.7×10^6 times for etoposide, 5.4×10^3 times for cisplatin, 1.1×10^5 times for carboplatin and 25 times for melphalan.

CHLA-20 was derived from a patient after treatment with doxorubicin, cyclophosphamide, cisplatin and teniposide. As shown in Table 1, IC_{90} values for this cell line were higher than clinically achievable levels for doxorubicin, cisplatin and carboplatin. The sensitivity to almost all cytotoxic agents tested in this study was decreased in CHLA-20 compared to CHLA-15, a DX cell line from the same patient. For instance, the IC_{90}s of CHLA-20 were greater than the IC_{90} values of CHLA-15 by 436 times for doxorubicin, 3839 times for etoposide, 5 times for cisplatin and 3.5 times for carboplatin.

DISCUSSION

Neuroblastoma initially responds to chemotherapy and then recurs and is incurable by chemotherapy [1, 2]. Development of chemotherapy refractory disease after initially successful chemotherapy points to the selection of drug-resistant tumour cells. Drug resistance in cancer has been largely investigated by selection of resistant variant cell lines with continuous or intermittent exposure of cells to the chemotherapeutic agents [3, 18] or by studying the association of drug resistance genes with clinical outcome [9, 19, 20]. In the present work we investigated whether neuroblastoma cells treated in patients and then established as cell lines had a sustained drug-resistant phenotype and whether drug resistance correlated with the treatment patients had received. Neuroblastoma cell lines utilised in the study were derived from patients treated with combination chemotherapy regimens and were not selected for resistance to one particular agent. Resistance was defined for 5 cytotoxic agents commonly used for neuroblastoma by comparing cell kill ability of the drug to the clinically achievable concentrations of the agent.

Cell lines established from patients at the time of diagnosis prior to chemotherapy exposure were mostly sensitive to all 5 cytotoxic agents tested in this study. Drug resistance was detected in cell lines isolated during disease progression in patients with neuroblastoma, and the highest resistance was demonstrated in cell lines obtained from patients treated with intensive marrow-ablative therapy and bone marrow transplantation. Three of five post-BMT cell lines yielded IC_{90} values to most cytotoxic agents that are clinically unachievable and many fold higher than clinically achievable drug concentrations: 51–255 times for etoposide, 13–719 times for doxorubicin and 25–78 times for cisplatin. In the current Childrens Cancer Group consolidation protocols for neuroblastoma, doxorubicin is omitted and cisplatin is replaced by carboplatin. Consideration should also be given to substituting novel non-cross-resistant agents for etoposide in the consolidation phase of treatment, since such high resistance shown to etoposide in cell lines exposed to the drug in patients suggests that etoposide may only contribute to drug-related toxicities for many patients during the consolidation phase.

Drug-resistance patterns expressed by neuroblastoma cell lines correlated with the treatment intensities patients were exposed to. The PD-Ind cell line SMS-KANR, SMS-KCNR and SMS-LHN, were established from patients treated with only cyclophosphamide and doxorubicin [11, 12] and they were sensitive to all cytotoxic agents tested in this study. Higher IC_{90} values and various levels of resistance to all drugs were seen in cell lines SK-N-BE(2), CHLA-20 and LA-N-6. SK-N-BE(2) was derived from a patient treated with vincristine and radiation and CHLA-20 from a patient treated with cisplatin and teniposide in addition to cyclophosphamide and doxorubicin [10]. A cell line with the highest drug resistant phenotype among PD-Ind cell lines (LA-N-6) was isolated from a patient who received multiple courses of chemotherapy containing cyclophosphamide, doxorubicin, teniposide, etoposide, vincristine and dacarbazine due to progressive disease [12]. Thus, expression of the drug-resistance phenotype in neuroblastoma cell lines appears to increase with the dose intensity of the chemotherapy given to the patients. Resistance to melphalan, a drug only used for BMT patients, was found only in two cell lines from the post-BMT group. Very high ($>10^4$-fold) resistance to etoposide was observed only in cell lines derived from patients exposed to etoposide or teniposide. Some degree of etoposide resistance was also seen in the SK-N-BE(2) derived from a patient treated with vincristine.

This is the first study of drug sensitivity for cell lines obtained from patients who underwent intensive myeloablative chemotherapy and BMT. Very high drug resistance was seen in both post-allo-BMT and post-ABMT cell lines. One of two post-BMT cell lines established after allogeneic BMT and two of three cell lines established after autologous BMT showed high resistance to all 5 cytotoxic agents tested in this study. The high sustained drug resistance in most of these post-BMT cell lines points to intrinsic alterations in tumour cells as a mechanism for post-BMT relapse and suggests that the use of drug resistance modulating agents and/or drugs that are not cross-resistant with agents used for induction chemotherapy will improve pre-BMT conditioning regimens.

A lower degree of resistance was seen for alkylators such as carboplatin and melphalan. Carboplatin resistant cell lines showed IC_{90} values 1.2–8.8 times greater than the CSS for neuroblastoma patients and cisplatin IC_{90} values were 1.4–78 times greater than the CSS for neuroblastoma patients. Thus, at the clinically achievable levels carboplatin was a more efficacious drug than cisplatin *in vitro*. In the panel of 17 cell lines, only two demonstrated resistance to 210 mg/m^2 of melphalan. These were post-BMT cell lines obtained from patients who received melphalan as a part of a conditioning regimen for BMT.

A number of studies have used cell lines first selected *in vitro* for drug resistance and then maintained without the selecting agent to monitor reversal of drug-resistant phenotype and associated genetic changes. Reversal of the resistant phenotype is noted whether or not concomitant genetic alterations occur [21–23]. By contrast, our panel of cell lines showed sustained drug resistance to various classes of cytotoxic agents which correlates with the therapies given to the patients. This makes these cell lines ideal for studying the molecular aspects of drug resistance. Two genes, MDR1 [24, 25] and the recently discovered *MRP* [26], are thought

to mediate resistance to epipodophyolotoxins, vinca alkaloids and anthracyclines. High resistance to doxorubicin and etoposide seen in cell lines isolated from patients treated with the drugs, and a tendency to develop etoposide resistance in cell lines of patients treated with vincristine or teniposide and doxorubicin, suggest that MDR1 and *MRP* might be one mechanism of doxorubicin and etoposide resistance.

The basis for resistance to alkylating agents is not as well defined as that for natural product drugs such as doxorubicin and etoposide, although a number of studies have identified several mechanisms that are likely involved. Changes associated with resistance to platinum compounds include increased DNA repair [27, 28], reduced drug accumulation (non-*MDR1* mediated) [29] and increased cellular inactivation [21, 30]. These various mechanisms are currently being investigated in this cell line panel.

Finally, these cell lines provide a unique panel for preclinical studies to identify non-cross-reacting agents for new clinical trials. They should help in designing clinical strategies to overcome drug resistance developed during the course of treatment by identifying optimal agents for use during consolidation therapy.

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