NF-κB Activation Mediates Doxorubicin-induced Cell Death in N-type Neuroblastoma Cells

Received for publication, September 7, 2001, and in revised form, October 10, 2001
Published, JBC Papers in Press, October 25, 2001, DOI 10.1074/jbc.M108674200

Xin Bian‡, Linda M. McAllister-Lucas‡, Feng Shao§, Kurt R. Schumacher‡, Zhiwei Feng‡,
Alan G. Porter¶, Valerie P. Castle†**, and Anthony W. Opipari, Jr.‡‡

From the Departments of ‡Pediatrics, §Biological Chemistry, and ††Obstetrics and Gynecology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0938 and the ¶Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Republic of Singapore

Neuroblastoma is the most common extracranial solid tumor of childhood. N-type neuroblastoma cells (represented by SH-SY5Y and IMR32 cell lines) are characterized by a neuronal phenotype. N-type cell lines are generally N-myc amplified, express the anti-apoptotic protein Bcl-2, and do not express caspase-8. The present study was designed to determine the mechanism by which N-type cells die in response to specific cytotoxic agents (such as cisplatin and doxorubicin) commonly used to treat this disease. We found that N-type cells were equally sensitive to cisplatin and doxorubicin. Yet death induced by cisplatin was inhibited by the nonselective caspase inhibitor z-Val-Ala-Asp-fluoromethylketone or the specific caspase-9 inhibitor N-acetyl-Leu-Glu-His-Asp-aldehyde, whereas in contrast, caspase inhibition did not prevent doxorubicin-induced death. Neither the reactive oxygen species nor the mitochondrial permeability transition appears to play an important role in this process. Doxorubicin induced NF-κB transcriptional activation in association with IκBα degradation prior to loss of cell viability. Surprisingly, the antioxidant and NF-κB inhibitor pyrrolidine dithiocarbamate blocked doxorubicin-induced NF-κB transcriptional activation and provided profound protection against doxorubicin killing. Moreover, SH-SY5Y cells expressing a super-repressor form of IκB were completely resistant to doxorubicin killing. Together these findings show that NF-κB activation mediates doxorubicin-induced cell death without evidence of caspase function and suggest that cisplatin and doxorubicin engage different death pathways to kill neuroblastoma cells.

Neuroblastoma (NB),¹ the most common malignant sympathetic nervous system tumor of childhood, arises from the neural crest (1, 2). Despite the array of chemotherapeutic agents presently available and current strategies employing intensive myeloablative chemotherapy with autologous bone marrow transplantation, most patients with high risk NB die of their disease (3, 4). To improve this situation, the basis for treatment failures as well as the mechanisms that underlie successful responses to chemotherapy in NB cells must be understood. The molecular response of the tumor cells to cytotoxic agents has become the focus of these efforts because it is clear that these pathways can lead to tumor cell death, whereas their absence or failure leads to resistant disease (5).

Two major pathways control the death response to cytotoxic agents, both of which rely on the eventual activation of downstream effector caspases. One pathway that leads to caspase activation is initiated by engagement of cell surface death receptors such as Fas/CD95/Apo-1, TNFR1, and TRAIL receptors (6). Engagement of Fas/CD95/Apo-1 by FasL/CD95L or specific agonistic antibodies transduces a signal that leads to the recruitment of the adapter protein FADD/MORT-1 (7) and procaspase-8, resulting in formation of the death-inducing signaling complex (8, 9), which leads to caspase-8 activation via autoproteolytic processing. A second major apoptosis-signaling pathway functions independent of surface receptor activation. In this pathway, diverse pro-apoptotic signals eventually provoke a change in mitochondrial function and the release of pro-apoptotic mediators including cytochrome c. Cytochrome c release initiates formation of apoptosome, a complex that includes procaspase-9, dATP, and Apaf-1. This allows proteolytic activation of caspase-9 as a result of induced proximity (10). In both the death receptor/caspase-8 and the mitochondria/caspase-9 pathways, the eventual activation of effector proteases caspases 3, 6, and 7 results in the dismantling of the cell as diverse cellular substrates are proteolized (11, 12).

Understanding how these elements of the death machinery function in NB pathogenesis, particularly in the response of NB tumors to cytotoxic drugs, is complicated by the fact that the cells that comprise NB tumors are heterogeneous. Heterogeneity is observed in the histologic appearance of NB tumor specimens and is reflected in the phenotypes of cell lines isolated from NB tumor specimens. In vitro culture conditions allow two predominant phenotypes to emerge: N- and S-types, of which N-type cell lines are the most common. N-type cells grow as poorly attached aggregates of small round cells, exhibit neurite-like processes, and possess enzymatic activities associated

¹ The abbreviations used are: NB, neuroblastoma; CDDP, cisplatin; Dox, doxorubicin; ROS, reactive oxygen species; MPT, mitochondrial permeability transition; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; FBS, fetal bovine serum; MEM, minimal essential medium; Ac-LEHD-ChO, N-acetyl-Leu-Glu-His-Asp aldehyde; Ac-DEVD-ChO, N-acetyl-Asp-Glu-Val-Asp-aldehyde; z-VAD-fmk, z-benzoyloxy carbonyl-Val-Ala-

Asp-fluoromethylketone; PI, propidium iodide; PDTC, pyrrolidine dithiocarbamate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

This paper is available on line at http://www.jbc.org
with neurotransmitter synthesis (13). The N-myc oncogene is frequently amplified in N-type lines, and these cell lines are tumorigenic when xenografted into immunodeficient mice (14, 15). In contrast, S-type cells grow flat, do not display neuritic processes, and are more adherent to substrate in culture. S-type cells have little or no ability to synthesize neurotransmitters, are not N-myc-amplified, and do not form tumors in mice (13–15).

NB cell apoptosis has been most thoroughly characterized in S-type cell lines. The S-type cell line SH-EP1 undergoes apoptosis in response to many anticancer agents, and these effects are blocked by Bel-2 (16). SH-EP1 cells express Fas/C95, and treatment with CDDP, Dox, or etoposide (VP-16) induces apoptosis that is associated with up-regulation of Fas/C95 and activation of caspase-8 (17). Whether this mechanism appropriately characterizes the arguably more malignant N-phenotype and, more generally, NB tumor behavior is uncertain. Recent studies have identified potentially important variations in the expression and function of elements of the apoptosis signaling pathways between S- and N-type NB cell lines. For instance, N-type cells express higher levels of Bel-2, and increased expression is associated with a drug-resistant phenotype (18, 19). N-type cells fail to express caspase-8 as a result of silencing through DNA methylation, which is implicated in the relative resistance of some lines to Dox (20). However, the Fas/C95 pathway appears to be less important in these cells. Some N-type cells are resistant to Fas-mediated apoptosis (21), potentially as a result of expression of Bel-2 and the caspase-8 inhibitory protein cellular FLICE-inhibitory protein or downregulation of caspase-8 (22). The N-type SH-SY5Y cell line treated with staurosporine undergoes apoptosis associated with activation of caspase-3 (23) that does not require the Fas/C95–L-receptor pathway (24). Despite all of these findings many N-type cell lines are sensitive in vitro to cytotoxic agents (19, 25). The present study was designed to determine the mechanism by which specific cytotoxic agents kill N-type NB cell lines in the hopes of defining elements of the death machinery that are functional and can therefore be targeted to treat high risk malignant NB tumors.

**EXPERIMENTAL PROCEDURES**

**Materials**—All tissue culture supplies and reagents including minimal essential medium (MEM), fetal bovine serum (FBS), OPTI-MEM, Geneticin (G418), and LipofectAMINE PLUS were purchased from Invitrogen. The caspase inhibitors Ac-LEHD-CHO, Ac-DEVD-CHO, and z-VDAD-fmk were obtained from Alexis Biochemicals (San Diego, CA). Rabbit polyclonal anti-I-Bα and I-Bβ antibodies were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase and rabbit anti-caspase-3 polyclonal antibodies were purchased from BD Transduction Labs (San Diego, CA). Polyclonal anti-caspase-9 antibody was purchased from Cayman Biotech (Ann Arbor, MI). Monoclonal anti-caspase-8 antibody was a gift from Dr. Marcus Peter (University of Michigan, Ann Arbor, MI).

**Cell Culture and Transfection**—Human NB cell lines SH-EP1, SH-SY5Y, and IMR32 were cultured in MEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified 5% CO2 incubator. SH-SY5Y cell lines stably expressing dominant negative I-Bα (I-BαM) as well as vector-transfected cells were generated as described previously (26) and maintained in routine MEM supplemented with 500 μg G418. Transfections were carried out using LipofectAMINE PLUS (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were seeded in tissue culture plates to achieve 50% confluence. Twenty-four hours later, the cells were transfected using a mixture of DNA and LipofectAMINE PLUS in OPTI-MEM (Invitrogen). Eight hours following transfection, the cells were transfected with MTT dye (1 mg/ml) at 37 °C for 4 h and lysed in a buffer containing 10% w/v SDS, 50% v/v N,N-dimethylformamide (pH 4.5). Absorbance at 600 nm (OD600) was determined for each well using an ELX 80 automated microplate reader (Biotek Instruments, Winskosi, VT). In assays of cell death inhibition, the cells were preincubated with one of the following caspase inhibitors: 100 μM z-VAD-fmk, 200 μM Ac-DEVD-CHO, or 50 μM Ac-LEHD-CHO in MEM supplemented with 5% PBS prior to the addition of Dox or CDDP. After subtraction of background absorbance, the OD600 of the treated cells with Dox was divided by that of the untreated cells to obtain the percentage of viable cells. In some experiments, cell viability was determined by trypan blue exclusion. For these assays, cells (2 × 105 cells/well) were plated on 24-well plates and treated with Dox at multiple time points to ensure that the images were not saturated.

**5-Aza-2′-deoxycytidine Treatment**—The NB cell lines SH-SY5Y and IMR32 that show no expression of caspase 8 were subjected to demethylating agent 5-aza-2′-deoxycytidine treatment. The cells were plated into 60-mm tissue culture dishes and incubated with 0.5, 1.5, 5, and 10 μM 5-aza-2′-deoxycytidine for a week. At days 4 and 7, cell lysates were collected in SDS sample buffer and subjected to immunoblot analysis.

**Flow Cytometric Analysis for Cell Membrane Permeability and Apoptosis**—The cells were plated at a density of 2 × 106 cells/well in 24-well plates and cultured overnight. The cells were then treated with Dox for designated time points and harvested by trypsinization. Cell viability was determined by trypan blue exclusion. For these assays, cells (2 × 105 cells/well) were plated on 24-well plates and treated with Dox at multiple time points to ensure that the images were not saturated.

**Determination of NF-κB-Dependent Reporter Gene Activation**—SH-SY5Y and IMR32 cells were transfected with 1 μg of the reporter plasmid pBVIX-Luc as described above. To assure identical transfection efficiency in control and treated cells, the cells were replated 12 h after

**A Dominant Role for NF-κB in Neuroblastoma Cell Death**

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
transfection into 12-well plates and after attachment were treated with or without Dox (0.5 \( \mu g/ml \)) and CDDP (3 \( \mu mol/liter \)) for the designated times. The cells were harvested by trypsinization, and luciferase activity was determined according to the manufacturer's instructions using the dual luciferase reagent kit (Promega, Madison, WI) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). An aliquot of the same samples was subjected to protein concentration determination (Bio-Rad). The NF-\( \kappa B \)-dependent luciferase activity was then normalized by protein concentration.

RESULTS

NB Cells Are Killed in the Presence or Absence of Caspase-8—One S-type (SH-EP1) and two N-type (SH-SY5Y and IMR32) NB cell lines with differences according to type in morphology, expression of neurochemical markers, and N-myc amplification were selected to compare their sensitivities to killing by CDDP and Dox (13–15). Viability after exposure to these agents was measured by the MTT assay. Time course (Fig. 1, A and B) and dose-response analysis (data not shown) revealed no meaningful differences between these cell lines with respect to either CDDP or Dox. The lack of a difference with respect to Dox was surprising. In part, a difference was expected because Dox killing of NB cells has previously been shown to operate through Fas/CD95-induced caspase-8 activation (17), and many N-type cell lines do not express caspase-8 (19). Moreover, the higher level of Bcl-2 found in the N-type lines used compared with the S-type SH-EP1 cells would predict greater resistance to both of these agents.

To definitively exclude the involvement of caspase-8 in the death of these particular N-type cells, we examined whether caspase-8 is expressed or whether the gene is functionally silenced. Caspase-8 protein was present in the SH-EP1 cells but was not detected by immunoblotting in either the SH-SY5Y or IMR32 cells, despite their sensitivity to Dox (Fig. 1C). By way of comparison, all three cell lines express caspase-9 and caspase-3, although the levels of caspase-9 are relatively low in SH-SY5Y cells (Fig. 1C). We further confirmed that the lack of caspase-8 expression results from DNA methylation by treating the cells with 5-aza-2'-deoxycytidine (0–10 \( \mu M \)) for 4 and 7 days.

CDDP and Dox Killing Are Differentially Sensitive to Caspase Inhibition—Having found that Dox and CDDP each kill N-type cells in the absence of caspase-8, we sought to determine whether there is any evidence of caspase involvement in this process. We first examined treated cells to determine whether pro-caspase-3 becomes processed. Caspase-3 activation is an important indicator of apoptosis because of its apical position in the execution arm of caspase function and its regulation by initiator caspases 8 and 9. A substantial portion of pro-caspase-3 is processed to its active forms (17- and 11-kDa) in N-type cells treated with CDDP (Fig. 2A). In contrast, treatment with Dox results in minimal processing (Fig. 2A) even after 72 h of treatment (data not shown). When caspase activity was inhibited using z-VAD-fmk, CDDP killing was almost entirely blocked (Fig. 2B). Identical results were obtained when a caspase-3-selective inhibitor (Ac-DEVD-CHO) was used (data not shown). Similar experiments in which caspase-9 was selectively inhibited using Ac-LEHD-CHO demonstrated almost complete protection against CDDP (Fig. 2C).
A Dominant Role for NF-κB in Neuroblastoma Cell Death

Dox-induced Death Is Blocked by PDTC and Is Independent of the MPT or ROS—Whereas prior work with the S-type SH-EP1 cell line demonstrated that the mechanism of Dox-induced cell death depends on signaling from Fas/CDDP leading to caspase-8 activation, disruption of the Δψm, cytochrome c release, and caspase-3 activation (17, 27), the results presented above caused us to suspect that an alternative pathway(s) might function in the N-type cells. In other well characterized models, Dox-induced death requires the generation of ROS (28) or collapse of the MPT (29). To determine the potential involvement of these signals in N-type cells, we looked for evidence of an increase in ROS and collapse of the MPT over the first 8 h after exposure of SH-SY5Y and IMR32 cells to Dox. Using flow cytometry to detect signals from the fluorescent indicators 3,3′-dihexyloxacarbocyanine iodide to measure Δψm, dihydroethidium to detect superoxide, and dichlorodihydrofluorescein diacetate to detect other ROS, we found no evidence of changes in these cells within this time period (data not shown).

NF-κB activation has also been implicated in Dox signaling (30), although in many cases NF-κB activation protects cells against death (31). To evaluate the potential involvement of NF-κB in Dox-induced N-type cell death, we blocked NF-κB activation by pretreating cells with the NF-κB inhibitor and antioxidant PDTC. PDTC has at least two chemical properties: a chelating activity for heavy metals and an antioxidant activity of its dithiocarboxy group. It appears that PDTC functions to suppress a reaction required for the release of IκB from NF-κB, which may involve a ROS. PDTC has no effect on the DNA binding properties of NF-κB, the nuclear uptake of NF-κB, the amount of NF-κB-IκB complex, or the release of IκB (32). After 48 h of treatment with Dox in the presence or absence of PDTC, the cells were evaluated by flow cytometry to determine viability on the basis of plasma membrane permeability to PI. PI permeability provided a more direct measure of cell death or survival than MTT conversion, and preliminary experiments demonstrated that it correlated well with morphologic evidence of death. As shown in Fig. 4, PDTC completely blocked cell death induced by treatment with Dox for 24 h in both SH-SY5Y and IMR32 cells. In contrast, PDTC had no effect on CDDP-induced death (data not shown). To further define whether the mechanism of Dox resistance by PDTC involves general antioxidant properties or a specific inhibitory action against NF-κB activation, other antioxidants were studied as potential inhibitors. SH-SY5Y cells were pretreated for 30 min with either one of the antioxidants (vitamin C, vitamin E, and Trolox) or 1 mM cyclosporin A to inhibit the MPT, prior to Dox exposure. Viability was determined on the basis of PI exclusion. Table I summarizes the results of these studies. 51% of SH-SY5Y cells treated with Dox alone demonstrate loss of membrane integrity by 24 h. Pretreatment with cyclosporin A to inhibit the MPT has no effect on viability. Similarly, none of the other antioxidants (vitamin C, vitamin E, and Trolox) prevented cell death like PDTC. Thus only PDTC was able to block this process. Similar results were obtained with IMR32 cells (data not shown). These results suggest the possibility that NF-κB activation is involved in Dox-induced cell death, whereas we found no convincing evidence to implicate either the collapse of Δψm or an increase in ROS.

Dox Induces IκBα Degradation and NF-κB Activation Prior to the Onset of Cell Death—Although PDTC is recognized as an inhibitor of NF-κB, its mechanism of action is not well defined, and its antioxidant properties make it unlikely to be entirely specific for NF-κB. As such, further experiments were necessary to definitely implicate NF-κB in this process. First, considering that IκBα levels are of paramount importance in the

### Figure 2
CDDP but not Dox killing of N-type NB cells is blocked by specific caspase inhibitors. A, immunoblot analysis of SH-SY5Y cells treated with control medium, 0.5 μg/ml Dox, or 3 μmol/liter CDDP up to 24 h. The processed forms of caspase-3 migrate at 11 and 17 kDa as indicated (B and C). Viability of SH-SY5Y and IMR32 cells after treatment for 24 h with 0.5 μg/ml Dox or 3 μmol/liter CDDP in the presence or absence of 100 μM z-VAD-fmk (B) or 200 μM Ac-LEHD-CHO (C). Viability was determined by MTT. The values are reported as the means ± S.D. of three representative experiments.
regulation of NF-κB activity under most conditions (33), we 
determined the level of I-κBα after treatment with Dox. In 
SH-SY5Y cells treated with Dox, I-κBα levels decreased 
significantly between 4 and 8 h after treatment (Fig. 5A), which 
is before significant loss of cell viability (Fig. 1A). Similar results 
were obtained with IMR32 cells, although the degradation of 
I-κBα occurred earlier, within 4 h of exposure (data not shown). 
These same membranes were also immunoblotted to detect 
I-κBα, but no appreciable change in I-κBα levels was detected 
(Fig. 5A). Treatment of these lines with CDDP over an identical 
time course demonstrated no change in the levels of either 
I-κBα or I-κBβ (data not shown).

NF-κB transcriptional activity was then directly measured 
in cells after treatment with Dox. This was accomplished by 
transiently transfecting SH-SY5Y and IMR32 cells with a lu-
ciferase reporter plasmid that contained within its promoter 
region six tandemly placed NF-κB consensus-binding sites. 
NF-κB-dependent transcriptional activation was detected 
within 8 h of exposure to Dox, but when cells were pretreated 
with PDTC, there was no luciferase response to Dox (Fig. 5B).

In accord with the findings above, CDDP treatment did not 
increase NF-κB transcriptional activity (Fig. 5B). These results 
suggest Dox induces NF-κB activation in a time-dependent 
manner and suggest that this activation is associated with 
degradation of I-κBα before the onset of cell death.

NF-κB Activation Is Essential for Dox-induced NB Cell 
Death—To confirm that NF-κB activation is required for Dox-
induced NB cell death, we utilized SH-SY5Y cells stably trans-
fected to express a dominant negative mutant of I-κBα, which 
functions as a super-repressor of NF-κB activation (26). These 
cells (designated I-κBαM/SH-SY5Y) and corresponding vector 
transfected controls were treated with Dox and then studied to 
determine I-κBα levels as well as viability. Vector control cells 
responded to Dox with evidence of I-κBα degradation (Fig. 6A).

As anticipated, in the I-κBαM/SH-SY5Y cells there was no 
change in the level of I-κBα following Dox treatment (Fig. 6A).
The NF-κB-sensitive reporter plasmid was transfected into 
each of these cell lines, and the luciferase response following 
Dox treatment was measured. The vector control line re-
sponded similarly to the parental cell type with a >10-fold 
induction of luciferase activity at 8 h, whereas the I-κBαM/SH-
SY5Y cells showed no induction (Fig. 6B). As well, suppression 
of NF-κB caused the cells to resist Dox killing. I-κBαM/SH-
SY5Y cell viability measured by trypan blue exclusion in the 
absence of Dox was 85 ± 9%, whereas control cell viability was 
99 ± 2%. 24 h after Dox treatment I-κBαM/SH-SY5Y viability 
remained at 87 ± 2%, whereas vector control cell viability was 
54 ± 4%. Morphologic changes were obvious in control cells 
within 24 h of Dox treatment as the cells became rounded and 
detached from the tissue culture plate (Fig. 6C, left panels). The 
I-κBαM/SH-SY5Y cells displayed morphology after treatment 
indistinguishable from untreated cells (Fig. 6C, right panels). 
These results confirm that I-κBα degradation and subsequent 
NF-κB activation is required for Dox-induced cell death in 
these N-type NB cell lines.

DISCUSSION
To define the mechanisms contributing to the development of 
NB drug resistance and treatment failure, it is necessary to 
first understand the pathways that mediate the death response 
to CDDP and Dox used in the treatment of this disease. For our 
studies, we focused on the response of cell lines derived from 
primary tumor specimens that display the N phenotype. The 
importance of studying these responses in N-type cells stems 
not only from the fact that the N phenotype is the most common 
to emerge from high risk tumor explants but also because 
N-type cells display morphologic and biologic characteristics of 
aggressive tumor cell behavior. In the present study, we found 
that N-type cells are as sensitive to treatment with Dox and 
CDDP as an S-type cell line. This finding was somewhat unex-
pected because S- and N-type cells differ in the expression of 
several elements of the cell death machinery. In particular 
N-type cells express higher levels of Bcl-2 (18) and fail to 
express caspase-8 secondary to DNA methylation (20). The
N-type lines under study here fulfill both of these characteristics. These results mean that caspase-8 is not required for killing by either agent and that even the relatively high level of Bcl-2 is insufficient to block death induced by CDDP or Dox.

Consistent with the results reported in other tumor models, we found that caspases are activated by CDDP in N-type NB cells, and we showed they are required for CDDP-induced cell death. CDDP has been shown previously to kill cells by forming DNA adducts causing G₂ cell cycle arrest (34). In HeLa cells and ovarian and squamous carcinoma cell lines the apoptotic pathway mediating CDDP killing requires caspase-9 (35–37), and in accord with these studies we found that a selective caspase-9 inhibitor strongly protected NB cells from CDDP killing. In part, the mechanism by which caspase-9 initiates this process is through inactivation of the nuclear transport system, which increases the diffusion limit of the nuclear pores, allowing caspase-3 and other molecules to enter the nucleus (38).

In contrast, our results with Dox fail to demonstrate an important role for caspases in the death response to this agent. Dox killed these cells even though (a) they do not express caspase-8, (b) there was only minimal pro-caspase-3 process-

---

**Table I**

| Treatment                      | Percentage of dead |
|--------------------------------|--------------------|
| Dox 0.5 μg/ml                  | 51 ± 1             |
| Dox 0.5 μg/ml and CsA 1 μM     | 56 ± 9             |
| Dox 0.5 μg/ml and vitamin C 100 μM | 44 ± 8          |
| Dox 0.5 μg/ml and vitamin E 100 μM | 46 ± 7          |
| Dox 0.5 μg/ml and Trolox 100 μM | 47 ± 5             |
| Dox 0.5 μg/ml and PDTC 100 μM | 17 ± 2             |

---

**Fig. 4. PDTC inhibits Dox-induced cell death.** SH-SY5Y treated for 24 h with either control medium (A and B) or 0.5 μg/ml Dox (C and D) in the absence (A and C) or presence of 100 μM PDTC (B and D). The cells were stained to determine plasma membrane permeability to PI and analyzed by flow cytometry. The numbers indicate the percentage of cells permeable to PI. The data presented are representative of multiple experiments with similar findings.
How does Dox act to kill cells? Despite extensive clinical experience with this agent against a wide range of malignant disorders, some of the most fundamental aspects of its mechanism of action are not defined. For example, the direct molecular target, which it binds to in cells, is ambiguous. Although anthracyclines are known to intercalate with DNA, the tumoricidal actions of Dox may not even require that the agent enter cells. For example, covalent linkage of Dox to agarose beads has revealed that even in this non-cell-permeable form, it effectively kills human leukemia cells in culture (40). Above all, however, Dox is believed to induce DNA damage. This action itself has been attributed to at least two mechanisms. In the first, it has been shown that Dox can accept an electron to become a highly reactive semiquinone free radical that reacts with molecular oxygen to form superoxide. The ROS generated subsequently damages DNA (28). In our experiments, we found no evidence that superoxide or other ROS are generated in the nuclear enzyme DNA topoisomerase II (41). The drug is found in covalent complexes with this enzyme and DNA that are associated with double strand breaks.

Our experiments were primarily directed at uncovering Dox-induced signals in N-type cells, which then engage the death response. We found that neither of the most prominent initiator caspases (caspase-8 and -9) was required for Dox killing, which immediately distinguished the death mechanism in N-type cells from that reported for the S-type SH-EP1 NB cell line as well as for leukemic cells. In these other examples, Fas/CD95-dependent caspase-8 activation and further mitochondrial perturbation are of paramount importance (17, 27).

Mechanistically, our data point to the fact that NF-κB plays a key role in mediating Dox-induced death of N-type NB cell lines. NF-κB is activated in cells after exposure to cytotoxic agents including ligands of the cell surface death receptors

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Dox regulates NF-κB activation in SH-SY5Y and IMR32 cells. A, SH-SY5Y cells were treated with 0.5 μg/ml Dox for the indicated times. Total cell lysate (30 μg) was resolved by SDS-PAGE and then immunoblotted to detect I-κBα or I-κBβ. The same blot was similarly probed to detect GADPH to confirm equivalent protein loading. A marked reduction of I-κBα is observed by 8 h without a significant change in I-κBβ. B, SH-SY5Y and IMR32 cells were transiently transfected with the pBVIx-Luc luciferase reporter plasmid, which contains six tandem NF-κB consensus elements within the promoter sequence. 24 h after transfection cells were treated for 4 or 8 h with 0.5 μg/ml Dox (white bars), Dox plus 100 μM PDTC (black bars), or 3 μmol/liter CDDP (gray bars). Luciferase activity was determined in three separate samples as the fold induction relative to untreated cells. The results are presented as the means ± S.D. from three separate experiments.

![Figure 6](http://www.jbc.org/)

**Fig. 6.** I-κBαM/SH-SY5Y cells are resistant to Dox-induced death. A, I-κBαM/SH-SY5Y and control vector transfected cells were treated with 0.5 μg/ml Dox for 0–16 h. Total cell lysates (30 μg) were resolved by SDS-PAGE and then immunoblotted to detect I-κBα. B, I-κBαM/SH-SY5Y (black bars) and vector control cells (white bars) were transiently transfected with the pBVIx-Luc luciferase reporter plasmid as described. 24 h following transfection, the cells were treated for 4 or 8 h with 0.5 μg/ml Dox. Luciferase activity was determined in three separate samples as the fold induction relative to untreated cells. The results are presented as the means ± S.D. from three separate experiments. C, 1.2 × 10⁶ I-κBαM/SH-SY5Y and control transfected cells were plated in 96-well culture plates in 10% FBS-supplemented MEM. 24 h later, growth medium was replaced with medium containing 0.5 μg/ml Dox, and the cells were cultured for a further 24 h. The cells were photographed (400×) using interference contrast microscopy. Cell viability was measured by trypan blue exclusion and is shown.
such as tumor necrosis factor and Fas, as well as by genotoxic agents and chemotherapeutic drugs. In many instances, NF-κB activation mediates resistance against cell death or protection from apoptosis (42–45). Indeed, it is well established that Dox and other anthracyclines induce NF-κB activation. As examples, inhibition of NF-κB enhances apoptosis induced by Dox in T leukemia cell lines (46) and in pancreatic carcinoma cells (47). NF-κB suppresses tumor necrosis factor-α-induced apoptosis through the transcription of gene products that function to block apoptosis, such as the cellular inhibitors of apoptosis (cIAP1, cIAP2, and XIAP), the tumor necrosis factor receptor-associated factors TRAF1 and TRAF2, the Bcl-2 homologue A1/Bfl-1, and the A20 zinc finger protein (43, 48–52).

Our data provide compelling evidence for an opposing function of NF-κB in N-type NB cells. In our model, drug-induced NF-κB activation actually mediates killing by Dox. This conclusion was first established by our results showing that Dox induced IκB degradation and NF-κB-dependent transcriptional activation prior to cell death. Second, the small molecule antioxidant PDTC suppresses Dox-induced IκBα degradation, NF-κB activation, and cell death. Furthermore, a highly specific NF-κB inhibitor (IκBαM) prevented IκBα degradation and cell death. Other recent studies have found evidence that NF-κB is a mediator of cell death in PC12 cells exposed to dopamine (53), in neurons undergoing focal ischemia (54), in Jurkat T cells exposed to either VP-16 or UV irradiation, and in hepatocellular carcinoma cell lines treated with Dox (55). Of particular note, Ryan et al. (56) have shown that p53-induced apoptosis requires NF-κB activation. Further, they demonstrated that mouse embryo fibroblasts derived from p56-deficient mice that are sensitized to undergo p53-dependent apoptosis by E1A transformation completely resist Dox-induced death, whereas E1A-transformed wild-type p56 controls were killed. In this context it is worthwhile noting that most NB tumors have wild-type p53, that the particular N-type cells used in our experiments have wild-type p53, and that Dox has been shown to increase p53 levels in SH-SY5Y cells (57). Thus our results with N-type cells appear to offer strong support for the hypothesis advanced by Ryan et al. (56) that inhibition of NF-κB in tumor cells that retain wild-type p53 diminishes rather than augments a therapeutic response. Finding that NF-κB mediates Dox-induced death of N-type cells opens a new opportunity for experimental strategies to treat this disease. For example mitozantrone has been shown to induce NF-κB in HL-60 cells by targeting topoisomerase II (58). This and other agents with the capacity to activate NF-κB should be carefully considered as candidates for further study in NB. Conversely, resistance to Dox (and potentially other drugs) may result from disturbances of this signaling pathway.

NF-κB activation has been observed in NB cells as part of the response to agents that cause cellular differentiation. SH-SY5Y NB cells when induced to differentiate with retinoic acid or 12-O-tetradecanoylphorbol 13-acetate transiently activate NF-κB before the morphological signs of differentiation (26). In this case NF-κB activation was associated with a significant reduction in the amount of IκBα, and cells transformed with IκBαM failed to differentiate into neuronal cell types when treated with retinoic acid or 12-O-tetradecanoylphorbol 13-acetate. In our experiments we found a reduction in IκBα, with no detectable change in IκBβ, suggesting that the signaling in SH-SY5Y cells that leads to NF-κB activation may vary depending on the conditions applied. These differences may provide some specificity to the signal response along with the myriad other signals that are concurrently generated by these agents.

We have yet to define the exact signals that lie upstream and downstream of NF-κB activation in response to Dox in these cells. Given the findings of Ryan et al. (56), it will be reasonable to determine whether NF-κB is activated by a p53-dependent mechanism, which also involves signals through the MAPK cascade. This is presently being explored using a series of p53 mutants and specific inhibitors of MEK activity. It is tempting to speculate on the mechanism by which NF-κB mediates the death response to Dox. One possibility is NF-κB activates Fas-mediated apoptosis. Support for this notion is provided by studies of T cells and T cell lines indicating that DNA-damaging agents activate the stress-activated protein kinase/c-Jun N-terminal kinase pathway to activate AP-1 and NF-κB resulting in expression of Fas/CD95-L and Fas-mediated apoptosis (59). This possibility seems unlikely in our system because N-type cells do not express caspase-8, and caspase-mediated death was not a primary mechanism of Dox-induced death. Another possibility is suggested by studies on endothelial cell death induced by hypoxia (60) and on hydrogen peroxide-induced apoptosis of HeLa cells (61). In both systems, NF-κB is induced and increases p53 expression. Hypoxia-induced cell death is additionally associated with a decrease in Bcl-2 expression (60). In any case, efforts are underway in our laboratory to identify the genes induced by NF-κB specifically following Dox treatment to define this portion of the signaling cascade. It will be important to determine whether the pro-survival genes associated with NF-κB expression in other cell types are absent from this expression profile.

REFERENCES

1. Saxen, L., and Saxen, E. (1960) Cancer Genet. Cytol. 13, 899–906
2. Machlin, G. A. (1989) in Neuroblastoma Clinical and Biological Manifestations (Pochetly, C., ed) pp. 195–231, Elsevier Biomedical, New York
3. Frappaz, D., Michon, J., Coze, C., Berger, C., Plouvier, E., Lasset, C., Bernard, J. L., Stephan, J. L., Boullot, E., Budon, M., Combaret, V., Fourquet, A., Philip, T., and Zuckier, J. M. (2000) J. Clin. Oncol. 18, 468–476
4. Schmitt, M. L., Lukens, J. N., Seeger, R. C., Brodeur, G. M., Shimada, H., Gerbing, R. B., Stram, D. O., Perez, C., Haase, G. M., and Matthyss, K. K. (2000) J. Clin. Oncol. 18, 1260–1268
5. Kaufmann, S. H., and Earnshaw, W. C. (2000) Exp. Cell Res. 256, 42–49
6. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
7. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
8. Schneider, P., and Tschopp, J. (2000) Pharm. Acta Helv. 74, 281–286
9. Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., and Peter, M. E. (1998) Eur. J. Biochem. 254, 439–450
10. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
11. Green, D. R. (1998) Cell 94, 695–698
12. Bzdikardjai, I., Oliver, H., van der Heijden, M., Lun, X., and Wang, X. (1999) Ann. Rev. Cell Dev. Biol. 15, 269–290
13. Ross, R. A., Spengler, B. A., and Biedler, J. L. (1983) J. Natl. Cancer Inst. 71, 1213–1247
14. Ross, R. A., Joh, T. H., Reis, D. J., Spengler, B. A., and Biedler, J. R. (1980) in Advances in Neuroblastoma Research (Evans, A. E., ed) pp. 151–160, Raven Press, New York
15. Ambros, I. M., and Ambros, P. F. (2000) in Neuroblastoma (Brodeur, G. M., Sawada, T., Tsuchida, Y., and Voute, P. A., eds) pp. 233–235, Elsevier Science Publishers B.V., Amsterdam
16. Doh, M., Nunez, G., Merchant, A. K., Maybaum, J., Rode, C. K., Bloch, C. A., and Castle, V. P. (1994) Cancer Res. 54, 3253–3259
17. Fulda, S., Sieverts, H., Friesen, C., Herr, I., and Debatin, K. M. (1997) Cancer Res. 57, 3823–3828
18. Dole, M. G., Jasty, R., Cooper, C. M., Thompson, C. B., Nunez, G., and Castle, V. P. (1995) Cancer Res. 55, 2576–2582
19. Lasorella, A., Iavarone, A., and Israel, M. A. (1995) Cancer Res. 55, 4711–4716
20. Beug, H., Wei, T., Valentin, Y. S., Keppler, D., Kress, L., and Ito, K. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 459–463
21. Kottula, V., Vassilopoulos, D., Wellmann, A., Motsiades, N., and Tsokos, M. (1997) Proc. Am. Assoc. Cancer Res. 38, 194
22. Pouliaki, V., Motsiades, N., Romero, M. E., and Tsokos, M. (2001) Cancer Res. 61, 4864–4872
23. Ponnambalam, R., McGinnis, K., Nadimpalli, R., Gilbertson, R. B., and Wang, K. J. (1997) J. Neurochem. 68, 2328–2337
24. Lopez, E., and Ferrer, I. (2000) Brain Res. Mol. Brain Res. 85, 61–67
25. Tieu, K., Zhou, D. M., and Nusseblad, C. (1999) J. Neurosci. Res. 58, 426–435
26. Feng, Z., and Porter, A. G. (1999) J. Biol. Chem. 274, 30341–30344
27. Fulda, S., Susin, S. A., Kroemer, G., and Debatin, K. M. (1998) Cancer Res. 58, 4453–4460
28. Lowy, J. W., Chen, H. H., Plambeck, J. A., and Acton, E. M. (1982) Biochem. Pharmacol. 31, 575–581
29. Decaudin, D., Geley, S., Hirsch, T., Castedo, M., Marchetti, P., Macho, A., Koller, R., and Kroemer, G. (1997) Cancer Res. 57, 62–67
A Dominant Role for NF-κB in Neuroblastoma Cell Death

30. Boland, M. P., Foster, S. J., and O’Neill, L. A. (1997) J. Biol. Chem. 272, 12952–12960
31. Baldwin, A. S. (2001) J. Clin. Invest. 107, 241–246
32. Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992) J. Exp. Med. 175, 1181–1194
33. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
34. Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992) J. Exp. Med. 175, 1181–1194
35. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
36. Ormerod, M. G., Orr, R. M., and Peacock, J. H. (1994) Br. J. Cancer 69, 93–100
37. Horky, M., Vojtesek, B., Vacha, J., and Wesierska-Gadek, J. (2001) J. Cell Sci. 114, 663–670
38. Kuwahara, D., Tsutsumi, K., Kobayashi, T., Hasunuma, T., and Nishioka, K. (2000) Cancer Lett. 148, 65–71
39. Asselin, E., Mills, G. B., and Tsang, B. K. (2001) Cancer Res. 61, 1862–1868
40. Faleiro, L., and Lazebnik, Y. (2000) J. Cell Biol. 151, 951–959
41. Wang, J. C. (1996) Annu. Rev. Biochem. 65, 635–692
42. Yamamoto, Y., and Gaynor, R. B. (2001) J. Clin. Invest. 107, 135–142
43. Wang, C. Y., Guttridge, D. C., Mayo, M. W., and Baldwin, A. S., Jr. (1999) Mol. Cell. Biol. 19, 5923–5929
44. Huang, T. T., Wuerzberger-Davis, S. M., Seufzer, B. J., Shumway, S. D., Kurama, T., Boothman, D. A., and Miyamoto, S. (2000) J. Biol. Chem. 275, 9501–9507
45. Wang, C. Y., Guttridge, D. C., Mayo, M. W., and Baldwin, A. S., Jr. (1999) Mol. Cell. Biol. 19, 5923–5929
46. Jeremias, I., Kupatt, C., Baumann, B., Herr, I., Wirth, T., and Debatin, K. M. (2001) Blood 91, 4624–4631
47. Arlt, A., Vorndmann, J., Breitenreicher, M., Folsch, U. R., Kalthoff, H., Schmidt, W. E., and Schafer, H. (2001) Oncogene 20, 859–868
NF-κB Activation Mediates Doxorubicin-induced Cell Death in N-type Neuroblastoma Cells
Xin Bian, Linda M. McAllister-Lucas, Feng Shao, Kurt R. Schumacher, Zhiwei Feng, Alan G. Porter, Valerie P. Castle and Anthony W. Opipari, Jr.

J. Biol. Chem. 2001, 276:48921-48929.
doi: 10.1074/jbc.M108674200 originally published online October 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108674200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 26 of which can be accessed free at http://www.jbc.org/content/276/52/48921.full.html#ref-list-1