Doxorubicin Requires the Sequential Activation of Caspase-2, Protein Kinase C δ , and c-Jun NH₂-terminal Kinase to Induce Apoptosis[□]

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Here, we identified caspase-2, protein kinase C (PKC) δ , and c-Jun NH₂-terminal kinase (JNK) as key components of the doxorubicin-induced apoptotic cascade. Using cells stably transfected with an antisense construct for caspase-2 (AS2) as well as a chemical caspase-2 inhibitor, we demonstrate that caspase-2 is required in doxorubicin-induced apoptosis. We also identified PKC δ as a novel caspase-2 substrate. PKC δ was cleaved/activated in a caspase-2–dependent manner after doxorubicin treatment both in cells and in vitro. PKC δ is furthermore required for efficient doxorubicin-induced apoptosis because its chemical inhibition as well as adenoviral expression of a kinase dead (KD) mutant of PKC δ severely attenuated doxorubicin-induced apoptosis. Furthermore, PKC δ and JNK inhibition show that PKC δ lies upstream of JNK in doxorubicin-induced death. *Jnk*-deficient mouse embryo fibroblasts (MEFs) were highly resistant to doxorubicin compared with wild type (WT), as were WT Jurkat cells treated with SP600125, further supporting the importance of JNK in doxorubicin-induced apoptosis. Chemical inhibitors for PKC δ and JNK do not synergize and do not function in doxorubicin-treated AS2 cells. Caspase-2, PKC δ , and JNK were furthermore implicated in doxorubicin-induced apoptosis of primary acute lymphoblastic leukemia blasts. The data thus support a sequential model involving caspase-2, PKC δ , and JNK signaling in response to doxorubicin, leading to the activation of Bak and execution of apoptosis.

INTRODUCTION

The anthracyclin doxorubicin (DXR) is a major antitumor agent used in the treatment of a variety of human cancers. Its intracellular effects include free radical formation, inhibition of DNA topoisomerase II, and DNA intercalation, resulting in inhibition of DNA replication and strand break-related DNA damage (Gewirtz, 1999). As with many other chemotherapeutic antitumor drugs, the ensuing induction of apoptosis seems to be the main antiproliferative effect. DXR-induced apoptosis typically involves the mitochondrial pathway. More specifically, we found that DXR induces Bak activation before cytochrome *c* (cyt *c*) release from mitochondria and effector caspase activation (Panaretakis *et al.*, 2002b). Accordingly, overexpression of the antiapoptotic Bcl-2 protein blocks DXR-induced Bak activation and apoptosis (Decaudin *et al.*, 1997; Panaretakis *et al.*, 2002b). However, despite its widespread use in the clinic, and the many

types of cellular damage DXR has been shown to cause, the apoptosis-inducing signaling upstream of the mitochondria is poorly characterized (Gewirtz, 1999).

The mitochondrial apoptosis pathway activated by many chemotherapeutic agents commonly involves stress-transducing kinases, culminating at the mitochondria. With the aid of proapoptotic Bcl-2 proteins such as Bak, Bax, and the BH3-only family members, cyt *c* and other apoptogenic factors such as SMAC and Htr2 will be released from the mitochondrial intermembrane space to the cytoplasm (Wei *et al.*, 2001). The release of these factors results in caspase activation (such as caspase-9 and -3) and an increase of their activity through inhibition of the inhibitors of caspases (such as IAPs).

An extra level of complexity was added recently to the stress-induced apoptotic signaling by the demonstration that some DNA-damaging agents cause caspase-2 activation upstream of Bak activation and the ensuing mitochondrial events (Lassus *et al.*, 2002; Robertson *et al.*, 2002). The proapoptotic protein caspase-2L (referred to here as caspase-2) is the prevailing isoform expressed in most tissues (Wang *et al.*, 1994), and it is the only procaspase present constitutively in the nucleus (Zhivotovsky *et al.*, 1999). Caspase-2 thus provides an important link between DNA damage and the engagement of the mitochondrial apoptotic pathway, but the pathways induced after caspase-2 activation and before

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the activation of the mitochondrial proapoptotic protein Bak are poorly understood.

Protein kinase C (PKC) δ is a proapoptotic kinase that has been associated with the response to DNA damage and apoptosis (Reyland *et al.*, 1999; Dal Pra *et al.*, 2000; Godbout *et al.*, 2002). PKC δ is a Ca^{2+} -independent member of a large superfamily of PKC isoforms that differ based on their requirement for lipid cofactors and Ca^{2+} for activation (Jaken, 1996). In some cell types, PKC δ overexpression can even induce apoptosis in the absence of additional stimuli (Emoto *et al.*, 1996; Ghayur *et al.*, 1996; Denning *et al.*, 1998; Reyland *et al.*, 1999). Furthermore, recent studies show that cells derived from PKC δ -null transgenic mice are defective in mitochondria-dependent apoptosis induced by various agents such as tumor necrosis factor- α , UV irradiation, and H_2O_2 (Leitges *et al.*, 2001). Proteolytic activation of PKC δ by downstream effector caspases, resulting in the generation of a 40-kDa active kinase domain, occurs in response to a variety of stimuli, including some DNA-damaging agents (Emoto *et al.*, 1996; Reyland *et al.*, 1999) and FAS ligand stimulation (Mizuno *et al.*, 1997; Frasch *et al.*, 2000). Also, when a construct encoding this 40-kDa active catalytic domain of PKC δ is transiently transfected into cultured cells, it rapidly induces apoptosis (Ghayur *et al.*, 1996; Mizuno *et al.*, 1997; Bharti *et al.*, 1998).

Activation of the stress-activated protein kinase (SAPK) pathways have long been associated with the apoptotic response induced by DXR and other DNA-damaging agents (Kharbanda *et al.*, 1995; Verheij *et al.*, 1996; Testolin *et al.*, 1997). The proapoptotic roles of the activated c-Jun NH₂-terminal kinase (JNK) isoforms are not clearly defined, but the phosphorylation of transcription factors such as c-Jun, ATF2 as well as pro- and antiapoptotic Bcl-2 family members such as Bim and Bcl-2 has been suggested to be of importance (Derijard *et al.*, 1994; Gupta *et al.*, 1995; Yamamoto *et al.*, 1999; Lei and Davis, 2003). Despite that DXR leads to the activation of JNK, the signaling cascade leading to this activation has not been delineated.

In previous studies, we identified the proapoptotic family members Bak and Bax as important mediators of DXR-induced apoptosis. In the present investigation, we have sought to define the key DXR-regulated upstream signaling events as well as examine the possibility whether these upstream signaling events belong to the same pathway or they are part of several pathways activated by DXR. Briefly, our data demonstrate the requirement and sequential action of caspase-2, PKC δ , and JNK for the execution of DXR-induced apoptosis.

MATERIALS AND METHODS

Patients

The study included leukemic cells from three patients diagnosed with pediatric acute lymphoblastic leukemia (ALL). The diagnosis was established according to the World Health Organization classification. All patients were informed of the investigational nature of this study, and informed consent was obtained from each patient in accordance with the local Hospital Clinic Ethical Committee (Karolinska Hospital, Stockholm, Sweden), which also approved the present study. Leukemic blasts from these three patients were isolated by density centrifugation on Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden).

Cell Lines, Culture Conditions, and Treatment

The wild-type (WT) Jurkat T-ALL cell line or Jurkat cells stably transfected with either a neo-vector or procaspase-2 antisense (*Casp-2/AS*) were cultured in RPMI 1640 medium (Invitrogen, Berlin, Germany) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Invitrogen), 2 mM L-glutamine, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 50 $\mu\text{g}/\text{ml}$ penicillin and maintained in a humidified incubator under 5% CO_2 at 37°C. U266, multiple myeloma cells, were cultured in the same conditions as Jurkat cells. Wild-type and *Jnk*- and reconstituted

mouse embryo fibroblasts (MEFs) cells were cultured in similarly supplemented DMEM in a humidified incubator under 5% CO_2 at 37°C. Cells were maintained in a logarithmic growth phase for all experiments.

Cells were treated with 60 ng/ml DXR (Adriamycin; Pharmacia & Upjohn, Stockholm, Sweden) for the indicated time points up to 24 h. The concentration of DXR was based on initial dose-response experiments and chosen to be clinically relevant (Gewirtz, 1999; Panaretakis *et al.*, 2002a).

Adenoviral Vectors and Infection of U266 Cells

Generation of the wild-type and kinase dead recombinant PKC δ adenoviruses has been described previously (Carpenter *et al.*, 2001). The kinase dead mutant K376R has been shown to function as an isoform-specific inhibitory kinase (Li *et al.*, 1995). The infection protocol was a modified version of that described previously (Matassa *et al.*, 2001). Briefly, U266 cells were infected with adenoviral wild-type PKC δ (adPKC δ -WT), dominant negative kinase dead (adPKC δ -KD), and a mock vector (adMXM) for 6 h in RPMI 1640 medium but with 1% serum. Coinfection of an adenoviral vector expressing green fluorescent protein (GFP) was used to select the U266 cells that have been infected. The adenoGFP construct was used at a ratio of 1:2 (1adGFP: 2adPKC δ). After the infection period, the cells were transferred to a larger volume in complete RPMI 1640 medium as described above. U266 cells were incubated for an additional 36 h in the presence or absence of DXR.

Inhibitors and Antibodies

The JNK inhibitor SP600125 (Calbiochem, San Diego, CA) was used at 10 μM . The PKC δ inhibitor rottlerin (Calbiochem) was used at 2 μM . The PKC α/β inhibitor Gö6976 (Sigma, St. Louis, MO) was used at 10 nM. The pancaspase inhibitor z-VAD-FMK (25 μM), the caspase-3 inhibitor z-DEVD-FMK (5 μM), and the caspase-2 inhibitor (z-VDVAD-FMK) (10 μM) were purchased from MP Biomedicals (Irvine, CA).

For analysis of Bak and Bax activation, we used antibodies that specifically recognize the active conformation of Bak (AM03, clone TC100; Calbiochem) and Bax (clone 6A7; BD Biosciences Pharmingen, San Diego, CA) as described previously (Panaretakis *et al.*, 2002a). The antibody against PKC δ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and against phospho-JNK (pTyr183/185) and phosphor-c-Jun (Ser63) from Cell Signaling Technology (Beverly, MA).

Assessment of Apoptosis

Redistribution of plasma membrane phosphatidyl serine (PS) is a marker of apoptosis and was assessed using Annexin V FLUOS (Roch Diagnostics, Mannheim, Germany) according to the manufacturer's protocol and as described previously (Panaretakis *et al.*, 2002a). The subsequent analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using the CellQuest Pro software (BD Biosciences).

Reduction in mitochondrial inner membrane potential, $\Delta\psi\text{m}$, is a typical feature of apoptotic cells. To detect DXR-induced changes in $\Delta\psi\text{m}$, cells were stained with tetramethylrhodamine ethyl ester perchlorate (Molecular Probes, Eugene, OR), and the assay was performed as described previously (Panaretakis *et al.*, 2002a).

In Vitro Caspase-2 Assay

Caspase activity was measured by cleavage of the caspase-2 substrate AcVDVAD-AMC (Peptide Institute, Osaka, Japan) in a fluorometric assay as described previously (Garcia-Calvo *et al.*, 1999). Briefly, aliquots containing 1×10^6 cells were washed once with ice-cold phosphate-buffered saline (PBS), pelleted, resuspended in 25 μl of PBS, and then transferred to a 96-well plate. Fifty microliters of freshly prepared substrate buffer (100 mM 2-(N-morpholino)ethanesulfonic acid [MES], 10% polyethylene glycol [PEG], 0.1% CHAPS, and 10 mM dithiothreitol [DTT], pH 6.5) containing the respective substrates were added per well. Cleavage of the fluorogenic peptide substrate was monitored by 7-amino-4-methylcoumarin liberation in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden). Experiments were performed in duplicates, and the activity was expressed as change in fluorescence units.

Western Blot Analysis

For Western blots, total cell extracts were prepared by direct lysis in a hot Laemmli buffer. Samples corresponding to 1×10^5 cells per well were separated on 10 or 12% SDS-PAGE followed by electroblotting to polyvinylidene difluoride-membranes (Roche Diagnostics) by semidry transfer. The filters were then probed with the appropriate primary antibody in 5% milk in PBS or Tris-buffered saline and 0.2% Tween 20 for 1 h at room temperature or overnight at 4°C and thereafter with a secondary antibody for 1 h. Protein bands were visualized using a SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL) according to the manufacturer's protocol. The images were captured using a LAS-I 000 from Fujifilm (Tokyo, Japan).

In Vitro Cleavage of PKC δ by Caspase-2

Bacterial expression plasmids containing active or inactive (mutant) recombinant caspase-2 were obtained from Professor Emad Alnemri (Kimmell

Figure 1. DXR requires caspase-2 to induce apoptosis in Jurkat cells. WT and AS2 Jurkat cells were cultured in the presence or absence of 60 ng/ml DXR for 24 h, and the relative activity of caspase-2 (A) toward a synthetic substrate was measured as described under *Materials and Methods*. D60, Jurkat cells treated with 60 ng/ml DXR. The bars represent the mean value of three independent experiments, and the error bars represent SD. Jurkat WT (D, i) and AS2 (D, ii) cells were treated with 60 ng/ml DXR for 24 h in the presence or absence of zVDVAD. Caspase-2 activity was measured (B) as well as two apoptotic markers: Annexin V positivity (C), D60, Jurkat cells treated with 60 ng/ml DXR for the indicated time; and Bak activation (D). (i) Gray line, WT, control cells; black line, WT, DXR-treated cells; dotted line, WT, zVDVAD-treated cells; and dash and dot line, WT, DXR + zVDVAD-treated cells; (ii) gray line, AS2, control cells; black line, AS2, DXR-treated cells; dotted line, AS2, zVDVAD-treated cells; and dash and dot line, AS2, DXR + zVDVAD-treated cells. The bars represent the mean value of three independent experiments, and the error bars represent SD. The flow cytometry histograms are representative of three independent experiments. After treatment with 60 ng/ml DXR for 24 h, WT and AS2 Jurkat cells were stained for Annexin V positivity and the activated form of Bak. Annexin V positivity (E, i) and Bak (E, ii) activation were analyzed by flow cytometry. (i) Dash and dot line, WT, control cells; black line, WT, DXR-treated cells; dotted line, AS2, control cells; and gray line, AS2, DXR-treated cells; (ii) gray line, WT, control cells; dash and dot line, WT, DXR-treated cells; dotted line, AS2, control cells; and black line, AS2, DXR-treated cells. The results are representative of three independent experiments.

Cancer Center, Thomas Jefferson University, Philadelphia, PA) and were overexpressed in the *Escherichia coli* strain BL21 (DE3) as C- or N-terminal His6-tagged proteins by using pET-21a or pET-28a vector (Novagen, Madison, WI) and purified by standard Ni²⁺-affinity chromatography. Recombinant PKC δ protein was purchased from Upstate Biotechnology (Charlottesville,

VA). In total, 100 ng of PKC δ was incubated with 10 ng and 20 ng of caspase-2 in a final volume of 50 μ l of assay buffer (1 mM MES, pH 6.5; 10% PEG, 0.1% CHAPS, and 10 mM DTT) for 2 h at 37°C. The reaction was terminated by the addition of Laemmli buffer, and proteins were analyzed by SDS-PAGE and immunoblotting, as described above.

Figure 2. Caspase-2 is required for protein kinase C δ cleavage in vitro and in vivo in Jurkat cells. (A) Immunoblot analysis of protein kinase C δ cleavage after 6, 16, and 24 h of treatment with DXR in Jurkat WT and AS2 cells. C, control; D60, 60 ng/ml DXR-treated cells; protein kinase C δ -FL, protein kinase C δ full length; and protein kinase C δ -CL, protein kinase C δ cleaved. Tubulin was used as loading control. The results shown are representative of three independent experiments. (B) Immunoblot analysis of protein kinase C δ cleavage by recombinant caspase-2 after 2 h at 37°C. The lanes were loaded with the following: Ctrl, untreated WT Jurkat cells; D60, WT Jurkat cells treated with DXR 60 ng/ml for 20 h; protein kinase C δ , recombinant protein kinase C δ alone; protein kinase C δ + WTC2, protein kinase C δ was incubated with 10 or 20 ng of WT recombinant caspase-2; and protein kinase C δ + mtC2, protein kinase C δ was incubated with mutant caspase-2. The results shown are representative of three independent experiments.

Immunostaining

Cells were cytospun onto glass slides, fixed in 4% paraformaldehyde for 20 min, and permeabilized using digitonin in PBS for 10 min. Stainings with the antibodies against cyt c (clone 6H2.B4; BD Biosciences Pharmingen) and PKC δ (Santa Cruz Biotechnology) were performed for 1 h at room temperature, followed by incubation with a secondary fluorescein isothiocyanate-conjugated antibody (anti-rabbit for PKC δ) and Texas Red-conjugated antibody (horse antimouse for cytochrome c). Slides were mounted using Vectashield with 4,6-diamidino-2-phenylindole for the staining of nuclei (Vector Laboratories, Burlingame, CA) before viewed with a Zeiss Axioplan 2 imaging microscope.

RESULTS

DXR Requires Caspase-2 to Induce Apoptosis in Jurkat Cells

It has been shown previously that caspase-2 is an initiator caspase for some types of DNA-damaging agents. Its role in DXR-induced apoptosis has not been investigated.

The effects of DXR treatment on caspase-2 activity were investigated in Jurkat cells. Already after 6 h of culture, there

Figure 3. DXR induces protein kinase C δ cleavage mainly through caspase-2. Immunoblot analysis of DXR-induced protein kinase C δ cleavage in the presence or absence of caspase-2 (zVDVAD) and caspase-3 (zDEVD) inhibitor after 24 h of treatment. The lanes were loaded with the following: Ctrl, untreated WT Jurkat cells; D60, WT Jurkat cells treated with 60 ng/ml DXR; D60 + VDVAD, WT Jurkat cells pretreated with VDVAD followed by DXR 60 ng/ml; D60 + DEVD, WT Jurkat cells pretreated with DEVD followed by DXR 60 ng/ml; VDVAD, WT Jurkat cells treated with VDVAD; and DEVD, WT Jurkat cells treated with DEVD. Tubulin was used as loading control. The results shown are representative of three independent experiments.

was a slight induction of caspase-2 activity, which increased with time (Figure 1A). To confirm the involvement of caspase-2, a specific caspase-2 inhibitor, zVDVAD, was used. Jurkat cells were pretreated for 1 h with 10 μ M zVDVAD and then cotreated with 60 ng/ml DXR for up to 24 h. As expected, zVDVAD treatment effectively blocked caspase-2 activity at all time points examined (Figure 1B). Pretreatment with the caspase-2 inhibitor blocked approximately half of the DXR-induced increase in Annexin V-positive cells (Figure 1C). Similarly, DXR-induced Bak activation was blocked to a similar extent by zVDVAD (Figure 1D, i).

To further strengthen the notion that caspase-2 activation is important in DXR-induced apoptosis, we used Jurkat cells stably transfected with an antisense construct for caspase-2 (denoted as AS2 cells). Although these cells are not void of caspase-2 activity (Robertson *et al.*, 2002), the effect of DXR on caspase-2 activation was severely attenuated (Figure 1A). Fully in line with the data obtained using chemical caspase-2 inhibition, AS2 cells demonstrated a significant resistance to DXR treatment, as demonstrated by Annexin V and Bak stainings (Figure 1E, i and ii, respectively). Furthermore, the chemical caspase-2 inhibitor, as expected, had no effect on the AS2 Jurkat cells (Figure 1, C and D, ii).

DXR-induced Apoptosis Requires PKC δ

Although caspase-2 has an established role in some types of apoptosis, the key downstream substrates are unclear. One of the recently identified stress-activated kinases is the PKC family member PKC δ , which also has been shown to be a caspase-3 substrate. To determine whether PKC δ is cleaved/activated in a caspase-2-dependent manner, protein lysates were prepared from WT and AS2 Jurkat cells treated with DXR for 6, 16, and 24 h. After Western blotting, the filter was probed with an anti-PKC δ antibody and both the full-length

Figure 4. Rottlerin partly blocks Annexin V positivity and Bak activation in DXR-treated WT Jurkat and U266 cells. Jurkat WT (i) and AS2 (ii) cells were pretreated with rottlerin for 1 h followed by addition of 60 ng/ml DXR (D60) for 24 h and then analyzed for Annexin V positivity (A) and Bak activation (C) by flow cytometry: (i) gray line, WT, control cells; black line, WT, DXR-treated cells; dotted line, WT, rottlerin-treated cells; and dash and dot line, WT, DXR + rottlerin-treated cells; (ii) gray line, AS2, control cells; black line, AS2, DXR-treated cells; dotted line, AS2, rottlerin-treated cells; and dash and dot line, AS2, DXR + rottlerin-treated cells. U266 cells were pretreated with rottlerin for 1 h followed by addition of 60 ng/ml DXR (D60) for 48 h and then analyzed for Annexin V positivity (D) by flow cytometry. WT Jurkat cells were pretreated with either rottlerin or Gö6976 for 1 h followed by treatment with 60 ng/ml DXR for 24 h and then analyzed for Annexin V positivity (B) by flow cytometry. The bars represent the mean value of three independent experiments, and the error bars represent SD. The flow cytometry histograms are representative of three independent experiments.

and the cleaved fragments were thus visualized. Considerable cleavage of the full-length PKC δ to its active form could be clearly observed after 16 h and was further enhanced by 24 h of DXR treatment of WT Jurkat cells. This active fragment was absent after cotreatment of WT Jurkat cells with DXR and the caspase-2 inhibitor zVDVAD and clearly decreased in the AS2 cells, indicating that caspase-2 activity is involved in PKC δ cleavage (Figure 2A).

To determine whether PKC δ is a substrate for caspase-2, we performed an *in vitro* cleavage assay. Recombinant PKC δ was incubated with either wild-type or mutant recombinant caspase-2, and the samples were analyzed by Western blotting. PKC δ was cleaved by caspase-2 *in vitro* (Figure 2B). The cleaved fragment corresponded to the 40-kDa active PKC δ , suggesting that caspase-2 is generating a PKC δ -cleaved fragment similar to that cleaved by caspase-3.

It has been published previously that caspase-3 cleaves PKC δ both *in vitro* and in cell lines. To exclude the possibility that caspase-3 and not caspase-2 is responsible for the observed PKC δ cleavage that we observe in Jurkat cells, we pretreated WT Jurkat cells with either zDEVD (5 μ M) or

zVDVAD (10 μ M) and analyzed for DXR-induced PKC δ cleavage after 24 h of treatment (Figure 3). The caspase-3 inhibitor blocks partially the DXR-induced PKC δ cleavage, whereas the caspase-2 inhibitor blocks it completely. However, the caspase-3 inhibitor did block the 20-fold DXR-induced caspase-3 activity (data not shown). These data suggest that caspase-2 is primarily responsible for the PKC δ cleavage induced by DXR in Jurkat cells without excluding the possibility that caspase-3 also cleaves PKC δ as part of a second wave later in the apoptosis process.

To evaluate the functional significance of caspase-2-mediated PKC δ cleavage, we examined whether inhibition of PKC δ activity has any effect on the apoptosis induced by DXR. WT and AS2 Jurkat cells as well as U266 cells were pretreated with 2 μ M rottlerin (PKC δ inhibitor) 1 h before the addition of DXR. Similar to caspase-2 inhibition, rottlerin was able to block approximately half of the Annexin V positivity as well as Bak activation induced by DXR in WT Jurkat cells (Figure 4, A and C, i, respectively), whereas rottlerin had little effect on Annexin V positivity or Bak activation in DXR-treated AS2 cells (Figure 4, A and C, ii,

Figure 5. Effect of the kinase dead protein kinase C δ mutant on DXR-induced apoptosis. U266 cells were coinfectd with adenoGFP and adPKC δ -WT, adPKC δ -KD, or adMXM, and the protein kinase C δ protein expression (A) as well as the effect of these mutant on DXR-induced mitochondrial depolarization as a measurement of cell death (B) was analyzed; D60, U266 cells treated with 60 ng/ml DXR for the indicated time. The immunoblot is a representative result of three independent experiments; protein kinase C δ -FL, protein kinase C δ full length. The bars represent the mean value of two independent experiments, and the error bars represent SD.

respectively). In U266 cells, PKC δ inhibition by rottlerin blocked more than half of the DXR-induced apoptosis (Figure 4D). To verify that PKC δ and not other PKC isoforms such as PKC α/β are involved in the execution of apoptosis initiated by DXR, we compared the inhibitory effect of rottlerin to that of Gö6976, a specific inhibitor of typical PKCs, i.e., PKC α and PKC β . The 10 nM Gö6976 concentration that we have used has been demonstrated to not inhibit PKC δ or any of the other atypical PKC family members (Martiny-Baron *et al.*, 1993). Inhibition of these typical PKCs with 10 nM Gö6976 even enhanced DXR-induced Annexin V positivity, suggesting that the other PKCs do not play a role in the execution of DXR-induced apoptosis (Figure 4B).

Because chemical inhibitors also may exert nonspecific effects, we decided to further confirm the involvement of PKC δ by using an adenoviral construct that expresses a dominant inhibitory form of PKC δ (adPKC δ -KD). Due to the extremely low infectivity of the Jurkat cells used, we used the U266 cell line that we demonstrated behaves in a

Figure 6. Effects of combination of caspase-2 and protein kinase C δ inhibitors on DXR-treated Jurkat cells. WT and AS2 Jurkat cells were treated with 60 ng/ml DXR (D60) for 24 h in the presence or absence of 2 μ M rottlerin and 10 μ M zVADVAD. The effect of the inhibitors was assessed as alterations in Annexin V positivity. The bars represent the mean value of three independent experiments, and the error bars represent SD.

similar manner in response to DXR. U266 cells were infected for 6 h with adenoviruses encoding wild-type PKC δ (adPKC δ -WT), dominant negative kinase dead PKC δ (adPKC δ -KD), or a mock vector (adMXM), and expression of PKC δ protein was analyzed by immunoblotting (Figure 5A). AdPKC δ -WT and adPKC δ -KD were abundantly expressed in U266 cells infected with these constructs compared with the cells infected with either adGFP or with adMXM alone, which expressed endogenous levels of PKC δ . AdPKC δ -WT-, adPKC δ -KD-, and adMXM-infected U266 cells were coinfectd with adGFP, and the GFP-positive cell population was assayed for mitochondrial depolarization as a marker of DXR-induced cell death. We have previously demonstrated that the amount of mitochondrial depolarization fully correlates with apoptotic cell death as measured by other markers such as caspase-3, Annexin V, and cyt c release (Panaretakis *et al.*, 2002b). In line with the effect induced by rottlerin, the U266 cells infected with the adPKC δ -KD demonstrated a decrease in DXR-induced mitochondrial depolarization and apoptosis compared with mock virus-infected cells (Figure 5B).

To further assess the presence of caspase-2 and PKC δ in the same pathway, we analyzed Annexin V positivity levels after pretreatment of WT Jurkat cells with rottlerin in combination with zVADVAD 1 h before the addition of DXR. The combination of rottlerin and zVADVAD failed to block DXR-induced Annexin V positivity any further than the inhibitors independently (Figure 6). The above-mentioned data suggest that caspase-2 and PKC δ are in the same pathway and that caspase-2 acts upstream to cleave/activate PKC δ .

DXR-induced Apoptosis Requires JNK

It has been demonstrated previously that DXR activates JNK, although the functional significance of this has not been determined. To investigate the involvement of JNK in DXR-induced apoptosis, a specific inhibitor of JNK, SP600125, was used. Jurkat WT and AS2 cells were pretreated with 10 μ M SP600125 for 1 h, followed by the addition of 60 ng/ml DXR for up to 24 h. SP600125 was found to largely block DXR-induced apoptosis in WT Jurkat cells,

Figure 7. SP600125 partly blocks DXR-induced apoptosis. WT and AS2 Jurkat cells were treated with 60 ng/ml DXR for 24 h in the presence or absence of SP600125. The inhibition in DXR-induced apoptosis was assessed by flow cytometry as changes in Annexin V positivity (A) and Bak activation (B, i and ii). (i) Gray line, WT, control cells; black line, WT, DXR-treated cells; dotted line, WT, SP600125-treated cells; dash and dot line, WT, DXR+SP600125-treated cells; (ii) gray line, AS2, control cells; black line, AS2, DXR-treated cells; dotted line, AS2, SP600125-treated cells; and dash and dot line, AS2, DXR + SP600125-treated cells. (C) WT and jnk1/jnk2 null- and jnk1-reconstituted MEF cells were cultured in the presence or absence of DXR 60 ng/ml (D60) for 48 h. The alterations in apoptosis were assessed as changes in Annexin V positivity. The bars represent the mean value of three independent experiments, and the error bars represent SD. The flow cytometry histograms are representative of three independent experiments. (D) WT and AS2 Jurkat cells were treated with DXR 60 ng/ml (D60) for 24 h in the presence or absence of SP600125, and the activity of caspase-2 was measured. The bars represent the mean value of three independent experiments, and the error bars represent SD.

both as Annexin V positivity as well as Bak activation (Figure 7, A and B, i, respectively). In contrast, SP600125 did not have any effect on Annexin V positivity and Bak activation in AS2 Jurkat cells treated with DXR (Figure 7, A and B, ii, respectively). The importance of JNK in DXR-induced apoptosis was further demonstrated with the resistance to DXR of MEFs lacking both jnk1 and jnk2 (MEFs DKO) compared with wild-type and jnk1 reconstituted MEFs (Figure 7C).

To determine the relation between JNK-activation and caspase-2 in this system, we also analyzed the effect of the JNK inhibitor on DXR-induced caspase-2 activity. SP600125 was found to have only a minor inhibitory effect on caspase-2 activity, suggesting that JNK is not situated upstream of caspase-2 in the DXR-induced signaling cascade (Figure 7D).

To establish the relationship between JNK and PKC δ cleavage/activation, we investigated the effect of SP600125 on PKC δ cleavage in DXR-treated WT Jurkat cells. SP600125

exerted no inhibitory effect on PKC δ cleavage (Figure 12). As expected, in DXR-treated AS2 cells there was little PKC δ cleavage, which was not affected by the addition of SP600125 (Figure 8).

To further confirm that caspase-2, PKC δ , and JNK are activated upstream of the mitochondria, we analyzed the effect of their inhibitors on the DXR-induced cyt c release. WT Jurkat cells were treated with either zVDVAD, rottlerin, or SP600125 followed by DXR for 24 h, and immunostainings for cyt c were made. Thereafter, the number of cells that have released cyt c was counted (Figure 9). The data lend further support to the notion that caspase-2, PKC δ , and JNK are active upstream of the mitochondria, because their inhibition leads to the significant decrease of the DXR-induced cyt c release. Fully in line with previous studies, the percentage of cells that have released cyt c from the mitochondria correlated very well with the percentage of cells that

Figure 8. SP600125 does not block DXR-induced protein kinase C δ cleavage. Immunoblot analysis of protein kinase C δ cleavage in Jurkat WT and AS2 cells treated with DXR for 20 h in the presence or absence of SP600125. C, control; D60, 60 ng/ml DXR-treated cells; protein kinase C δ -FL, protein kinase C δ full length; and protein kinase C δ -CL, protein kinase C δ cleaved. Tubulin was used as loading control. The results shown are representative of three independent experiments.

have activated Bak, making Bak activation a reliable measurement of events occurring upstream of the mitochondria.

We then examined whether rottlerin and zVDVAD have any effect on the activation/phosphorylation of JNK. WT-Jurkat cells were pretreated with rottlerin and zVDVAD, respectively, followed by DXR for 24 h. DXR induces phospho-JNK activation as demonstrated by Western blotting, and this activation was largely inhibited by the caspase-2 and PKC δ inhibitors (Figure 10A). We further confirmed that PKC δ is situated upstream of JNK activation by infecting U266 cells with a kinase dead PKC δ construct and examined the levels of c-Jun phosphorylation, a known downstream target of JNK. U266 cells were infected for 6 h with adenoviruses encoding wild-type PKC δ (adPKC δ -WT), dominant negative kinase dead PKC δ (adPKC δ -KD), or a mock vector (adMXM), and expression of PKC δ protein was analyzed by immunoblotting (Figure 10B, i). In line with the inhibitory effect induced by rottlerin, the U266 cells infected

Figure 9. Effect of caspase-2, protein kinase C δ , and JNK inhibitors on DXR-induced cyt c release. WT Jurkat cells were pretreated with zVDVAD, rottlerin, or SP600125 for 1 h followed by 60 ng/ml DXR for 24 h. Cyt c release was quantified by counting 500 cells from each slide. The bars represent the mean value of two independent experiments, and the error bars represent SD.

with the adPKC δ -KD demonstrated a large decrease in DXR-induced phosphorylation of c-Jun compared with mock virus-infected cells (Figure 10B, ii).

WT-Jurkat cells also were pretreated with rottlerin and zVDVAD, respectively, followed by DXR for 4, 8, 12, and 16 h, and the levels of JNK activation were determined by flow cytometry (Figure 10C). JNK is activated already after 2 h, and this induction is sustained and increased after 8 h up to 16 h. JNK activation by DXR is blocked by rottlerin and zVDVAD at all time points, further demonstrating that caspase-2 and PKC δ are operating upstream of JNK. In contrast to the phosphorylated form of JNK, total protein levels of JNK1/2 were not altered by DXR (Figure 10D).

To further analyze whether these enzymes lie in the same pathway, the effects of combinations of caspase-2, PKC δ , and JNK inhibitors were examined. Combining SP600125 and zVDVAD or SP600125 and rottlerin had only a minor additive effect on inhibition of apoptosis induced by DXR compared with SP600125 only (Figure 11, A and B).

DXR is one of the main treatment modalities against ALL. To examine the clinical relevance of our findings, we pretreated leukemic blasts derived from ALL patients with rottlerin, zVDVAD, or SP600125 to analyze whether these chemical inhibitors were able to inhibit DXR-induced apoptosis *ex vivo* after exposure for 24 h (Figure 12). In this small group of patients, all the inhibitors were able to block DXR-induced apoptosis to some degree with varying potencies.

DISCUSSION

Although DXR is a backbone agent in the treatment of a large number of common malignant diseases, the molecular mechanisms underlying its cytotoxic effects remain largely unknown, especially so the critical signaling from the initial cellular insults to the engagement of the mitochondrial apoptosis pathway. Furthermore, differences within this mediating signaling most probably explain much of the cellular variability in the response to DXR. Herein, we have therefore explored the intracellular pathways in DXR-induced proapoptotic events acting upstream of the mitochondria.

Our results show that DXR activates caspase-2 upstream of Bak activation and the execution of apoptosis in Jurkat cells and that caspase-2 is a critical component of the DXR-induced apoptotic machinery. These data parallel the involvement of caspase-2 as an initiating caspase in the proapoptotic action of some other DNA-damaging agents such as etoposide (Robertson *et al.*, 2002). The participation of caspase-2 in DXR-induced apoptosis varies from cell line to cell line. For example, in U266 cells, DXR does not require caspase-2 to induce its proapoptotic effects (our unpublished data). Others also have shown the cell-context specific nature of caspase-2 requirement for genotoxic stress-induced apoptotic effects (Lassus *et al.*, 2002).

Bid is one of the known substrates of caspase-2 in the proapoptotic signaling induced by some chemotherapeutic agents (Lassus *et al.*, 2002; Robertson *et al.*, 2002). In fact, ectopically expressed caspase-2 could trigger the translocation of Bid to mitochondria and release of cyt c (Paroni *et al.*, 2001). The evidence mentioned above indicates one way by which caspase-2 could activate the effector caspases is through cleavage of Bid. The participation of Bid in our system was examined in a previous study from our laboratory by investigating the sensitivity of bid^{-/-} cells to doxorubicin (Panaretakis *et al.*, 2002b). The data clearly showed that bid did not have a regulatory role in DXR-induced apoptosis, and therefore it is unlikely that caspase-2 is transducing the DXR-mediated apoptotic signals via Bid cleav-

Figure 10. Effect of rottlerin and zVDVAD on DXR-induced JNK activation. (A) Immunoblot analysis of JNK phosphorylation in Jurkat WT pretreated with VDVAD or rottlerin for 1 h followed by the addition of DXR for 24 h. WT-C, control; WT-D60, 60 ng/ml DXR-treated cells; WT-D60 + VDVAD, WT Jurkat cells pretreated with VDVAD and 60 ng/ml DXR; WT-D60 + rottlerin, WT Jurkat cells pretreated with rottlerin and 60 ng/ml DXR; VDVAD, WT Jurkat cells treated with VDVAD; and rottlerin, WT Jurkat cells treated with rottlerin. Tubulin was used as loading control. The results shown are representative of two independent experiments. (B) U266 cells were infected with adPKC δ -WT, adPKC δ -KD, and adMXM, and the protein kinase C δ protein expression (i) as well as the effect of these mutant on DXR-induced phosphorylation of c-Jun (ii) was analyzed. D60, U266 cells treated with 60 ng/ml DXR for the indicated time. The immunoblot is a representative result of two independent experiments; protein kinase C δ -FL, protein kinase C δ full length. (C) WT-Jurkat cells were treated with 60 ng/ml DXR (D60) for the indicated time points in the presence or absence of rottlerin or zVDVAD, and the phospho-JNK levels were assessed by flow cytometry. The bars represent the mean value of two independent experiments, and the error bars represent SD. (D) Total protein levels of JNK were assessed by Western blotting in WT-Jurkat cells treated with 60 ng/ml DXR for the indicated time points. The result shown is representative of two independent experiments.

age. In this study, we suggest that an alternative pathway exists, namely via the caspase-2-dependent cleavage and activation of PKC δ to lead to the activation of Bak, cyt c release, and effector caspase-activation.

We initially assumed that the partial nature of the resistance to DXR in the AS2 Jurkat cells is due to incomplete down-regulation of caspase-2 protein levels as demonstrated in previous investigations by using these cells (Robertson *et al.*, 2002). However, the very efficient chemical inhibition of caspase-2 activity by zVDVAD in WT Jurkat cells had a similar incomplete effect. Moreover, chemical inhibitors of the other two enzymes involved, JNK and PKC δ , led to a similar incomplete inhibition of DXR-induced apoptosis, suggesting that several proapoptotic pathways are induced simultaneously by DXR, which would be in line with the multitarget nature of this drug (Gewirtz, 1999).

In this study, we have discovered another major downstream target for caspase-2, namely, PKC δ . Its cleavage is attenuated in the antisense caspase-2 Jurkat cells and is completely blocked by the caspase-2 inhibitor zVDVAD. Furthermore, caspase-2 was found to directly cleave PKC δ in vitro, in a similar manner to caspase-3 (Ghayur *et al.*, 1996). PKC δ is a known caspase-3 substrate and in this study, we demonstrate for the first time that PKC δ also is cleaved by caspase-2. The optimal recognition motif for the group II caspases (caspase-2, caspase-3, and caspase-7) is DEXD (Mancini *et al.*, 2000). In fact, it has been shown before that caspase-2 and caspase-3 have similar substrates spec-

ificities. α II Spectrin and Golgin are two proteins that are cleaved on the same site by caspase-2 and caspase-3 (Mancini *et al.*, 2000; Rotter *et al.*, 2004).

We have demonstrated previously that DXR is a potent inducer of apoptosis as well as Bak activation in U266 cells (Panaretakis *et al.*, 2002b). Furthermore, inhibition of PKC δ or JNK in U266 cells had similar effects compared with Jurkat cells. The similarities between Jurkat and U266 cells allowed us to use U266 for the adenoviral experiments because the Jurkat cells used in this study were not possible to infect.

Inhibition of PKC δ activity either by chemical inhibition or by using a kinase dead mutant furthermore resulted in a considerable resistance to DXR-induced cell death. These data indicate that PKC δ is an important regulator in the execution of the DXR-induced apoptotic program.

The mechanism of activation of PKC δ by various proapoptotic and antiapoptotic stimuli involves a series of post-translational modifications (phosphorylations and cleavage) (for reviews on PKC δ , see Basu, 2003; Jackson and Foster, 2004). Although distinct tyrosine and serine/threonine phosphorylations can lead to either an anti- or proapoptotic response, cleavage of PKC δ to its 40-kDa, fully active catalytic fragment is the only definite modification that leads to a proapoptotic response as demonstrated by Emoto *et al.* (1996). Data supporting this hypothesis come mainly from experiments in which overexpression of this fragment by itself induces chromatin condensation and DNA fragmentation (Ghayur *et al.*, 1996).

Figure 11. Effects of combination of SP600125-zVDVAD and SP600125-rotterlin on DXR-induced apoptosis. WT and AS2 Jurkat cells were treated with 60 ng/ml DXR (D60) for 24 h in the presence or absence of SP600125 + rotterlin (A) and SP600125 + zVDVAD (B). Changes in DXR-induced apoptosis were assessed by flow cytometry as alterations in Annexin V positivity. The bars represent the mean value of three independent experiments, and the error bars represent SD.

The previous proposition that caspase-3 is the only caspase that could cleave PKC δ posed a problem as to how such a downstream effector caspase could activate an upstream regulator of apoptosis. Furthermore, expression of a kinase dead mutant inhibited both caspase-9 and caspase-3 activation, further suggesting that an alternate, upstream caspase could be responsible for the cleavage of PKC δ (Matassa *et al.*, 2001). In this study, we demonstrate that the initiator caspase-2 has the ability to cleave, and therefore activate, PKC δ .

The downstream proapoptotic targets of PKC δ include c-Abl, Lamin B, and lipid scramblase and PKC δ activation causes disassembly of nuclear lamins and inhibition of DNA-PK expression (Bharti *et al.*, 1998; Cross *et al.*, 2000; Frasnch *et al.*, 2000; Godbout *et al.*, 2002). Another putative downstream target in response to DNA-damage is JNK (Yoshida *et al.*, 2002).

We found that JNK is activated in response to DXR as was described for cells exposed to various DNA-damaging agents (Kharbanda *et al.*, 2000; Yoshida *et al.*, 2002; Besirli and Johnson, 2003). However, the mechanism of activation of either JNK directly or of the SAPK cascade is poorly described. The fact that DXR-induced apoptosis and Bak activation are severely attenuated in cells treated with the

Figure 12. Effects of rotterlin, zVDVAD and SP600125 in DXR-treated patient samples. Leukemic blasts from three different ALL patients were pretreated for 1 h with rotterlin, zVDVAD, and SP600125 followed by 100 ng/ml DXR (D100) for 24 h. The inhibition in DXR-induced apoptosis was assessed by flow cytometry as changes in Annexin V positivity.

JNK inhibitor SP600125 supports a key role of JNK activation also in DXR-induced apoptosis. jnk1 and jnk2 DKO MEFs were almost completely resistant to DXR-induced apoptosis, further emphasizing the importance of JNK in this respect. The relationship between JNK activation and the other proapoptotic enzymes analyzed in this study was evaluated. The fact that there is no clear effect of the JNK inhibitor SP600125 in AS2 cells, or cells cotreated with inhibitors to caspase-2 or PKC δ , strongly indicates that JNK acts in the same pathway. Furthermore, JNK inhibition had no effect on DXR-induced PKC δ cleavage or phosphorylation, whereas both rotterlin and zVDVAD inhibit the DXR-induced activating phosphorylation of JNK. In addition, the DXR-induced phosphorylation of c-Jun, a known downstream target of JNK was partially blocked by the dominant negative form of PKC δ , further demonstrating that protein kinase C δ is upstream of JNK phosphorylation and activation. These data further indicate that JNK is important at a step downstream of caspase-2 and protein kinase C δ . It is not known whether protein kinase C δ directly activates JNK by phosphorylation or by activating the SAPK cascade. This needs to be established in future studies.

A minor decrease in caspase-2 activity was noted, however, after SP600125 treatment. One likely explanation for this could be that the amplification of initiator caspases by effector caspases, such as caspase-3, is diminished by JNK inhibition.

Primary ALL samples were used to confirm the clinical importance of protein kinase C δ , caspase-2, and JNK in the execution of DXR-induced apoptosis. It is conceivable that these proteins are required *in vivo* for DXR to exert its antineoplastic effects, and deactivating mutations in these proteins may reflect one of the mechanisms of resistance against this drug.

In summary, the present investigation proposes a model for DXR-induced cytotoxicity, where a significant part of the apoptosis-inducing capacity of this drug is exerted by a sequential activation of caspase-2, protein kinase C δ , and JNK, leading to Bak activation and apoptosis. This characterization of the molecular background to DXR-induced ap-

apoptosis will ultimately lead to an optimized clinical use of this important chemotherapeutic compound in terms of selection of sensitive patients, combination with other drugs, and overcoming resistance as well as greater understanding of the major apoptosis pathways.

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