Drug-induced Caspase-3 Activation in a Ewing Tumor Cell Line and Primary Ewing Tumor Cells

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Abstract. *Ewing sarcoma is a rare malignancy occurring in childhood and adolescence which has a prognosis of about 50% long-term event-free survival, depending on risk factors such as tumor volume, initial metastases at the time of diagnosis and tumor response to presurgical chemotherapy. In order to tailor therapy to the individual patient, new prognostic factors need to be identified. In this study, we showed that etoposide and actinomycin D kill Ewing tumor cells (RD-ES cell line) mainly via a caspase-dependent mechanism. Both drugs induced a significant caspase-3 activation, which can be detected with a new caspase-3 assay. Dose-response analyses showed that induction of cell death and caspase-3 activation is mediated by similar concentration ranges of both drugs. In addition to the cell line, caspase-3 activation was also shown during drug-mediated stimulation of freshly isolated cells from tumor biopsies. In conclusion, actinomycin D and etoposide stimulated caspase-3 activation in a Ewing tumor cell line and in freshly isolated tumor cells, causing drug-induced cell death. Thus, determination of caspase-3 activation might be a suitable method for drug sensitivity testing in patients with Ewing tumors before initiating treatment. This assay could therefore help individualize therapy in future tumor patients.*

Ewing tumors are a rare type of solid malignancy located primarily in bone tissue, the annual incidence of which is estimated at 0.6 per million in the general population (1). Treatment consists of primary chemotherapy followed by resection of the tumor and/or irradiation of the sites (2-5). Known prognostic factors include tumor volume, tumor site and histological response to chemotherapy (6). Depending on these three factors, post-surgical treatment is currently being investigated in a European multicenter study. It varies in intensity, *e.g.* from either one or two chemotherapy regimens for good responders to conventional *vs.* high-dose chemotherapy and stem cell rescue for poor responders (7). However, it is not clear which patients might benefit from which options. Thus, in an optimized treatment situation, patients should receive only those drugs which have previously shown effectiveness against their own tumor. However, unlike leukemia treatment protocols (8), so far no reliable *in vitro* assay for drug sensitivity testing in solid tumors is available.

Several anticancer drugs, such as cisplatinum, have been shown to induce tumor cell death by activation of caspases and subsequent induction of apoptosis (9). Similarly, actinomycin D and etoposide, which are commonly used in the treatment of Ewing tumors, stimulate caspase-dependent cytotoxicity in different tumor models such as glioblastoma, small cell lung cancer and lymphoma (10-13). However, the cytotoxicity mechanism is dependent on the cell system in question. Although these drugs are widely used in the treatment of Ewing tumor cells, there are no reports on how actinomycin D or etoposide facilitate the death of Ewing tumor cells.

In order to assess the potency of caspase-3 activation for *in vitro* drug sensitivity testing in a solid tumor model, we investigated: 1) whether etoposide and actinomycin D induce caspase-dependent cytotoxicity in a Ewing tumor cell line; and 2) whether drug-induced caspase-3 activation can be determined in a dose-dependent fashion in freshly isolated Ewing tumor cells from biopsies.

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Materials and Methods

Patients. Primary tumor cells used for *ex vivo* studies were isolated from fresh biopsy specimens of two patients with Ewing tumors. The patients and/or parents gave informed consent.

Preparation of biopsies. Biopsies were mechanically fragmented below particles 3x3x3 mm in size. The biopsy fragments were repeatedly incubated for 10-15 minutes at 37˚C with an enzyme cocktail consisting of 50 U/ml collagenase III (Biochrom, Berlin, Germany), 150 U/ml collagenase IV (Sigma, Deisenhofen, Germany), 200 U/ml hyaluronidase (Sigma) and 100 U/ml DNAse I (Sigma) in HBSS (Hank's buffered salt solution) until a single cell suspension was obtained. The cells were then washed with HBSS, resuspended in cell culture medium and immediately used for caspase activation testing. Cell viability was tested by Trypan blue exclusion after each enzyme isolation passage. A total of 30-35 x 10^6 viable single tumor cells was thus collected. For testing in the caspase assay, a density of 1x10^6 cells/ml was required.

Cell line and culture conditions. The human Ewing's sarcoma cell line RD-ES was obtained in 1984 from the primary tumor of a 19-year-old Caucasian male (15). The cells, which grow in adherent monolayers or loosely adherent clusters or clumps, were cultured in RPMI 1640 medium (Gibco BRL, Karlsruhe, Germany) containing 15% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all additives from Biochrom Seromed, Berlin, Germany) at 37˚C in a humidified 5% CO_2 atmosphere.

Drugs. Freshly prepared stock solutions of actinomycin D (Act) and etoposide (VP-16) were provided by the central pharmacy of the Leipzig University Medical Center, Germany, at the following concentrations: VP-16 (1 mg/ml), Act (0.5 mg/ml). For cytotoxicity testing, fresh stock solutions and dilutions in culture medium were used. For the caspase assay, these drugs and dilutions in culture medium were titrated into 96-well plates and stored until use at -20˚C.

Determination of apoptosis and cell death. Apoptosis and cell death were assayed after culturing for 24 h using an Annexin V-FITC staining kit (Immunotech, Coulter Inc., Marseille, France) and flow cytometry. After harvesting the cells with trypsin-EDTA and then stopping trypsinization with culture medium, the cells were washed in PBS in an ice-bath and resuspended in binding buffer with >1.5 mM calcium. The cells were then stained with Annexin V-FITC (25 µg/ml) and propidium iodide (250 µg/ml) and analysed by flow cytometry (Epics XL, Beckman Coulter, Krefeld, Germany). The fraction of vital cells was determined as the double-negative cell fraction, *i.e.*, the proportion of cells which stained positive neither for AV nor for PI. Results were evaluated using Expo 32 software (Beckman Coulter, Krefeld, Germany).

General caspase inhibitor assay. The general caspase inhibitor z-VAD-fmk (R&D Systems, Wiesbaden, Germany) was reconstituted in DMSO to yield a 20 mM stock solution and aliquots stored at -20˚C. The inhibitor was used at a concentration of 100 µM of z-VAD-fmk. Cells were preincubated with z-VAD-fmk or an equal volume of DMSO as a solvent control for 1 h before stimulation with the drugs. The cells were then stimulated with or without VP-16 (10 µg/ml) and Act (0.5 µg/ml). AV/PI staining and flow cytometry was carried out 24 h after addition of drugs. Results were expressed in cytotoxicity rates defined as 100% – % vital cells (= AV-/PI-).

Total specific cytotoxicity: *cytotoxicity in the presence of a drug + DMSO (solvent control) minus cytotoxicity in the presence of culture medium + DMSO*

Specific caspase-independent cytotoxicity: *cytotoxicity in the presence of a drug + z-VAD-fmk (as caspase inhibitor) minus cytotoxicity in the presence of culture medium + z-VAD-fmk*

Specific caspase-dependent cytotoxicity: *specific total cytotoxicity minus specific caspase-independent cytotoxicity*

Determination of drug-induced caspase activity. Drug-induced apoptosis by caspase activation was determined using a new caspase-3 assay (international patent application number PCT/EP00/02174; Evotec Technologies BmbH., Erkrath, Germany). Cells were plated on drug-precoated 96-well plates at a density of 10^6/ml and incubated at 37˚C in a humidified 5% CO_2 atmosphere. All trials were run in triplicate. Cells plated in culture medium only served as zero controls. After overnight incubation (16-18 h), cells were lysed with 100 µl/well of cell lysis buffer supplemented with 20 mM DTT immediately before use. Ten µl of a fluorigenic 1 mM caspase-3-specific substrate Ac-DEVD-AMC (Bachem, Heidelberg, Germany) was added to each well. Caspase activity was determined immediately at 360 nm excitation and 465 nm emission wavelength using a fluorescence plate reader (Tecan Inc., Salzburg, Austria). The plates were read for kinetics over 1 h at 13 intervals. The fluorescence signal is directly proportional to the caspase activity in each sample and expressed as mean slope per hour (MSH) of the data points.

Determination of IC_{50} values. A new data evaluation software was written for determination of the IC_{50} values of the anticancer drugs. The IC_{50} value is defined as the drug concentration where 50% of the maximum fluorescence intensity (=caspase activation) in a sample is reached. Briefly, enzyme saturation curves were obtained by plotting the mean of the MSH values against the drug concentrations to find the optimum curve fit. Furthermore, the ratio of the area under the curve:plateau and the turning point of each curve were calculated. From the entire experimental series of a drug, only those curves which met the following criteria were chosen for calculation of the IC_{50} values:

A. Main criteria (all criteria must be met):
1. A reasonable curve fit is obtained.
2. The IC_{50} value is >0.
3. The curve defines one single turning point.
4. The ratio of the area under the curve:plateau is >1.

B. Minor criteria (must be met in increasing order):
The following minor criteria were applied to the curves which met the main criteria in this order:
1. lowest error score;
2. highest plateau value;
3. greatest area under the curve.

Statistical analysis. Student's *t*-test for paired or unpaired samples was used.

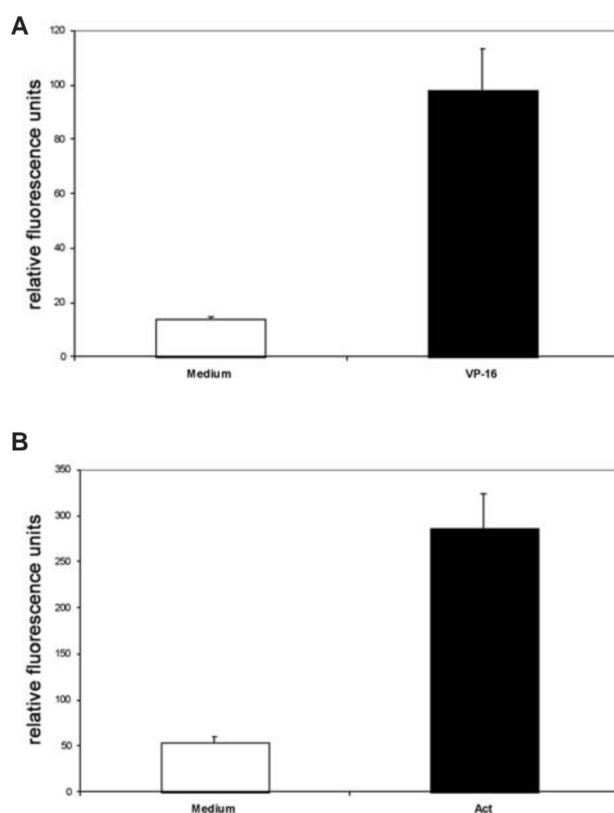

Figure 1A and 1B. *Drug-mediated activation of caspase-3 in RD-ES cells. RD-ES cells were plated out in 96-well plates (100 µl/well) at a density of 1x10⁶/ml. Cells were stimulated in triplicate with VP-16 (10 µg/ml), Act (0.5 µg/ml) or with culture medium only. After culturing for 16 hours under standard conditions, cells were assayed for caspase-3 activation. Shown are the relative fluorescence units of caspase activation in the presence of a drug (solid bars) or medium (white bars) of three independent experiments (mean and SEM). VP-16 and Act induced significant caspase-3 activation ($p<0.05$, t-test for paired samples).*
A: VP-16-induced caspase activation
B: Act-induced caspase activation

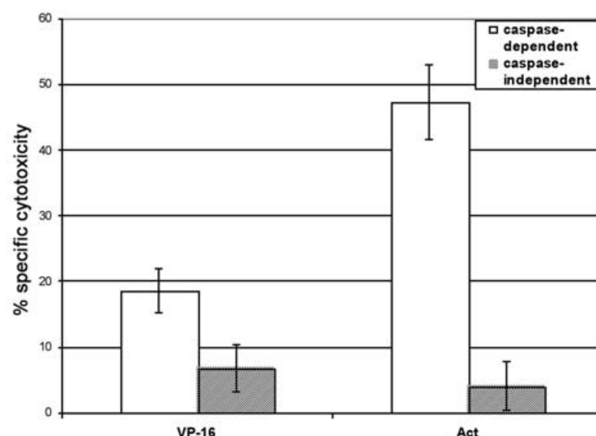

Figure 2. *Drug-induced caspase-dependent and caspase-independent cytotoxicity in RD-ES cells. RD-ES cells were plated out in 24-well plates at a density of 1x10⁶/ml. Z-VAD-fmk (100 µM) or DMSO only (solvent control) were added to the cultures. After preincubating for 1 hour at 37˚C and 5% CO_2, cells were stimulated with VP-16 (10 µg/ml), Act (0.5 µg/ml) or with culture medium. After culturing for 24 hours under standard conditions, cells were stained with AV/PI and analysed by flow cytometry. Results are expressed as specific caspase-dependent (white bars) or specific caspase-independent (grey bars) cytotoxicity of the different stimuli as defined in Materials and Methods. VP and Act induced a significant caspase-dependent cytotoxicity ($p<0.05$).*

Results

Since caspase-induced apoptosis of tumor cells is an essential mechanism of drug-induced cytotoxicity, we first analysed drug-induced caspase-3 activation by using a novel caspase-assay (see Materials and Methods). Compared to medium control, VP-16 and Act induced a significant activation of caspase-3 in RD-ES cells (Figure 1A and 1B).

Next, we investigated whether caspase activation is an important mechanism of VP-16- or Act-induced cytotoxicity in Ewing tumor cells. To that end, the Ewing tumor cell line RD-ES was stimulated with VP-16 and Act. Next, drug-induced cell death was assayed with AV/PI staining. Preincubation of cells with z-VAD-fmk, a general caspase inhibitor, could separate drug-induced cytotoxicity into caspase-dependent and caspase-independent. VP-16- and Act significantly induced caspase-dependent cytotoxicity, indicating that cytotoxic effects of the drugs are induced mainly by caspase-dependent mechanisms. In contrast, Act and VP-16 induced only marginal caspase-independent cytotoxicity (Figure 2).

VP-16 and Act induced caspase activation and caspase-dependent cytotoxicity in RD-ES cells. However, the maximum level of caspase activation varied between experiments. In order to establish an assay system for prospective drug sensitivity testing, a reliable expression system is required. Therefore, dose-response analyses were run and IC_{50} values for caspase activation and cytotoxicity rates were determined. There were no significant differences between the IC_{50} values of the caspase and the cytotoxicity assay, indicating a mutual relationship between caspase activation and drug-induced cell death (Figure 3).

Finally, we investigated whether drug-induced caspase activation could be determined in primary tumor cells isolated from fresh tumor biopsy specimens at the time of diagnosis. Tumor cells were collected from biospsies of Ewing tumor patients, isolated from the biopsies (see Materials and Methods) and then stimulated with various doses of Act or VP-16 in the caspase assay. After 16 h of culture, IC_{50} values of caspase activation were determined (Table I). The results of these experiments show that

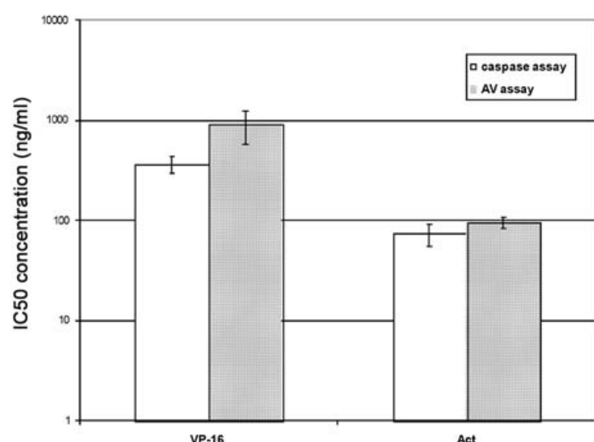

Figure 3. *Drug-induced caspase activation in comparison to drug-mediated cytotoxicity.RD-ES cells were stimulated with VP-16 (0-50 μg/ml) or Act (0-4 μg/ml). After culturing for 24 hours under standard conditions, cells were stained with AV/PI and analysed by flow cytometry. Caspase activation was simultaneously determined after 16 hours. Results of dose-response analyses are expressed as IC_{50} concentrations. Shown are the mean and SEM of three experiments. Differences between IC_{50} values of caspase activation (white bars) and AV assays (grey bars) were not significant ($p>0.05$, t-test for unpaired samples).*

caspase activation can be determined from primary tumor cells obtained from fresh biopsy specimens similar to the Ewing tumor cell line. However, IC_{50} values of tumor cells taken from biopsies are quite different from those of the tumor cell line. While IC_{50} values for Act seemed to be lower, values for VP-16 were about 100 times higher for the tumor biopsy cells.

Discussion

We could show that actinomycin D and etoposide activate caspase-3 in Ewing tumor cells. Apoptosis is caused by activating caspase-3, amongst many other caspases. Caspase-3, one of the downstream effector caspases, works as a key trigger of apoptosis (15), which eventually results in cell death.

We could show that both drugs induced death of the tumor cells mainly by caspase-dependent mechanisms. Inhibition of caspase activity by z-VAD-fmk significantly reduced the cytotoxic effects of actinomycin D and etoposide. Miller *et al.* (16) and Xiang *et al.* (17) give evidence for a clear separation of these divergent intracellular cytotoxicity pathways by applying z-VAD-fmk and showing that z-VAD-fmk could not prevent apoptosis induced by overexpression of Bax, although caspase-3 activation and nuclear fragmentation were clearly blocked, suggesting a caspase-independent death pathway downstream of Bax. Similarly, in a recent study by Stahnke *et al.* (18) on peripheral blood lymphocytes of chemotherapy patients, *in vitro* apoptosis could generally not be inhibited by z-VAD-fmk, thus indicating additional involvement of caspase-independent cell death.

Table I. *Caspase activation in tumor cells isolated from fresh tumor biopsy specimens.*

IC_{50} values of caspase activation in ng/ml

	VP-16	Act
Patient 1	3078	8.3
Patient 2	15844	5.8

Act and VP-16 induced significant cytotoxicity in the Ewing tumor cell line RD-ES. However, dose-response experiments revealed similar IC_{50} values of these drugs for cytotoxicity and caspase activation, suggesting a direct corrrelation between caspase activation and cell death in this model. Since different mechanisms apart from apoptosis might be involved in drug-induced cell death, this relationsip seems to be very important for a prospective clinical application.

Peak plasma levels of 21 μg/ml were found after intravenous infusion of 100 mg/m^2 VP-16, and 600 ng/ml after 24 hours (20). Thus, etoposide concentrations which cause half maximum caspase activation (IC_{50} values) are within the range of plasma levels seen in patients treated with conventional doses of etoposide.

The pharmacokinetics of actinomycin D show peak plasma concentrations of 100-500 ng/ml and a rapid elimination to below 50 ng/ml within 4 hours. However, drug concentrations in different cells, such as granulocytes or lymphocytes, remain above 100 ng/ml for up to 48 hours. Thus, the IC_{50} concentrations found in our experiments with actinomycin D are comparable to the *in vivo* pharmacological range measured following conventional treatment (21).

Similar to the results with the Ewing tumor cell line, activation of caspase-3 was also observed in tumor cells isolated from fresh biopsy specimens, indicating that our protocol might be useful for determining drug sensitivity in solid tumors. However, results of drug sensitivity testing in leukemia treatment protocols show that therapeutic consequences can only be drawn from a prospective study where results of *in vitro* testing are correlated with the event-free survival rates of patients (8).

In conclusion, we could show that etoposide and actinomycin D activated caspase-3 in a Ewing tumor cell line as well as in freshly isolated Ewing tumor cells. The *in vitro* cytotoxic drug concentrations were within the

pharmacological range seen in patients treated with these drugs. If further research based on this study shows a correlation between the *in vitro* caspase assay and the *in vivo* tumor response to anticancer drugs, this assay might prove a useful tool for tailoring the therapeutic strategy to patients with Ewing tumors.

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