Chemotherapy-induced Apoptosis of S-type Neuroblastoma Cells Requires Caspase-9 and Is Augmented by CD95/Fas Stimulation*

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Stromal or S-type tumor cells are a distinct lineage found in neuroblastoma tumors and have an important role in the biology of this disease. Anticancer agents induce apoptosis through death receptor- and mitochondria-initiated pathways. The object of this work was to determine the involvement of these pathways in the response to doxorubicin (Dox) and cisplatin (CDDP) in S-type neuroblastoma cells. Both drugs activated caspase-9 and caspase-3 but not caspase-8. Caspase-9-specific inhibition blocked S-type cell death induced by Dox. SH-EP1 cells transfected to express dominant negative mutant caspase-9, but not those expressing DN caspase-8, were resistant to Dox- and CDDP-induced apoptosis. The lack of caspase-8 involvement in chemotherapy-induced death was not the result of an intrinsic inability of these cells to activate this enzyme because when they were treated with tumor necrosis factor-related apoptosis-inducing ligand, caspase-8 was activated. We also found that both drugs up-regulated CD95/Fas expression but that CD95/Fas signaling was not necessary for cell killing. Experiments testing the response of chemotherapy-treated cells to agonists of the CD95/Fas receptor established that Dox and CDDP treatment sensitizes cells to CD95/Fas killing. Together, these results are consistent with a model in which caspase-9 is of central importance in the death mechanism utilized by these drugs in S-type cells. Although the death response is not dependent on CD95/Fas, concomitant stimulation of this receptor amplifies the death response in drug-treated cells.

Neuroblastoma (NB) is a malignant tumor of neural crest origin that arises from postganglionic sympathetic neuroblasts. NB tumors consist of two principal neoplastic cells: neuroblastic and stromal. Cell type heterogeneity is observed in the morphological appearance of NB tumors and reflected in the phenotypes of cell lines isolated from tumor specimens. When tumor explants are placed in culture, two predominant phenotypes emerge: stromal (S-) and neuronal (N-) types.

Recently, our laboratory defined a difference in the function of NF-κB, related to survival, between certain N- and S-type cell lines. In the S-type cells examined, including SH-EP1 cells, NF-κB p50/p50 homodimers and p65/p50 heterodimers are constitutively active (1). Pharmacologic or molecular inhibition of NF-κB results in apoptosis, demonstrating that its activity is required for survival probably through the transcriptional up-regulation of survival genes. In the N-type cells tested, NF-κB is not constitutively active, rather NF-κB activity is induced by cytoxic drugs and is necessary for the drugs to kill (2). These differences among NB cell lines point to the need to understand death signaling in a context that considers the stromal or neuroblastic phenotype of cells.

N-type cells express enzymes for the synthesis of neurotransmitters, grow as poorly adherent aggregates of small round cells with neuritic processes, and have cell surface receptors characteristic of embryonic neuronal precursors (3–7). In contrast, S-type cells are devoid of the enzymatic machinery for neurotransmitter synthesis, grow flattened and adherent to substrate, and are highly resistant to complement-mediated lysis (8). S-type cells, which can transdifferentiate into N-type cells, may be more able to persist after treatment with cytotoxic drugs (9). The expression of membrane-bound complement inhibitors, in particular CD59, is one specific mechanism by which these cells survive cytoxic therapy (8, 10). These characteristics support an argument that S-type cells potentially give rise to chemoresistant clones in NB tumors and therefore underscore the need to define therapeutic mechanisms that can effectively target these cells.

Doxorubicin (Dox) and cisplatin (CDDP) were the focus of this study because they are cytotoxic drugs that are components of all standard chemotherapeutic protocols to treat NB. Previous work indicated that S-type cells respond to Dox and CDDP with increased expression of CD95/Fas, increased expression of FasL mRNA, and activation of pro-caspase-8 (11), advancing the idea that these drugs kill S-type cells via CD95/Fas and caspase-8 activation. More recently, this hypothesis was challenged by data showing that disrupting CD95/Fas death receptor signaling by expressing a dominant negative (DN) mutant form of the CD95/Fas-associated death domain protein (FADD), did not reduce SH-EP1 killing by Dox (12).

In this report, we provide the results of experiments designed to elucidate the importance of CD95/Fas, caspase-8, and caspase-9 in chemotherapy-induced S-type cell death. The results indicate that caspase-9 is a necessary part of the death response to both Dox and CDDP. In drug-treated cells,
Caspase-9 Mediates Chemotherapy-induced S-type NB Cell Death

MATERIALS AND METHODS

Cell Lines—The S-type cell lines SH-EP1, SK-N-AS, and LA-1–SS were used in this study (13–15). Cells were propagated in minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified 5% CO2 incubator at 37 °C. The human promyelocytic leukemia cell line HL-60, used as a control in several experiments, was maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum.

Caspase Activation Determined by Flow Cytometry—2 × 106 cells were plated onto 24-well dishes. 24 h later, cells were treated with 1 µg/ml TRAIL (Genentech, South San Francisco), 0.5–1 µg/ml Dox (Aldrich), or 10 µg/ml CDDP (Bristol-Myers Squibb Co.) for 4–24 h. At serial time points, cells were harvested by trypsinization and incubated with CaspTag, caspase-3, -8, or -9-selective substrates (Intergen, Purchase, NY) for 30 min. Cells were then washed, and caspase activity was determined by measuring fluorescent intensity with flow cytometry using a FACScaliber (BD Biosciences). The data were analyzed using CellQuest software (BD Biosciences).

Generation of SH-EP1 Cells Stably Expressing DN Caspase-8 or -9—SH-EP1 cells were transfected with expression plasmids encoding hemagglutinin (HA)-tagged DN caspase-8 (pcDNA3-HA-DN-caspase-8 (16, 17), DN caspase-9 (pcDNA3-HA-DN-caspase-9) (18), or vector control (pcDNA3). Transfections were carried out using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. 48 h after transfection, cells were cultured in selective medium containing 0.5 mg/ml Geneticin (Invitrogen). Individual colonies were propagated separately, and transfectants expressing high levels of DN caspase-8 or DN caspase-9 detected by immunoblot analysis were chosen for subsequent study.

Immunoblotting—Cells were harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol. Protein was quantified using a Bio-Rad assay kit. 50–100 µg of protein from whole cell lysates was resolved electrophoretically on 15% SDS-polyacrylamide gels and transferred to Hybond-P membrane (Amersham Biosciences) by electroblotting. Membranes were blocked and incubated with primary antibody. Primary antibodies used in this study were specific for HA (1:1,000, Roche Applied Science), caspase-3 (1:1,000, BD Biosciences), caspase-8 (1:1,000, Cell Signaling Technology, Beverly, MA), caspase-9 (1:2,000, Stressgen, Victoria, BC), active caspase-8 (1:1,000, Cell Signaling Technology), and pCaspase-9 (Ch11, 1:500, Upstate Biotechnology, Lake Placid, NY), FasL (1:1,000, Pharmingen), or glyceraldehyde-3-phosphate dehydrogenase (1:2,000, Chemicon International Inc., Temecula, CA). After incubation with peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), antigen-antibody complexes were detected using enhanced chemiluminescence (ECL, Amersham Biosciences) followed by exposure of membranes to Kodak XAR film (Eastman Kodak).

Cell Viability—Briefly, 1 × 104 cells were plated in 96-well tissue culture dishes. 24 h later, culture medium was supplemented as indicated with Dox, CDDP, recombinant soluble human TRAIL, or vehicle control. To test the functional importance of caspase-9 in S-type cell death, SH-EP1, SK-N-AS, and LA-1–SS were preincubated with 100 µM caspase-9-specific inhibitor Ac-LEHD-CHO for 1 h prior to the addition of 0.5 µM/ml Dox. To evaluate the CD95/Fas signaling pathway, cells were preincubated with 1 µg/ml anti-FasL antibody NOK-1 (Pharmingen) prior to treatment with Dox, CDDP, or 32 µmol/liter resveratrol.

RESULTS

Caspase Activation in SH-EP1 Cells—We first determined whether active caspase-8 or active caspase-9 provides a sufficient signal to trigger SH-EP1 cell apoptosis. Overexpression of procaspase-8 or -9 after single gene transfection is one means of selectively activating these enzymes (23). We found that transfection of either caspase-8 or -9 is cytotoxic to SH-EP1 cells. Within 24 h after transfection, a marked loss of viability occurred in SH-EP1 cells expressing either caspase-8 or caspase-9 (Fig. 1). Immunoblot analysis confirmed the expression and processing of caspase-8 and -9 in the appropriate transfected cells.
To determine the specific caspases involved in drug-induced apoptosis, SH-EP1 cells were treated with Dox or CDDP and then probed with fluorogenic caspase substrates to measure caspase activity selectively (caspase-3, -8, and -9) as a function of time. As shown in Fig. 2, no caspase activity was detected in untreated cells (<10% of cells fluorescent). Furthermore, increases in caspase activity (~3, -8, or -9) were not detected before 8 h in Dox-treated cells and before 14 h in CDDP-treated cells (time points not shown). Caspase-3 and caspase-9 became active 8 h after treatment with Dox and 14 h after treatment with CDDP (Fig. 2, fluorescence in 30–45% of cells). No increase in caspase-8 activity was detected at these same time points (only 4–13% of cells positive). To ensure that this assay had adequate sensitivity to detect caspase-8 activity, SH-EP1 cells were treated with TRAIL, a ligand of death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5, both of which are coupled to caspase-8 (24). As expected, when these cells were exposed to the caspase-8-selective substrate, increased fluorescence was detected within 4 h (34% of cells positive). Together, these results show caspase-9, but not caspase-8, being activated by Dox and CDDP. Moreover, although these drugs do not activate caspase-8, this caspase is responsive to death receptor (TRAIL) stimulation in these cells.

To confirm the results from these activity assays, namely that caspase-9 is activated upstream of caspase-8 or -3, we monitored caspase-3, -8, and -9 activation in response to Dox treatment as a function of time using immunoblotting to detect directly the active, processed forms of these caspases. Consistent with our findings above, the active form of caspase-9 was detected as early as 8 h after Dox treatment, whereas the processed forms of caspase-3 and -8 were not detected until 12 h. Immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) demonstrates equivalent protein loading.

Caspase-9 Is Required for Drug-induced Death of SH-EP1 Cells—To test whether caspase-9 activity is necessary for drug-induced apoptosis in S-type cell lines, a caspase-9-selective peptide inhibitor (Ac-LEHD-CHO) was added to cells prior to treating with Dox. As shown in Fig. 4A, the Dox-induced death of SH-EP1, SK-N-AS, and LA-1–5S cells was significantly blocked when these cells were pretreated with Ac-LEHD-CHO. To confirm these findings further, SH-EP1 cells were stably transfected to express DN mutant caspase-9. This mutant protein specifically inhibits the processing of substrates by wild-type caspase-9 (18). Vector controls as well as DN mutant caspase-8 cells were also generated. The mutant proteins also contain a C-terminal HA tag which allowed their expression to be verified by immunoblotting to detect HA (Fig. 4B). Clones stably expressing these DN mutants were tested to determine their response to Dox and CDDP. As shown in Fig. 4C, Dox treatment caused vector control and DN mutant caspase-8 cells to appear apoptotic within 24 h, with membrane blebbing, nuclear condensation, and cell shrinkage. In contrast, DN mu-

![Fig. 2. Caspase activation in response to TRAIL, Dox, and CDDP. SH-EP1 cells were treated with 1 μg/ml TRAIL, 0.5 μg/ml Dox, or 10 μg/ml CDDP. At the indicated times, caspase-selective fluorescent substrates were added, then after 30 min cells were released by trypsin and analyzed by flow cytometry. Numbers indicate percentage of cells with fluorescent intensity above control. Data are representative of three or more experiments.](http://www.jbc.org/)

![Fig. 3. Timing of caspase processing in response to Dox treatment. Immunoblot detecting processed forms of caspase-3, -8, and -9 in SH-EP1 cell extracts prepared at the indicated times during 0.5 μg/ml Dox treatment. The processed form of caspase-9 was detected as early as 8 h, whereas the active forms of caspase-3 and -8 were not detected until 12 h. Immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) demonstrates equivalent protein loading.)](http://www.jbc.org/)
tant caspase-9 cells retained a normal appearance during Dox treatment. Identical differences in morphology were noted between these cell types after treatment with CDDP (data not shown).

The effects on survival were quantified using the MTT assay. Vector-transfected cells and cells expressing DN caspase-8 were equally sensitive to both drugs such that at the selected drug doses, 90% cell death was observed (Fig. 4). In contrast, cells expressing DN mutant caspase-9 were partially resistant to Dox and CDDP; at the same drug concentrations, fewer than 60% of cells were killed (p < 0.01). Additional control experiments were performed in which these cells were treated with TRAIL to ensure that DN caspase-8 expression was indeed sufficient to block death under conditions in which this caspase was known to be activated as an early signal (see Fig. 2). Vector-transfected and DN mutant caspase-9-expressing cells were equally sensitive to TRAIL (70 and 67% death), and, as expected, cells expressing DN mutant caspase-8 were highly resistant (90% viable).

CD95/Fas Signaling Is Not Required for Dox or CDDP Killing—The clear absence of a role for caspase-8 led us to predict that CD95/Fas is also not involved because caspase-8 plays a pivotal role in CD95/Fas-induced apoptotic signaling (17). Nevertheless, previous reports showing that SH-EP1 cells treated with Dox increase CD95/Fas and FasL mRNA expression (11) made it important for us to test directly whether CD95/Fas signaling was necessary for drug-induced death in these cells. Blocking CD95/Fas signaling using a monoclonal antibody specific for FasL, NOK-1, allowed this question to be addressed. CD95/Fas activation occurs when this receptor binds soluble or cell surface-associated FasL. NOK-1 binds FasL such that it blocks its interaction with CD95/Fas and inhibits receptor stimulation (25). As shown in Fig. 5A, adding NOK-1 to cells prior to treating with Dox had no effect on killing. In similar experiments, NOK-1 also did not change killing by CDDP (data not shown). To prove that NOK-1 was effective at blocking CD95/Fas signaling under the conditions used here, it was tested to demonstrate its ability to inhibit HL-60 cell killing by resveratrol. HL-60 cells are killed by resveratrol in a CD95/Fas-dependent process (20). As expected, resveratrol-induced HL-60 death was blocked by NOK-1 (Fig. 5B). From these results we conclude that signaling through CD95/Fas is not necessary for SH-EP1 cells to be killed by either Dox or CDDP.

Significance of CD95/Fas and FasL Expression in Cells Treated with Dox—The absence of a functional requirement for CD95/Fas signaling prompted further experiments to understand whether CD95/Fas has any significance in the chemotherapy response. We tested the SH-EP1 and SK-N-AS cells to establish whether CD95/Fas and FasL were actually expressed and how the level of expression was affected by drug treatment. CD95/Fas was detectable in untreated cells and increased dramatically in response to Dox (Fig. 6A). Cell-associated FasL was barely detectable prior to treatment and increased only
minimally (1.5-fold) in response to Dox. Soluble FasL in the conditioned medium of treated or untreated cells was not detected by immunoblotting (data not shown). The sizable increase in CD95/Fas expression compared with the low level and minimal induction of FasL suggested that the absence of CD95/Fas-induced death was potentially attributable to insufficient FasL expression.

Although CD95/Fas signaling is not required for SH-EP1 cell killing by Dox or CDDP, the high level induction of CD95/Fas hinted that it might contribute to the chemotherapy response if sufficient FasL was present. In vivo, the possibility exists for additional FasL to be supplied to S-type cells through their contact with other tumor cell types and tissue constituents that contain FasL. To test the hypothesis that Dox treatment in fact renders cells more sensitive to CD95/Fas death signaling, SH-EP1 cells were treated with Dox in the presence of recombinant soluble FasL as well as agonistic anti-CD95/Fas antibody (CH11 clone (26)). Following these treatments, viability was quantified using the MTT assay and analyzed to identify a cooperative effect between the receptor agonists and Dox, leading to increased killing. Concentrations of FasL and CH11 antibody were chosen such that when used independently, they produced no reduction in cell viability (Fig. 6B). In fact, when used as single agents, both agonists caused an apparent modest increase in viable cell number. This small effect is most likely the result of a slight positive modulation of the enzymatic activity measured by MTT. When Dox was added to cells in the presence of either FasL or CH11 antibody, cell killing was increased significantly compared with that caused by Dox alone (p < 0.01). Similar results were obtained when these agonists were combined with CDDP (data not shown). Taken together, these results show that SH-EP1 cells respond to Dox and CDDP with an increase in CD95/Fas expression. Even though signaling through this receptor is not necessary for either drug to kill these cells, the increased CD95/Fas receptor expression may be of therapeutic consequence because drug-treated cells become more sensitive to CD95/Fas-mediated death signals.

CD95/Fas signaling may contribute to the therapeutic response of S-type cells during chemotherapy. The possibility that this signaling mechanism contributes to the therapeutic response is dependent on the presence of FasL within NB tumors. Of note, a previous study suggested that FasL is indeed expressed in NB tumors and was most notably detected in high stage tumors (27). To confirm the results of this earlier study, we examined FasL protein expression by immunohistochemical detection in several NB tumors. Our experiments employed a monoclonal antibody (G274-4), which on the basis of a thorough evaluation and stringent criteria, specifically detects FasL in tissue sections (28). In control lymph node sections, the FasL-specific monoclonal antibody labeled germinal center cells, consistent with the previous description of the localized expression of FasL within germinal centers (29). Immunohistochemical staining of four separate NB tumor specimens showed high level expression in two tumors (Fig. 7) and scattered cells positive for FasL in the other two. These results are consistent with the previous study mentioned above which found that 17 of 35 specimens were immunoreactive for FasL. On this basis, it is plausible that signaling through CD95/Fas occurs in vivo to modulate the therapeutic response of S-type cells to chemotherapy. These results raise the exciting possibility that combining conventional agents with strategies that increase the presence of FasL in NB tumors might increase the effectiveness of already available drugs.

**DISCUSSION**

Histological examination of post-therapy tumor specimens indicates that residual NB deposits often lack neuroblastic elements and are composed primarily of stromal components (9). Stated differently, chemotherapy-induced differentiation to a stromal state may not eliminate disease, particularly if such lesions are able to repopulate the tumor with a chemoresistant clone. Studies attempting to define the mechanism(s) of NB drug resistance must consider whether an ineffective apoptotic response in stromal tumor cells gives rise to NB resistance. This and other evidence, suggesting that the S-type cells in NB tumors are critical to disease pathogenesis, underscores the possibility that effectively targeting this population of cells with cytotoxic therapy could be a way of increasing treatment success.

Because of direct physical and chemical interactions with several different molecular targets in cells (proteins, nucleic acids, lipid membranes), Dox and CDDP engage multiple, parallel upstream mechanisms to kill cells. Despite the complexity
of the circuitry involved, signaling responses eventually converge at a finite number of points which directly interface with the cellular death machinery; this includes caspases, mitochondria, membrane death receptors, stress-induced kinases, and transcriptional factors, such as NF-κB. Our work here first focused on identifying the caspase(s) that are required for SH-EP1 cell killing.

Caspases have an important role, consistent with classical tumor suppressor function, in the tumorigenesis of NB. Caspase-8 is genetically inactivated through DNA methylation and gene deletion in aggressive NB tumors (30–32). Its silencing in these tumors results in altered sensitivity to death receptor-induced apoptotic signals (31). Caspase-8 expression is also absent in small cell lung cancers, arguing that it may have a more generalized tumor suppressor function (33). It is less clear whether other caspases, including caspase-9, act as tumor suppressors. Interestingly, the gene encoding caspase-9 is located on chromosome 1p36.1, a region frequently undergoing loss of heterozygosity in NB. However, several studies have indicated that the remaining allele expresses a functional caspase-9 enzyme in these NB tumor cells (34). Thus, there is as of yet no evidence that caspase-9 functions as a classical tumor suppressor in NB. Its potential tumor-suppressor role in other cancers is also under investigation. In this respect, its expression has been found to be reduced in mice with a predisposition to colon cancer because of mutation of the adenomatous polyposis coli gene (35).

A large body of evidence points to the fact that Dox and CDDP trigger the mitochondrial permeability transition, which then leads to apoptosome formation and initiates caspase activity beginning with caspase-9 cleavage (36–39). These reports suggested that caspase-9 activation is an upstream signaling event and required for anticancer agent-induced apoptosis in human ovarian cancer, gliosarcoma, leukemia, lymphoma, and head and neck squamous carcinoma. Notwithstanding this, studies on the chemotherapy response of S-type cells have found that caspases are involved, but the data conflict with respect to the mechanistic importance of CD95/Fas signaling and caspase-8 activation (12).

We found that caspase-9 is critical for the death response of SH-EP1 cells to Dox and CDDP. It is activated within 8–14 h of drug exposure, and its function is necessary because cell death is blocked by the selective peptide inhibitor and expression of a DN caspase-9 mutant. A critical role for caspase-9 is in fact consistent with previous work showing that high level expression of Bcl-2 or Bcl-xL inhibits drug-induced death of S-type cells (40, 41). Bcl-2 and Bcl-xL act principally by preventing collapse of the mitochondrial permeability transition, thus they ultimately interfere with caspase-9 activation.

These results pose an important question: What upstream signals lie between drug treatment and caspase activation? In some instances, caspase-9 activation after chemotherapy is p53-dependent (36). On the other hand, there are also several examples of anticancer agents triggering apoptosis via caspase-9 independent of functional p53 (42, 43). Because wild-type p53 is expressed in most NB tumors, we tested whether p53 mediates the caspase-9 response to Dox and CDDP in S-type cells. SH-EP1 cells stably expressing the human papilloma virus E6 protein were found to have no detectable p53 but remained as sensitive as control cells to Dox and CDDP (data not shown). As such it is not likely that p53 is the upstream signal for caspase-9 activation in this model.

Our data also show that caspase-8 is not activated, and moreover, that specific inhibition of caspase-8 does not limit killing by these drugs. In similar experiments described by Fulda et al. (11), Dox and CDDP treatment was observed to cause caspase-8 activation. This difference in results may be explained by the fact that our experimental design focused on detecting the early caspase signals, such that caspase function was monitored during the first 8 h after drug treatment. In the experiments by Fulda and co-workers, cells were apparently analyzed 24–48 h after treatment. Because of cross-talk between effector and executionary caspases, analysis at these later times raises the possibility that the observed activation of caspase-8 was of secondary importance. We purposely focused on earlier times to define the proximal signals because these are more likely to have regulatory significance than caspase activation that occurs once cell death is already well under way and irreversible.

With separate experiments, we also showed that caspase-8 is functionally intact in SH-EP1 cells because it is activated by and mediates killing by TRAIL (see Fig. 2). This indicates that its absence from the early Dox or CDDP response is not the result of an inherent defect in its expression or in the processes leading to its activation in these cells. Rather, the cytotoxic drugs tested appear to act independently of the pathways that lead to its early activation.

CD95/Fas-mediated death signaling proceeds through the formation of the death-inducing signaling complex at the plasma membrane when this receptor is occupied by its ligand, FasL. The death-inducing signaling complex leads to recruitment of FADD, which interacts directly with caspase-8 through death effector domains. Caspase-8 is critical for coupling CD95/
Fas receptor activation to the executionary phase of cell death. Given the essential role for caspase-8 in CD95/Fas signaling, the lack of its involvement in Dox and CDDP-induced death is persuasive evidence that CD95/Fas is similarly not necessary for killing by these drugs.

Similar to previous studies, we found that Dox and CDDP both increase CD95/Fas protein levels in SH-EP1 cells (11). From earlier unpublished work, we realized that increased expression of CD95/Fas by itself is insufficient to induce apoptosis in SH-EP1 cells. To help understand the potential significance of up-regulation in the context of drug treatment, we tested whether blocking CD95/Fas signaling with antibody that neutralizes any FasL affects the response to Dox or CDDP and found that it had no effect. Interestingly, these results are consistent with findings by Hopkins-Donaldson et al. (12) showing that Dox-induced death of SH-EP1 cells was not inhibited by overexpression of a DN FADD mutant, which is an alternative approach to block CD95/Fas signaling.

Although CD95/Fas is not necessary for cell death in this model, its up-regulation appears still to have significance in the chemotherapeutic response of SH-EP1 cells. Treatment with Dox or CDDP increases the sensitivity of cells to FasL or agonistic anti-CD95/Fas antibody. Thus, the increase in CD95/Fas expression induced by drug treatment is associated with increased sensitivity to FasL killing. One explanation for these results is that the level of CD95/Fas expressed by untreated cells is below the threshold that allows a sufficient death signal, even when sufficient amounts of FasL are present. When high levels of CD95/Fas are induced by the drugs, the cells acquire increased sensitivity and respond when FasL is added. Mechanisms other than receptor up-regulation could be contributing to the increased sensitivity of treated cells to FasL. For example, in cardiac myocytes, reactive oxygen species that are produced in response to Dox reduce the expression of c-FLIP, a protein that blocks CD95/Fas-induced caspase-8 activation (44). A further implication of our data is that the level of FasL made by the SH-EP1 cells, even when treated with the cytotoxic drugs, is insufficient to stimulate CD95/Fas signaling adequately.

Collectively, these results depict a response of S-type NB cells to Dox and CDDP which is principally mediated by caspase-9 activation. Our findings suggest the prospect that the overall drug response is modulated by CD95/Fas-dependent signals, which undoubtedly takes on greater significance outside monocultures of SH-EP1 cells. In vivo, CD95/Fas signaling may have increased significance as FasL is present in serum, on the surface of lymphocytes, and as we and others have shown, it is expressed in NB tumors (27). Therapeutic strategies to increase the presence of FasL in the NB tumor microenvironment may complement the tumoricidal mechanism of conventional therapies against the stromal component of NB tumors.

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