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Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 activation in a Fas-independent way

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Abstract It has recently been proposed that doxorubicin (DOX) can induce apoptosis in human T-leukemia cells via the Fas/FasL system in an autocrine/paracrine way. We show here that treatment of Jurkat cells with either anti-Fas antibodies, anthracyclin drugs or actinomycin D induces the activation of CPP32 (caspase-3) and apoptosis. However, DOX treatment did not induce the expression of membrane FasL or the release of soluble FasL and co-incubation with blocking anti-Fas antibodies prevented Fas-induced but not DOX-induced apoptosis. All the morphological and biochemical signs of apoptosis induced by anti-Fas or DOX can be prevented by Z-VAD-fmk, a general caspase inhibitor. DEVD-cho, a specific inhibitor of CPP32-like caspases which completely blocks Fas-mediated apoptosis, prevented drug-induced nuclear apoptosis but not cell death. We conclude that: (i) DOX-induced apoptosis in human T-leukemia/lymphoma is Fas-independent and (ii) caspase-3 is responsible of DOX-induced nuclear apoptosis but other Z-VAD-sensitive caspases are implicated in cell death.

Key words: Fas; Caspase; CPP32; Chemotherapeutic drug; Apoptosis; Leukemia

1. Introduction

The mechanism of the cytotoxicity of anthracyclin antibiotics on tumor cells has long been a matter of debate. Recently it has been proposed that these and other cytotoxic drugs exert their effects by inducing the resident program of cell death known as apoptosis [1,2]. One of the most studied apoptotic pathways is that triggered by the ligation of the plasma membrane molecule Fas (APO-1/CD95). When Fas is trimerized by its natural ligand, FasL, either in soluble form [3] or expressed in the membrane of effector cells [4], or by cytotoxic agonistic antibodies [5], several intracellular adapter proteins are recruited to the clustered receptors. These molecules, known as FADD/MORT1 [6], bind to intracellular Fas domains, known as death domains, and recruit one or several cysteine proteases with Asp specificity (caspases), such as caspase-8 (FLICE/MACH/Mch-5) or caspase-10 (FLICE2/Mch-4) [7–9]. The recruitment of these proteases induces their autocatalytic processing and activation, which finally leads to the cleavage and activation of CPP32-like proteases, the apoptotic executioner.

It has recently been proposed [10] that doxorubicin- (DOX) and methotrexate-induced apoptosis in human T-leukemic cells occurs through the up-regulation of membrane Fas and the induction of FasL expression, leading to Fas/FasL interaction and cell death. However, this mechanism is difficult to apply to leukemic cells like L1210 and K562 cells that do not express functional Fas [11,12] but are yet sensitive to toxicity elicited by anthracyclin drugs [13,14]. We have recently found that several Jurkat-derived sublines which have lost the expression of membrane Fas and cytosolic caspase-3 are also resistant to DOX-induced toxicity (Martinez-Lorenzo et al., submitted). The resistance in this case could be due a priori to the lack of expression of either Fas, CPP32 or both. We show here that anthracyclin drugs as well as actinomycin D induce apoptosis in T-leukemia cells upon activation of the CPP32 protease. All the morphological and biochemical signs of apoptosis induced by these drugs can be prevented by treatment of cells with Z-VAD-fmk, a general caspase inhibitor, which prevents CPP32 processing and activation, irrespective of the expression of functional membrane Fas. Moreover, in Jurkat (CD95/Fas$^+$) cells DOX treatment does not induce the release of preformed FasL and co-incubation with blocking anti-Fas antibodies prevent Fas-induced but not DOX-induced apoptosis.

2. Materials and methods

2.1. Materials

DOX, daunorubicin, actinomycin D, methotrexate, vincristine and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were products from Sigma (Madrid, Spain). Cytotoxic monoclonal anti-human Fas IgM antibody (clone CH-11) was from UBI (Lake Placid, NY, USA), non-cytotoxic blocking mouse monoclonal anti-human Fas IgG1 antibody (clone SM1/23) was from Bender (Barcelona, Spain) and mouse IgG2a anti-human CPP32 (clone 19), which recognizes the 32 kDa proenzyme, from Transduction Laboratories (Lexington, KY, USA). *N*-Acetyl-Asp-Glu-Val-Asp aldehyde (DEVD-cho) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone (Z-VAD-fmk) were from Bachem (Bubendorf, Switzerland).

2.2. Cell proliferation and toxicity assays

The human T-cell leukemic cell lines Jurkat (clone E6.1) and MOLT-4, and the T-lymphoma HUT 78 were from the American Type Culture Collection. Cells were routinely cultured at 37°C in RPMI 1640 medium supplemented with 5% fetal calf serum, L-glutamine and penicillin/streptomycin (hereafter called complete medium), using standard cell culture procedures. In proliferation and toxicity assays, cells (5×10^5 cells/ml) were treated in complete medium with either cytotoxic IgM anti-Fas antibody (50 ng/ml) or the indicated drug in flat-bottom, 96-well plates (100 μl/well). For apoptosis inhibition assays, cells were preincubated for 3 h with 600 μM DEVD-cho

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Abbreviations: FasL, Fas ligand; PS, phosphatidylserine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; DOX, doxorubicin; DEVD-cho, *N*-acetyl-Asp-Glu-Val-Asp aldehyde; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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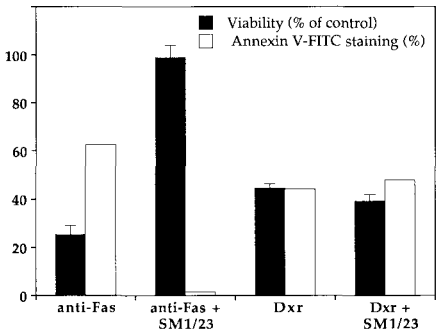

Fig. 1. Neutralizing anti-Fas antibody (SM1/23) prevents Fas-, but not DOX-induced apoptosis. Jurkat cells (5×10^5 cells/ml) were preincubated or not with 100 ng/ml of SM1/23 antibody for 30 min at 4°C, and then incubated for 21 h with 0.5 μM DOX or 50 ng/ml cytotoxic anti-Fas IgM antibody in the presence or absence of SM1/23 antibody, as indicated. Cell viability was determined by the MTT assay.

or for 1 h with 100 μM Z-VAD-fmk prior to the addition of drugs. Control cultures were treated with the appropriate amount of DMSO alone (0.1%, final concentration). Cell viability was determined by a modification of the MTT reduction method of Mosmann [15] or by the trypan blue exclusion test. Apoptosis was also microscopically evaluated by scoring the number of cells exhibiting a blebbing morphology, defined by the appearance of distinct protrusions of the plasma membrane and/or vacuolization. Chromatin condensation and nuclear fragmentation during apoptosis was evaluated by labeling nuclei with *p*-phenylenediamine (PPDA) in glycerol and visualized by fluorescence microscopy [16]. After treatments, cells (5×10^5) were fixed with 1% paraformaldehyde in PBS, pH 7.4 at room temperature for 15 min. Then, cells were washed with PBS and centrifuged onto round coverglasses, previously treated for 20 min with 100 μl of a sterile solution of poly-L-lysine in distilled water. Dried coverglasses containing fixed cells were placed onto a drop of PPDA stain (1 mg/ml PPDA in PBS/oxidized glycerol, 1/5), and analyzed by fluorescence microscopy.

The possible implication of FasL in DOX-induced apoptosis was evaluated by using the blocking anti-Fas antibody SM1/23. Cells (2.5×10^5 cells/ml) were preincubated at 4°C for 30 min in PBS+1% BSA, pH 7.4, containing or not (controls) 100 ng/ml of the SM1/23 antibody. Then cells were resuspended at 5×10^5/ml in complete medium containing the same amount of blocking anti-Fas antibody, and treated with 50 ng/ml of cytotoxic IgM anti-Fas antibody or 0.5 μM DOX at 37°C for 21 h. Then cells were collected by centrifugation and viability determined by the MTT assay and by trypan blue staining. The possibility that DOX could induce cell death via the release of soluble FasL was analyzed as follows: Jurkat cells were treated with 1 μM DOX in complete medium for 5 h. Cells were recovered by centrifugation, washed with cold RPMI medium, resuspended at 5×10^5 cells/ml in complete medium and cultured for 1, 24 or 48 h. Culture supernatants were obtained by centrifugation and their toxicity was evaluated in a sensitive bioassay using untreated Jurkat cells as targets [3].

2.3. Flow cytometry analysis

Fas membrane expression was analyzed with a FITC-labeled anti-human Fas antibody (Bender, Barcelona, Spain). Cells (5×10^5 in 50 μl) were incubated at 4°C with 5 μg/ml of the antibody in PBS containing 0.2% BSA and 0.02% sodium azide for 1 h. Cells were washed with PBS, fixed with 1% paraformaldehyde in PBS, pH 7.4, for 15 min and 5000 cells/sample analyzed in an Epics XL-MCL (Coulter, Spain) flow cytometer. Phosphatidylserine (PS) exposure during apoptosis was evaluated by annexin V-FITC staining [17]. Briefly, cells were washed with PBS and incubated in a solution of 0.5 μg/ml FITC-labeled annexin V in binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES/NaOH, pH 7.4) at 4°C for 30 min. Then, cells were centrifuged, washed, resuspended in 1 ml of binding buffer and analyzed by flow cytometry.

2.4. Analysis of CPP32 activation

CPP32 activation was evaluated by Western blot analysis of cell homogenates with a specific anti-CPP32 antibody. Jurkat cells (5×10^5 cells/ml) were incubated in complete medium with one of the following drugs: DOX (1 μM), daunorubicin (1 μM), actinomycin D (1 μg/ml), methotrexate (50 and 100 ng/ml) or vinblastine (10 ng/ml). At the times indicated, cell viability was determined by trypan blue staining and cells were recovered by centrifugation, washed twice with cold PBS and lysed in 1 ml of lysis buffer (0.15 M NaCl, 1 mM EDTA, 30 mM NaF, 10 μg/ml leupeptin, 1 mM PMSF, 50 mM Tris-HCl, pH 7.6, containing 1% Triton X-100) [18]. Solubilized proteins were resolved by SDS-10% PAGE and transferred to nitrocellulose membranes (Hybond-C extra, Amersham). Membranes were sequentially incubated with 50 ng/ml anti-CPP32 antibody and with 0.2 μg/

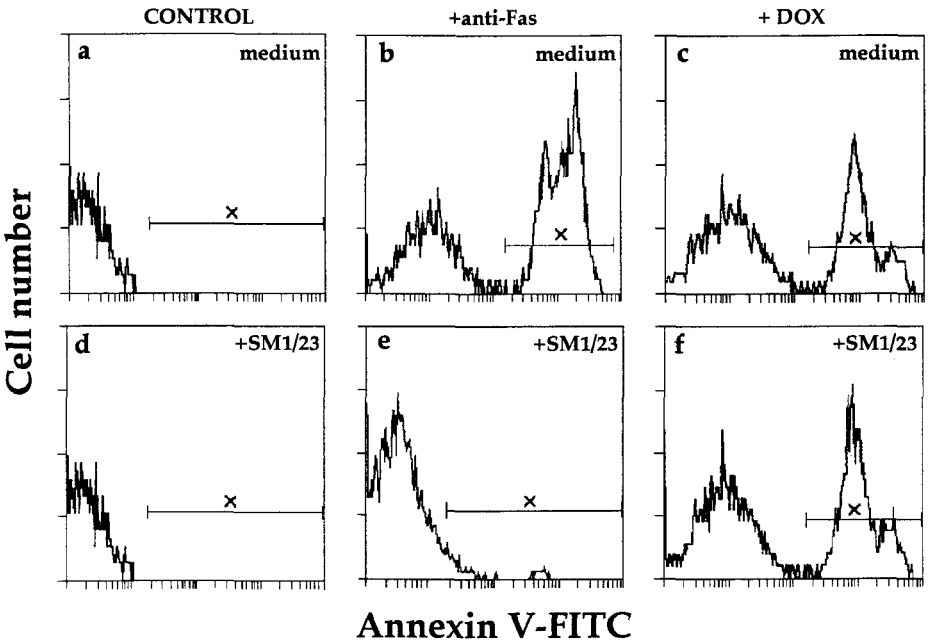

Fig. 2. Neutralizing anti-Fas antibody does not block DOX-induced PS exposure. Jurkat cells treated as in Fig. 1 were analyzed for PS exposure by measuring annexin V-FITC binding by flow cytometry. Cells were cultured in medium alone (a–c), or in medium containing 100 ng/ml anti-Fas IgG1 antibody (SM1/23) (d–f), and treated with 50 ng/ml anti-Fas antibody (b, e) or 1 μM DOX (c, f).

ml goat anti-mouse IgG coupled to alkaline phosphatase (Sigma) and revealed with the BCIP/NBT substrate.

3. Results

3.1. DOX-induced apoptosis does not involve Fas triggering

It has recently been proposed that the Fas/FasL system could be involved in the cytotoxicity exerted by several drugs, including DOX [10]. To test this hypothesis, apoptosis was induced in Jurkat cells by DOX treatment in the presence or absence of an anti-Fas blocking antibody (SM1/23). To assess the blocking activity of this antibody, cells were also treated with a cytotoxic monoclonal anti-human Fas IgM antibody (CH-11). After 21 h of incubation, cell viability was determined and the PS translocation analyzed by annexin V-FITC binding. As shown in Fig. 1, while all the cytotoxicity induced by CH-11 antibody was prevented by the blocking antibody, there was no inhibition of DOX-induced cell death. Treatment of Jurkat cells with CH-11 antibody or DOX induced the early exposure of PS on the cell membrane. PS exposure was totally inhibited by the blocking antibody in CH-11-treated cells while no inhibition could be seen in cells incubated with DOX (Fig. 2). We next tested if treatment of Jurkat cells with DOX induced the release of soluble FasL to the culture medium, which is the main mediator of activation-induced cell death in these cells [3]. Jurkat cells were treated for 5 h with 1 μM DOX, washed, and cultured in fresh medium for 1, 24 or 48 h and culture supernatants collected by centrifugation. Untreated Jurkat cells were resuspended in these supernatants and cell viability was determined by the MTT assay after 24 h of culture. Although cells pretreated with DOX died after 24–48 h of culture, none of their culture supernatants was toxic for untreated Jurkat cells (data not shown). We also evaluated the sensitivity of MOLT-4 and HUT 78 cells to DOX-induced apoptosis. According to previous data [19], MOLT-4 cells expressed Fas membrane levels similar to that of Jurkat cells (data not shown), but MOLT-4 cells were resistant to anti-Fas-induced apoptosis (Fig. 3). Resistance to Fas-mediated apoptosis was not reverted by co-treatment with cycloheximide, use of higher antibody concentrations and/or longer incubation times (data not shown). However, MOLT-4 cells were sensitive to DOX-induced apoptosis (Fig. 3). Moreover, the same concentration of DOX caused only a slight cytostatic effect on HUT 78 cells, which were almost as sensitive as Jurkat cells to anti-Fas-induced apoptosis (Fig. 3).

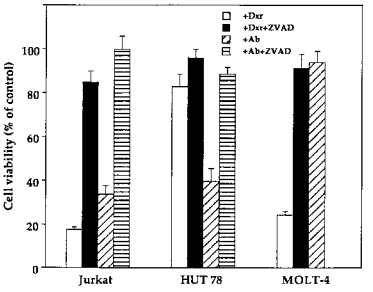

Fig. 3. Z-VAD-fmk prevents DOX- and anti-Fas-induced cell death. Jurkat, MOLT-4 or HUT 78 cells (5×10^5 cells/ml) were preincubated with 100 μM Z-VAD-fmk for 1 h, and then treated with 1 μM DOX or 50 ng/ml cytotoxic anti-Fas antibodies (Ab) for 16 h, as indicated. Cell viability was determined by the MTT assay. Cells treated with Z-VAD-fmk alone were used as controls.

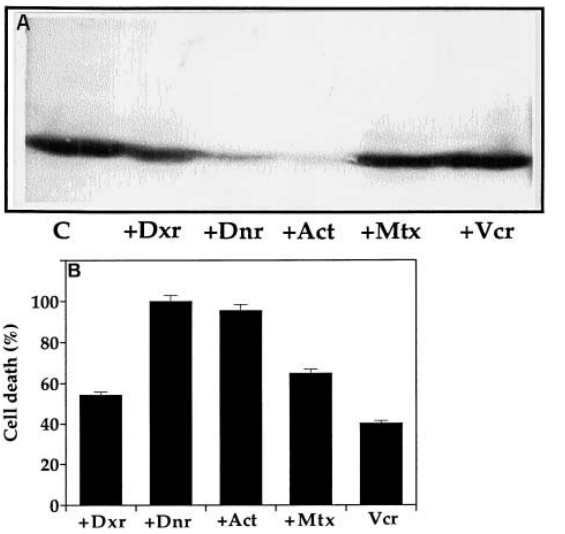

Fig. 4. DOX, daunorubicin and actinomycin D induce early CPP32 activation which correlates with cell death. A: Jurkat cells (5×10^5 cells/ml) were incubated for 16 h with 1 μM DOX, 1 μM daunorubicin or 1 μg/ml actinomycin D or for 24 h with 10 ng/ml vincristine or 100 ng/ml methotrexate, as indicated. Cell proteins were separated by SDS-10%PAGE and CPP32 activation analyzed by immunoblotting using an anti-CPP32 antibody. B: Cell viability of the corresponding cells was determined by the MTT assay.

3.2. DOX treatment induces early CPP32 activation which correlates with cell death

We studied the implication of CPP32 in cell death induced by a number of drugs. First, the type of cell death (apoptosis/necrosis) caused by the different drugs was analyzed by nuclear staining with PPDA. In the experimental conditions used, DOX, daunorubicin and actinomycin D caused chromatin condensation and nuclear fragmentation in Jurkat cells, while methotrexate and vinblastine did not (Fig. 6 and data not shown), although there was a considerable amount of cell death (Fig. 4). Longer times of incubation (48 h) were needed to detect chromatin condensation caused by methotrexate or vinblastine (data not shown). CPP32 activation was analyzed by Western blotting, using an anti-CPP32 antibody. After 16

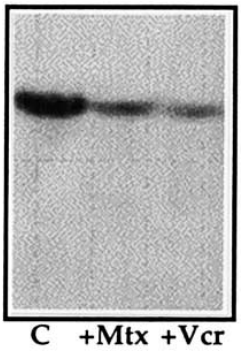

Fig. 5. Methotrexate and vincristine induce late CPP32 activation. Jurkat cells were incubated for 48 h with 50 ng/ml methothrexate or 100 ng/ml vincristine. CPP32 activation was determined as indicated in Fig. 4.

h incubation with either DOX, daunorubicin or actinomycin D, a clear activation of CPP32 (a reduced intensity of the 32 kDa band in the blot) was observed (Fig. 4A). This correlated with the extent of cell death induced by each of these drugs (Fig. 4B). Incubation of the cells for 24 h with methotrexate or vinblastine did not induce a significant activation of this caspase, though the extent of cell death was similar to that produced by DOX. However, at longer incubation times (48 h) with these drugs, which induced a virtually complete cell death, a late and partial activation of CPP32 was observed (Fig. 5).

3.3. *DOX-induced apoptosis is blocked by peptide inhibitors of caspases*

Since a significant correlation between DOX-induced death and CPP32 activation appeared, we further studied the implication of CPP32 in apoptosis induced by this drug. DOX-induced toxicity was fully inhibited by Z-VAD-fmk, a caspase inhibitor of wide specificity, in all cells tested (Fig. 3). Z-VAD-fmk also prevented Fas-mediated apoptosis in Jurkat and HUT-78 cells. Both DEVD-cho, which efficiently inhibits

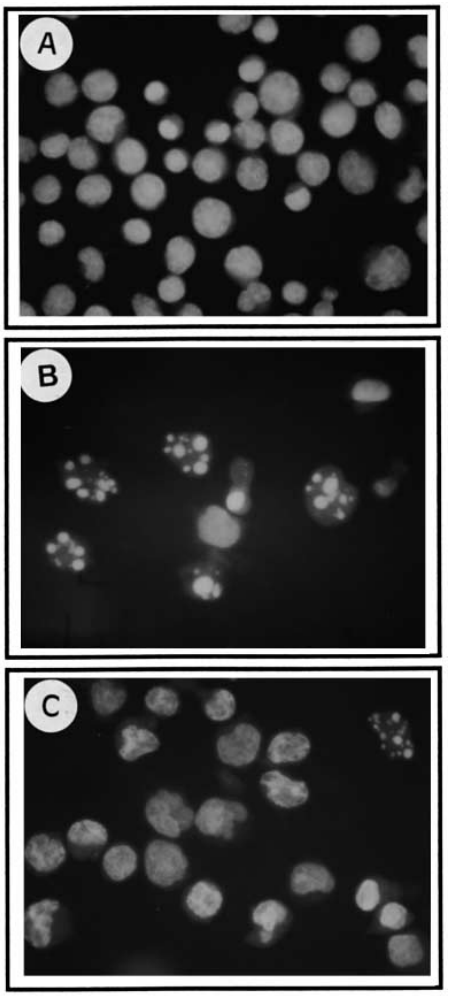

Fig. 6. DEVD-CHO inhibits DOX-induced nuclear apoptosis. Jurkat cells (5×10^5 cells/ml) were treated with 1 μM DOX for 16 h in the presence or absence of 600 μM DEVD-CHO. Cells were stained with PPDA and photographed under fluorescence illumination (magnification, ×280). A: Untreated cells. B: Cells treated with DOX. C: Cells treated with DEVD-CHO and DOX.

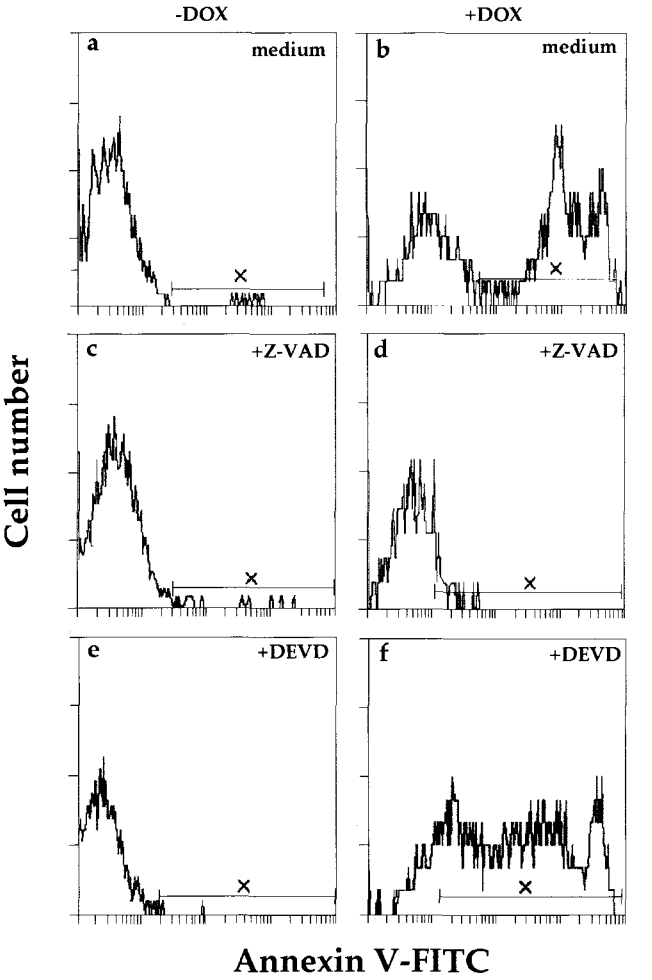

Fig. 7. Z-VAD-fmk, but not DEVD-CHO, inhibits DOX-induced PS exposure. Jurkat cells (5×10^5 cells/ml) were preincubated in complete medium containing: (a, b) DMSO alone; (c, d) 100 μM Z-VAD-fmk; or (e, f) 600 μM DEVD-CHO, and cultured for 16 h in the absence (a, c, e) or presence (b, d, f) of 1 μM DOX. Binding of annexin V-FITC was used as a measure of PS exposure.

CPP32-like protease activity, and Z-VAD-fmk prevented chromatin condensation and nuclear fragmentation induced by DOX (Fig. 6 and data not shown). Another hallmark of apoptosis, the blebbing of the cell membrane, was assessed by light microscopy. DOX treatment induced the appearance of a blebbing morphology in most Jurkat cells (62%). Addition of Z-VAD-fmk restored normal morphology (3% blebbing cells) but a significant proportion of cells treated with DOX in the presence of DEVD-cho still showed the characteristic blebbing phenotype (46% of cells).

The effect of caspase inhibitors on PS translocation induced by DOX was also analyzed by annexin-V-FITC staining (Fig. 7). PS translocation was completely blocked when DOX-treated cells were incubated with Z-VAD-fmk. However, DEVD-CHO did not prevent DOX-induced PS exposure (Fig. 7). These results suggest that CPP32-like proteases mediate DOX-induced nuclear apoptotic events, but other Z-VAD-inhibitable caspase(s) are involved in cell death.

4. Discussion

Doxorubicin and daunorubicin are currently used in the

treatment of acute leukemias, Hodgkin's disease, sarcomas and breast cancer. In spite of their generalized use, the mechanism by which they induce cell death remains poorly defined. DNA intercalation/binding, inhibition of topoisomerase II, free radical generation and damage to cell membranes have been proposed as mechanisms by which they could exert their cytotoxic effects [20]. However, it seems that DNA intercalation is not necessary for the drugs to induce cell death, since *N*-trifluoroacetyldoxorubicin-14-valerate or transferrin-DOX derivatives can induce cell death without association with DNA [21,22]. Recent work from different laboratories [10,23,24] indicates that DOX and daunomycin cause apoptosis in tumor cells. It has also been proposed that DOX-induced apoptosis is mediated by the activation of the Fas/FasL system [10]. Our present results show that, like FasL or agonist anti-Fas antibodies, DOX treatment induces CPP32 activation and apoptosis. However, co-incubation with blocking anti-Fas antibodies fully protected Jurkat cells from anti-Fas- but not from DOX-induced toxicity. This blocking antibody has also been proven to prevent the toxicity induced by soluble FasL secreted by PHA-stimulated Jurkat cells [3]. In addition, MOLT-4 cells, which express membrane Fas but are resistant to Fas-mediated apoptosis [19], were efficiently killed by DOX. In contrast, HUT 78 cells were comparatively insensitive to DOX but were as sensitive as Jurkat cells to Fas-induced apoptosis. Therefore, it can be concluded that DOX-induced apoptosis occurs independently of the activation of the Fas/FasL system since no correlation between sensitivity to Fas and DOX was found.

Inhibition of CPP32 activity with DEVD-cho prevented Fas-induced apoptosis, as already reported [5,25]. However, this peptide only blocked DOX-induced nuclear apoptosis, but not cell death. Both DOX- and Fas-induced apoptosis were blocked by Z-VAD-fmk, which prevents CPP32 activation by inhibiting the activity of FLICE/Mch-5 [9,26], and presumably of other caspases. This suggests that the execution of DOX-induced apoptosis is mediated by several caspases, CPP32 controlling only the formation of apoptotic nuclei. This could be achieved through the direct cleavage and activation of Mch-2 by CPP32 [27]. Mch-2 (caspase-6) [28] specifically cleaves nuclear lamins [29], which are responsible for the maintenance of nuclear shape and are also implicated in the interactions between the chromatin and nuclear matrix [30]. Daunorubicin, a related anthracyclin, and actinomycin D, an inhibitor of transcription, also caused the early activation of CPP32 which correlated with cell death. This was not the case for methotrexate and vincristine, drugs in which a delay between the onset of cell death and CPP32 activation was observed, reflecting probably their different intracellular pathways for apoptosis induction.

We have recently found that Jurkat-derived sublines that have lost membrane Fas expression were resistant to both anti-Fas and DOX-induced apoptosis. In these sublines, the loss of Fas was also accompanied by the loss of CPP32 expression and, presumably, of other Z-VAD-sensitive caspases (Martinez-Lorenzo et al., submitted). A similar phenomenon may have been the cause of the apparent causal link previously observed between Fas and DOX-induced apoptosis [10].

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