The CD95 (APO-1/Fas) System Mediates Drug-induced Apoptosis in Neuroblastoma Cells

Simone Fulda, Hauke Sieverts, Claudia Friesen, Ingrid Herr, and Klaus-Michael Debatin

ABSTRACT

Anticancer agents have been shown to trigger apoptosis in chemosensitive tumors such as neuroblastomas. We previously identified activation of the CD95 system as one of the key mechanisms for doxorubicin-induced apoptosis in leukemic T cells. Here, we report that therapeutic concentrations of doxorubicin, cisplatinum, and VP-16 led to induction of CD95 receptor and CD95 ligand (CD95-L) that mediated cell death in chemosensitive neuroblastoma cells. Using F(ab')2 anti-CD95 antibody fragments to interfere with CD95-L-receptor interaction markedly reduced apoptosis induced by those drugs in vitro. Cyclosporin A inhibited induction of CD95 mRNA and CD95-L mRNA and blocked drug-mediated apoptosis. Drug-induced apoptosis involved activation of caspases (interleukin 1β-converting enzyme/Ced-3-like proteases) and processing of the prototype caspase substrate PARP and was completely blocked by benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, a peptide inhibitor of caspases. In addition, neuroblastoma cells that were resistant to CD95-triggered apoptosis also displayed cross-resistance to chemotherapeutic agents. These data provide new clues for understanding the molecular requirements for drug-induced apoptosis in chemosensitive neuroblastoma cells by demonstrating that cell death was mediated via the CD95-L-receptor system and may open new avenues for targeting drug resistance of neuroblastoma.

INTRODUCTION

Neuroblastoma is the most common childhood extracranial malignant solid tumor arising from the sympathetic nervous system (1). Negative growth regulation may play a pivotal role in neuroblastoma growth because spontaneous regression is found in certain cases, which may be due to induction of apoptosis (1, 2). Maturation of neoplastic cells into terminally differentiated ganglion cells has also been reported to occur during spontaneous regression (2). In addition, programmed cell death is especially important in the development of the nervous system, in which a large number of newly generated neurons die by apoptosis, thus ensuring that the appropriate number of mature neurons survives (3).

CD95 is a M, 45,000 type I transmembrane cell surface receptor of the TNF7nerve growth factor receptor family (4-6). Ligation of CD95 by anti-CD95 antibody or by CD95-L rapidly induces apoptosis both in vitro and in vivo in sensitive cells (6). Although CD95 is expressed on a variety of normal and neoplastic cells, the key role of the CD95 pathway in negative growth regulation has mostly been studied within the immune system (7, 8). CD95 expression has been found in neuroblastoma tumor specimens, as well as in neuroblastoma cell lines (9). CD95-L is a type II transmembrane molecule of M, 40,000 and part of the TNF/nerve growth factor family that may also occur in a soluble form, released from the cell surface by cleavage of a metalloprotease (10, 11). CD95-L is predominantly expressed in activated T cells, but it has also been found to be constitutively expressed in a variety of tissues (8).

Neuroblastoma is a chemosensitive tumor, and polychemotherapy using cytostatic agents such as doxorubicin, cisplatinum, and VP-16 is considered an important and effective treatment modality (12, 13). Cytotoxic drugs successfully used in chemotherapy of neuroblastoma are thought to target diverse cellular elements in conferring a lethal effect in malignant cells (14-16). Recently, anticancer agents, irrespective of their intracellular target, have been shown to exert their biological effect by triggering a common final death pathway known as apoptosis in target cells, e.g., in leukemic and lymphoid cells (17, 18). Cellular changes indicative of apoptosis have also been observed in neuroblastoma cells after treatment with drugs such as doxorubicin, cisplatinum, cyclophosphamide, and VP-16 (19-21). Extensive studies of the biochemical and molecular pharmacology of drug-target cell interactions have been performed. However, the precise molecular requirements by which chemotherapeutic drugs initiate the apoptotic pathway are poorly defined. There is mounting evidence that anticancer treatment may kill through apoptosis by activating a signaling pathway in the target cell rather than by crippling cellular metabolism (22).

Previous work in our laboratory demonstrated that apoptosis induced by cytostatic drugs at therapeutic concentrations in leukemia cells activated a cell death mechanism that depended on CD95-L-receptor interaction (23). Upon treatment with doxorubicin and methotrexate, expression of CD95-L was induced that mediated apoptosis in an autocrine or paracrine manner (23). Similarly, autocrine suicide in activated T cells had been reported to occur via CD95-L-receptor interaction following production of CD95-L in response to T-cell receptor triggering (24). Because we found that neuroblastoma cells express CD95 and are susceptible to CD95-mediated apoptosis via an agonistic anti-CD95 monoclonal antibody, we asked whether apoptosis induced by cytotoxic drugs in neuroblastoma cells also involves the CD95-L-receptor system.

MATERIALS AND METHODS

Drugs. Doxorubicin (Farmitalia, Milano, Italy), cisplatinum (Sigma, Deisenhofen, Germany), and VP-16 (Bristol, Munich, Germany) were provided as pure substances and dissolved in sterile water (doxorubicin and cisplatinum) or DMSO (VP-16) prior to each experiment (1 mg/ml).

Cell Culture. Human neuroblastoma cell lines SH-EP1 (MYCN 1X; no Ip deletion), Kelly (MYCN 100-120X; 1p deletion), and LAN-5 (MYCN 25X; 1p deletion) were the generous gift of M. Schwab (25-27). SH-EP1 and LAN-5 cells, variants of SH-EP neuroblastoma cells that are resistant to anti-CD95, were generated by continuous culture in the presence of the agonistic anti-APO-1 antibody (4) for more than 2 months. Cells were maintained in monolayer culture in 75-cm2 tissue culture flasks ( Falcon, Heidelberg, Germany) in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10%...
Drug-induced apoptosis. SH-EP cells were determined by FACS analysis of propidium iodide-stained nuclei after treatment with doxorubicin, cisplatinum, or VP-16 at concentrations and time points indicated. Top (doxorubicin), ■ 0.01 µg/ml; □ 0.05 µg/ml; ▲ 0.1 µg/ml; ○ 0.5 µg/ml; □ 1 µg/ml; ◇ 5 µg/ml; ▲ 10 µg/ml. Middle (cisplatinum), ■ 0.5 µg/ml; □ 1 µg/ml; ◇ 5 µg/ml; ▲ 10 µg/ml; ○ 50 µg/ml; □ 100 µg/ml. Percentage of specific apoptosis was calculated as follows: 100 × (experimental apoptosis — spontaneous apoptosis in medium/100 — spontaneous apoptosis in medium). Data points, means of triplicates with SD of less than 10%. Similar results were obtained in three separate experiments.

Apoptosis was assessed by FACS analysis of propidium iodide-stained nuclei. Percentage of specific apoptosis was calculated as described in A. Columns, means of triplicates with SD of less than 10%. Similar results were obtained in three separate experiments.

Western Blot Analysis. Proteins for Western blot analysis were extracted from cells lysed for 30 min at 4°C in PBS with 0.5% Triton X (Serva) and 1 mM phenylmethylsulfonyl fluoride (Sigma) followed by high-speed centrifugation. Membrane proteins were eluted in PBS containing 0.1 M glycine (pH 3.0) and 1.5 M Tris (pH 8.8). Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL). Forty µg protein per lane were separated by 12% SDS-PAGE and electrophoresed onto nitrocellulose (Amer sham, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 h in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection was performed using mouse anti-CD95-L monoclonal antibody, mouse anti-CD95 monoclonal antibody, mouse anti-p53 monoclonal antibody (Transduction Laboratories, Lexington, KY), and rabbit anti-3-PARP polyclonal antibody (Enzyme Systems Products, Dublin, CA). ECL (Amersham) was used for detection.

RESULTS

Drug-induced Apoptosis. Incubation of SH-EP cells with doxorubicin caused cell death with morphological features typical of apoptosis, such as cell shrinkage, membrane blebbing, and nuclear condensation (data not shown). Optimal cell killing at 96 h was detected by trypan blue exclusion was achieved using 0.1—1 µg/ml doxorubicin, 5–50 µg/ml cisplatinum, or 50–100 µg/ml VP-16 (data not shown). We next determined whether drug-induced cell death involved apoptosis as measured by DNA fragmentation and changes of cell membrane structure and cell size. Quantification of drug-induced DNA fragmentation was performed by FACS analysis of propidium iodide-stained hypodiploid DNA of cells following drug treatment (28). Maximal DNA fragmentation at 96 h was seen at 0.5–1 µg/ml doxorubicin, 10 µg/ml cisplatinum, or 50–100 µg/ml VP-16, corresponding to the concentrations optimal for inducing
cell death (Fig. 1A). Drug-induced apoptosis was also assessed by annexin V staining and forward/side scatter analysis (31, 32), in addition to DNA analysis, leading to similar results (data not shown).

To test whether drug-induced apoptosis depended on protein synthesis and/or gene expression (33, 34), SH-EP cells were incubated with cycloheximide or cyclosporin A prior to addition of doxorubicin. Doxorubicin-induced DNA fragmentation was markedly inhibited in the presence of cycloheximide or cyclosporin A (Fig. 1B), suggesting that intact protein biosynthesis and de novo transcription were required for doxorubicin-triggered apoptosis.

**Activation of the CD95 System in Drug-induced Apoptosis.** Previous work in our laboratory showed that drug-induced apoptosis in leukemia cells involved CD95-L-receptor interaction (23). Because neuroblastoma cells expressed CD95 and were sensitive to CD95-triggered apoptosis, we investigated whether apoptosis following treatment with anticancer agents in neuroblastoma cells was mediated by the CD95-L-receptor system.

**Induction of CD95-L.** We first analyzed whether CD95-L was produced in response to drug treatment. Incubation with cytotoxic drugs induced CD95-L mRNA and protein expression, as shown by RT-PCR and Western blot, respectively. Maximal induction of CD95-L mRNA and protein was found at 24 h in SH-EP cells and at 48 h in LAN-5 and Kelly cells (Fig. 2A), corresponding to a delayed induction of apoptosis in these cell lines (data not shown). Highest CD95-L expression was observed at drug concentrations most effective in triggering apoptosis, i.e., 0.5–1 μg/ml doxorubicin, 10 μg/ml cisplatinum, and 100 μg/ml VP-16 (Fig. 2).

**Up-Regulation of CD95.** Because p53-mediated up-regulation of CD95 was previously demonstrated in some tumor cell lines (35–37), we studied whether or not expression levels of CD95 could also be modulated by cytotoxic drugs. CD95 expression was up-regulated up to 7-fold upon incubation with 5 μg/ml cisplatinum for 24 h, as assessed by FACS analysis (Fig. 3A). Increased levels of CD95 protein following treatment with cisplatinum, doxorubicin, and VP-16 were also found by Western blot analysis (Fig. 3B). To know whether increased expression of CD95 was regulated by de novo gene expression, RT-PCR was performed. Cisplatinum, VP-16, and doxorubicin caused a strong up-regulation of CD95 mRNA (Fig. 3B). Cyclosporin A, which blocked doxorubicin-mediated apoptosis, also inhibited induction of CD95 mRNA and CD95-L mRNA upon doxorubicin treatment.
Fig. 3. Drug-induced up-regulation of CD95. A. FACS analysis of CD95 expression. SH-EP cells were treated with 5 μg/ml cisplatinum for 24 h, stained with mouse antihuman CD95 monoclonal antibody, and analyzed by flow cytometry. Filled curve, stained cells treated with cisplatinum (+DDP); solid curve, stained control cells (−DDP); dotted curve, unstained control cells; broken curve, cisplatinum-treated cells stained with isotype-matched control antibody. Fluorescence intensity (abscissa) is plotted against cell counts (ordinate). All experiments were done in triplicate with SD of less than 10%. Similar results were obtained in three separate experiments. B, analysis of CD95 mRNA and protein expression. SH-EP and LAN-5 cells were treated for 24 h with 0.5 μg/ml doxorubicin (Doxo), 10 μg/ml cisplatinum (DDP), or 100 μg/ml VP-16 (+). CD95-L mRNA expression was determined by RT-PCR. CD95-L protein expression was analyzed by Western blot. C. inhibition of doxorubicin-induced up-regulation of CD95 mRNA and CD95-L mRNA by cyclosporin A (CsA). SH-EP cells were incubated with 0.5 μg/ml CsA for 1 h at 37°C prior to treatment with 0.5 μg/ml doxorubicin (D 0.5) for 24 h. CD95 and CD95-L mRNA expression were determined by RT-PCR. D. analysis of p53 protein expression by Western blot. SH-EP and LAN-5 cells were treated with 0.5 μg/ml doxorubicin (+) for indicated times. Forty μg protein of cell lysates per lane were separated by 12% SDS-PAGE. Immunodetection of p53 protein was performed by mouse anti-p53 monoclonal antibody and ECL.

Inhibition of Doxorubicin-induced Apoptosis by F(ab')2 Anti-CD95 Antibody Fragments. To see whether blockage of CD95 could mediate increased resistance to drug-induced apoptosis in neuroblastoma cells, we used F(ab')2 anti-CD95 antibody fragments previously shown to inhibit autocrine/paracrine T-cell death, as well as doxorubicin-triggered apoptosis, in T-ALL cell lines (23, 24). Treatment of SH-EP cells with F(ab')2 anti-CD95 prior to addition of doxorubicin, cisplatinum, and VP-16 markedly reduced drug-induced apoptotic cell death at concentrations most effective in triggering apoptosis in neuroblastoma cells (Fig. 4A). In addition, doxorubicin-induced apoptosis was markedly diminished in LAN-5 and Kelly cells following preincubation with F(ab')2 anti-CD95 (Fig. 4B). F(ab')2 FI123 (IgG3) control antibody fragments did not interfere with death signaling after drug treatment. This indicates that drug-triggered apoptosis was mediated via CD95-L-receptor interaction in neuroblastoma cells.
not shown). To explore whether a prototype caspase substrate, the nuclear enzyme PARP, was cleaved during doxorubicin-triggered apoptosis, we performed Western blot analysis. PARP was proteolytically processed to its characteristic Mr, 85,000 fragment (Fig. 6C). These data demonstrate that caspases functioned as death effector molecules during chemotherapy-induced apoptosis of neuroblastoma cells.

DISCUSSION

Apoptosis has been identified as a common response of chemosensitive neoplastic cells, including neuroblastoma, to a variety of drugs (17–21, 23). However, although p53 has been implicated in the process of drug-induced apoptosis (38), the precise molecular requirements that characterize a chemosensitive phenotype in tumor cells are not well understood. In particular, the effector pathways that lead to apoptotic death after chemotherapy in tumors such as neuroblastoma have not been elucidated.

Here, we report that the CD95-L-receptor system was activated by cytotoxic compounds and mediated apoptosis in neuroblastoma cells in vitro. Treatment with doxorubicin, cisplatinum, and VP-16, proven to be effective agents against neuroblastoma in vivo, induced CD95-L expression and triggering of apoptosis via autocrine or paracrine death. In addition, drug treatment up-regulated CD95, which might render neuroblastoma cells even more sensitive to CD95-L, which is simultaneously produced by the tumor cells. Inhibition of CD95 and CD95-L production by cyclosporin A reduced drug-triggered apoptosis. Cyclosporin A has previously been shown to inhibit CD95-based cytotoxicity by blocking CD95-L expression (23, 24, 39). A functioning CD95 signaling pathway was required in neuroblastoma target cells for the execution of cell death because blocking of the CD95 receptor by antagonizing antibody constructs strongly reduced apoptosis sensitivity and because CD95-resistant cells were cross-resistant to drug-triggered apoptosis. CD95-resistant neuroblastoma cells were found to have a defect in the CD95 system because they exhibited a...
strong reduction in CD95 expression and no up-regulation of CD95-L upon drug treatment. Furthermore, caspases were crucially involved in chemotherapy-triggered apoptosis of neuroblastoma cells, leading to cleavage of PARP and ultimately to cell death. PARP, an enzyme involved in DNA repair, has previously been shown to be proteolytically processed by CPP32 (40). Cleavage of PARP has also been observed following treatment with anticancer agents (41). Caspases have been identified as effector molecules in CD95-mediated death signaling (29), as well as in execution of cell death following stimulation through TNF receptor I and granzyme B (42, 43). Because upstream blockade of the CD95 pathway by antagonistic anti-CD95 antibody constructs strongly reduced the death signal given by the cytotoxic drugs used in our system, activation of caspases in neuroblastoma cells was most probably induced via activation of the CD95 pathway itself.

Molecular events leading to induction of CD95-L and CD95 might include stabilization and accumulation of wild-type p53 protein. Upon treatment with anticancer agents, p53 accumulated prior to maximal induction of CD95-L and CD95. Wild-type p53 has previously been shown to mediate up-regulation of CD95 in human tumor cell lines (35–37). Because the frequency of p53 mutations is generally considered to be very low in neuroblastoma (44), wild-type p53 may mediate apoptosis in neuroblastoma cells by inducing apoptosis-promoting molecules such as CD95. Recently, the sphingomyelin signal transduction pathway has been linked to drug-induced cell death (45, 46). In line with this, cross-linking of CD95 has also been shown to result in generation of ceramide (47). Ceramide production, as measured in daunorubicin-treated cells (45, 46), might be part of the CD95 signaling cascade initiated by cytotoxic agents.

For our studies, we primarily used SH-EP neuroblastoma cells, which represent a prototype chemosensitive neuroblastoma cell line. However, induction of CD95 and CD95-L upon drug treatment and CD95-mediated blockade of drug-induced apoptosis were also found in other neuroblastoma cell lines with different molecular characteristics such as overexpression of MYCN (LAN-5 and Kelly). The cytotoxic drugs used in this study are three of the first-line agents in the clinical management of neuroblastoma (12, 13). Drug concentrations most effective to induce apoptosis in our experiments are within the range of plasma concentrations that can be achieved in patients (48–50). Thus, the mechanism of cytotoxicity described here is probably of therapeutical relevance for neuroblastoma.

Taken together, the cytotoxic action of chemotherapeutic agents may interfere with apoptosis pathways at several levels, including triggering of CD95-L-receptor interaction, accumulation of wild-type p53 protein, and stimulation of signaling cascades such as the sphingomyelin pathway, ultimately leading to activation of death effector molecules, i.e., proteases of the ICE family. Our findings provide new insights into the molecular mechanisms leading to chemotherapyproduced apoptosis in chemosensitive neuroblastoma cells by demonstrating an important role for the CD95-L-receptor system. These data contribute to the mounting evidence that activation of the CD95 system is central to drug-mediated apoptosis in tumor cells of hematopoietic as well as of nonhematopoietic origin (23, 51). In a more general sense, our findings show that chemosensitivity of tumor cells, e.g., neuroblastoma cells, may depend on intact apoptosis pathways, such as the CD95 pathway, that are initiated by treatment with chemotherapeutic agents in vivo. Understanding the nature of the signaling pathway(s) in drug-induced apoptosis may be fundamental in determining drug sensitivity and overcoming drug resistance of tumor cells.

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Fig. 6. Drug-induced activation of caspases. A, analysis of ICE proteolytic activity. SH-EP cells were incubated with 0.05 μg/ml (■), 0.1 μg/ml (●), and 0.5 μg/ml (▲) doxorubicin for times indicated. ICE activity was analyzed as described using the fluorogenic ICE substrate DABCYL-YVADAPK-EDANS (29). Data points, means from three independent experiments done in triplicate. SDs were less than 10%. B, inhibition of drug-induced apoptosis by peptide inhibitor of caspases. SH-EP cells were treated with 0.5 μg/ml doxorubicin (Doxo), 10 μg/ml cisplatinum (DDP), and 100 μg/ml VP-16 for 48 h in the absence (■) or presence (▲) of 60 μM zVAD-fmk, a broad range inhibitor of caspases. Apoptosis was determined by FACS analysis of propidium iodide-stained nuclei. Specific apoptosis was calculated as described in Fig. 1A. Columns, means from three independent experiments done in triplicate. SDs were less than 10%. C, cleavage of PARP. Cells were treated with 0.5 μg/ml doxorubicin (+) for indicated times. Forty μg protein per lane isolated from cell lysates were separated by 12% SDS-PAGE. Immunodetection of PARP protein was performed by rabbit anti-PARP polyclonal antibody and ECL.

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