In Lymphatic Cells Par-4 Sensitizes to Apoptosis by Down-Regulating Bcl-2 and Promoting Disruption of Mitochondrial Membrane Potential and Caspase Activation

Simone Bohrer, Kai U. Chow, Florian Beske, Natasa Kukoc-Zivojnov, Elena Puccetti, Martin Ruthardt, Christine Baum, Vivek M. Rangnekar, Dieter Hoelzer, Paris S. Mitrou, Eckhart Weidmann

Department of Medicine III, Johann Wolfgang Goethe-University Hospital, 60590 Frankfurt am Main, Germany [S. B., K. U. C., F. B., N. K.Z., E. P., M. R., C. B., D. H., P. S. M., E. W.], and Department of Radiation Medicine, University of Kentucky, Lexington, Kentucky 40536 [V. M. R.]

ABSTRACT

Inhibition of apoptosis is a hallmark of malignancies of the hematopoietic system. Previous studies in nonhematopoietic cells demonstrated that the prostate-apoptosis-response-gene-4 (Par-4) is up-regulated in cells undergoing programmed cell death and that Par-4 exerts its proapoptotic effect by down-regulating Bcl-2. After showing the aberrant expression pattern of Par-4 in neoplastic lymphocytes as well as demonstrating inverse expression patterns of Par-4 and Bcl-2 in malignant cells of patients suffering from acute lymphocytic leukemia, we assessed the functional consequences of Par-4 overexpression during apoptosis in Jurkat T lymphocytes. We show that in lymphatic cells Par-4 overexpression decreases the level of Bcl-2, whereas Bax, the proapoptotic counterpart of Bcl-2, retains unaltered levels. Moreover, Par-4 overexpression is accompanied by cleavage of poly(ADP-ribose) polymerase (PARP). Despite these effects, overexpression of Par-4 alone is not sufficient to induce apoptosis but markedly increases the rate of apoptosis on treatment with different chemotherapeutic agents. On chemotherapeutic treatment Par-4 overexpression enhances disruption of mitochondrial membrane potential, PARP-cleaving activity, as well as activation of caspase-3. The hypothesis of caspase-dependency of Par-4-promoted apoptosis is additionally supported by demonstrating complete abrogation of programmed cell death after pretreatment with a broad spectrum caspase-inhibitor. On inhibition of caspase-3 overexpression of Par-4 enables lymphatic cells to alternatively activate caspases-9, -6, and -7 by diminishing the influence of the inhibitors of apoptosis proteins (IAPs) cIAP1 and XIAP.

Our study is the first to identify Par-4 as a proapoptotic protein in lymphatic cells, outlining a model of action evaluating the role of Bcl-2/Bax, as well as demonstrating the impact of Par-4 expression on PARP cleavage, disruption of mitochondrial membrane potential, caspase activation, and interactions with inhibitors of apoptosis proteins.

INTRODUCTION

Deregulation of apoptosis contributes to the pathogenesis of many diseases, among them autoimmunity, neurodegenerative disorders, and cancer (1). Alterations during apoptosis include changes in the expression levels of Bcl-2 family members, loss of MMP, activation of caspases, cleavage of PARP, and DNA fragmentation (1–3). The Bcl-2 family members are considered to be key regulators of apoptosis, some of them inhibiting apoptosis, among them Bcl-2 and Bcl-2 family members are considered to be key regulators of apoptosis. The atypical protein kinases, it also markedly lessened the susceptibility of the cells toward apoptotic stimuli (10).

Bcl-2 is a well-characterized proto-oncogene conferring longevity (11). Overexpression of Bcl-2 prolongs cell survival, a crucial event in leukemogenesis/lymphomagenesis, because longer living cells are subjected to more oncogenic insults promoting the emergence of a transformed phenotype (12). Secondly, Bcl-2 acts as multidrug resistance protein delaying or preventing apoptosis induced essentially by all of the chemotherapeutic agents used currently (4, 13).

Taking into account the described effect of Par-4 expression on regulation of Bcl-2, as well as the role of Bcl-2 in lymphatic cells, we previously screened different populations of normal and neoplastic lymphocytes for expression of Par-4. We demonstrated a deregulated expression of Par-4 in neoplastic lymphocytes, as well as an inverse expression pattern of Par-4 and Bcl-2 in lymphatic cell lines and in blast cells of patients suffering from acute lymphocytic leukemia (14).

To investigate the function of Par-4 in apoptosis of neoplastic lymphocytes we overexpressed Par-4 in the lymphatic cell line Jurkat and assessed the influence of Par-4 expression on major steps of apoptosis signaling. To the best of our knowledge this is the first study evaluating the functional relevance of Par-4 expression in lymphatic cells and outlining its impact on the major components of the apoptotic cascade.

MATERIALS AND METHODS

Cell Culture. Jurkat T cells were cultured at 37°C under 5% CO2 in RPMI 1640 (Life Technologies, Inc., Paisley, Scotland) supplemented with 10% FCS (Life Technologies, Inc., Inc.), 2 mM L-glutamine (Life Technologies, Inc.), and a 1% penicillin-streptomycin mixture (Life Technologies, Inc.). For all of the experiments cells were seeded in a concentration of 25 × 10⁶ cells/ml.

Antibodies. The antibody directed against bcl-2 was purchased from DAKO, Glostrup, Denmark; against caspase-6 from Becton Dickinson, Heidelberg, Germany; against caspase-7 from PharMingen, Heidelberg, Germany; against α/β-tubulin from Dün, Asbach, Germany; against XIAP, cIAP1, and cIAP2, from R&D Systems, Wiesbaden, Germany; and against par-4, bax, PARP, caspase-3 and -9, and the secondary alkaline-phosphatase conjugated antibodies from Santa Cruz Biotechnology, Santa Cruz, CA.

Construction of Plasmids and Stable Transfection. Par-4 cDNA had been subcloned in the EcoRI site of the pCB6 expression vector (15). Par-4 cDNA was excised by digestion at EcoRI and subcloned into the pCR II vector (Invitrogen, Carlsbad, CA). After digestion with SpeI and XhoI the obtained fragment was subcloned into the Zn2+-inducible pGMSVino vector (16). After electroporation, cells were selected by treatment with G418 (Serva, Heidelberg, Germany) and subcloned under limiting dilution conditions. Clones were tested for Par-4 expression by Western blotting without and with Zn2+ induction. Five clones were found to express the transgene, four of them exhibiting Par-4 expression in absence of Zn2+ induction, an effect caused by leakage of the promoter. Three of these clones were used as stably expressing clones. Jurkat cells were also transfected with the “empty” MT vector and in particular those of the protein kinase C isoforms (9). Thus, Par-4 hinders activation of the mitogen-activated protein kinases, enzymes critical for cell survival, thereby promoting apoptosis. Bcl-2 overexpression in these cells not only suppresses the inhibitory effect of Par-4 on the atypical protein kinases, it also markedly lessened the susceptibility of the cells toward apoptotic stimuli (10).
underwent the identical selection procedures. Two empty mock clones were used in all of the experiments as a negative control.

**Induction and Detection of Apoptosis.** Apoptosis was induced by incubation with 2 μg/ml doxorubicin (Pharmacia and Upjohn GmbH, Erlangen, Germany) or by incubation with 6 μg/ml cytosin-arabinoside (Upjohn GmbH, Heppenheim, Germany). Apoptosis was quantified by a FACScan flow cytometer (Lysis II; Becton Dickinson) after 24, 48, and 72 h using 7-AAD (Sigma Chemical Co., Deisenhofen, Germany). Error bars in the graphs represent SE of triplicates calculated using the GraphPad Prism Software (San Diego, CA).

**Analysis of MMP.** Changes in MMP were measured by flow cytometry using the intramitochondrial dye JC-1 (Alexis Biochemicals, Gruenberg, Germany) following the manufacturer’s instructions after 3, 18, and 24 h. Data were converted to density plots using CellQuest software (Becton Dickinson).

**Inhibition of Caspase Activity.** The broad spectrum caspase inhibitor zVAD-fmk and the caspase-3 inhibitor DEVD-CHO (Enzyme Systems Products, Dublin, CA) were used at a concentration of 50 μM following the manufacturer’s instructions.

**Measurement of Caspase-3 Activity.** Caspase-3 activity was assessed after 2, 6, 10, and 24 h using the Fluorometric Immunosorbent Enzyme Assay (Roche, Mannheim, Germany) following the manufacturer’s instructions. The caspase-3 fluorescence assay kit detects the shift in emission of fluorescence of AFC. AFC is conjugated to a specific tetrapeptide sequence (DEVD-AFC) emitting blue light. On proteolytic cleavage of the substrate, the liberated AFC emits a yellow-green fluorescence at 505 nm. Absorbance was measured using a multifunctional reader (Tecan, Crailsheim, Germany).

**Western Blot Analysis.** Cells (1 × 10^6) were pelleted and fractionated by SDS-PAGE (8–15% gradient gels), and proteins were transferred to a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with a 5% nonfat, dry milk and incubated with the primary antibody. Unbound antibody was removed by washing with Tris-buffered saline (pH 7.2) containing 0.5% Tween 20. The membrane was then incubated with the secondary alkaline-phosphatase-conjugated antibody. After extensive washing proteins were detected on addition of the staining substrates (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Boehringer Mannheim, Indianapolis, IN). Blots were “stripped” using mercaptoethanol 100 mM, 2 mM Tris-HCl (pH 6.8) and 2% SDS 20%.

**RESULTS**

**Generation of Par-4-overexpressing Jurkat Cell Clones.** To assess functional consequences of Par-4 expression in lymphatic cells we transfected the Par-4 cDNA under the control of a mouse metallothionein promoter (MT-vector) into Jurkat T cells and selected several clones by G418 treatment under limiting dilution conditions. No significant differences regarding cloning efficiency between mock- and Par-4-transfected cells were seen (data not shown). After G418 selection and limiting dilution, 5 of 20 Jurkat clones were positive for Par-4 expression as proven by Western blot analysis with an anti-Par-4 antibody (Fig. 1 and data not shown). Given the fact that 4 of these 5 clones exhibited a strong leakage of the promoter we used 3 of these cell clones as stably expressing Par-4 clones for all of the subsequent experiments. To avoid the bias of clonal variability, all of the experiments were carried out using 3 Par-4 overexpressing clones as well as two clones of Jurkat cells transfected with the empty MT mock-vector serving as negative controls (Fig. 1A). The generation of viable and proliferating cell clones stably expressing Par-4 provides evidence that Par-4 overexpression alone is not sufficient to induce programmed cell death in lymphatic cells.

**Ectopic Expression of Par-4 Sensitizes Jurkat T Cells to Apoptosis Induced by Chemotherapeutic Agents.** To additionally evaluate the effect of Par-4 overexpression on the rate of apoptosis of lymphatic cells, we assessed the rate of apoptosis in transfected Jurkat clones as compared with the mock-transfected Jurkat clones. As demonstrated in Fig. 2B by comparable rates of apoptosis in untreated cells, Par-4 overexpression itself, i.e., without the proapoptotic stimulus of a chemotherapeutic agent, does not alter the rate of apoptosis in Jurkat T-cells. 1-β-D-Arabinofuranosylcytosine as well as doxorubicin led to an increase in the number of apoptotic cells, an effect that was enhanced in Par-4-overexpressing cells. As shown in Fig. 2, C and D, the percentage of apoptotic cells in Par-4-expressing clones exceeded the percentage of apoptotic cells in the mock-transfected cells by 15–30%, an effect sustained over 72 h. Taken together these results demonstrate that in lymphatic cells overexpression of Par-4 itself does not lead to programmed cell death, but sensitizes cells toward treatment with different cytotoxic agents.

**Overexpression of Par-4 Down-Regulates Bcl-2 in Jurkat T Lymphocytes.** To elucidate the mechanisms by which overexpression of Par-4 facilitates apoptosis we assessed the expression levels of Bcl-2 and Bax. For assessment of a potential influence of Par-4 overexpression itself on the two Bcl-2 members, protein levels of Bcl-2 and Bax in the transfected Jurkat clones were evaluated before induction of apoptosis by chemotherapeutic treatment. In comparison with the mock clones, Par-4-overexpressing clones exhibited decreased cellular expression levels of Bcl-2 (Fig. 2B). Neither incubation with doxorubicin nor incubation with 1-β-D-arabinofuranosylcytosine led to an additional decrease of Bcl-2 levels (data not shown). Neither Par-4 expression alone nor concomitant incubation with 1-β-D-arabinofuranosylcytosine or doxorubicin led to a change in Bax expression levels (data not shown). These findings suggest that in lymphatic cells Par-4 functions upstream of Bcl-2 and Bax by down-regulating the expression level of Bcl-2 thus sensitizing cells toward a proapoptotic stimulus.

**On Chemotherapeutic Treatment Par-4 Enhances Disruption of MMP.** Because certain Bcl-2 family members, among them Bcl-2 and Bax, are considered to regulate apoptosis by preserving or disrupting the MMP (5), we tested the hypothesis that overexpression of Par-4 and the associated changes in the expression levels of Bcl-2 translate into premature and/or enhanced loss of MMP. As depicted in Fig. 3A, MMP is not changed in Par-4-overexpressing cells as compared with mock-transfected cells. In contrast, disruption of MMP on treatment with 1-β-D-arabinofuranosylcytosine or doxorubicin was augmented in Par-4-overexpressing clones in regard to the percentage of control clones experiencing disruption of MMP (Fig. 3, B and C). These data provide evidence that overexpression of Par-4 enhances

**FUNCTION of Par-4 IN NEOPLASTIC T LYMPHOCYTES**

**Fig. 1. Ectopic expression of Par-4 in Jurkat T lymphocytes and concomitant down-regulation in the expression levels of Bcl-2. A**, protein expression levels of Par-4 in two Jurkat clones stably transfected with the empty mock vector (MT-A24, MT-A30, mock) and in three Jurkat clones containing the Par-4 expression vector (MTPARB8, MTPARB310, Par-4). Nonspecific cross-reactivity of the Par-4 antibody was used as a control for equal protein loading (top lanes). **B**, expression of Bcl-2 in mock-transfected clones (mock) and Par-4-positive clones (Par-4). **C**, expression of tubulin serving as a loading control.
The Proapoptotic Function of Par-4 Is Suppressed by Inhibition of Caspases. Because the obtained results outlined a mechanism of Par-4-promoted apoptosis dependent on caspase activation, we additionally evaluated the role of caspases by incubating mock- and Par-4-expressing clones with the chemotherapeutic agents with and without the presence of the broad-spectrum caspase-inhibitor zVAD-fmk, a peptide considered to block all of the known caspases. As shown in Fig. 6, zVAD-fmk completely blocked apoptosis up to 48 h in all of the clones independent of the Par-4 expression level and the chemotherapeutic agent applied. After 72 h there was a slight increase in the number of apoptotic cells despite the presence of zVAD-fmk. This might be because of a mitochondrial dysfunction leading to cell death even without caspase activity, or there might be a decreased PARP-cleaving activity already in absence of chemotherapy-induced apoptosis (Fig. 4, A and C, control). On treatment with 1-β-D-arabinofuranosylcytosine and doxorubicin PARP-cleavage was enhanced in Par-4-overexpressing cells as compared with mock-transfected clones (Fig. 4, A–D, Ara-C, Doxo). This demonstrates that overexpression of Par-4 is accompanied by “spontaneous” PARP-cleaving activity, i.e., PARP-cleaving activity without a proapoptotic agent and secondly, that PARP-cleaving activity is augmented over time in Par-4-overexpressing Jurkat clones on incubation with different chemotherapeutic agents.

On Chemotherapeutic Treatment Par-4 Promotes Activation of Caspase-3. PARP cleavage is attributed to caspase activation, in particular to activation of caspase-3. Caspase-3 is not only known to cleave PARP but is also considered to be the central executioner caspase during apoptosis (17). To test the hypothesis that the above-described PARP-cleavage is attributable to an activation of caspase-3, we measured quantitative changes in caspase-3 activity by flow-cytometric analysis. Fig. 5A demonstrates that Par-4-overexpressing and control cells exhibited the same level of caspase-3 activity in the absence of a proapoptotic stimulus. After incubation with the different chemotherapeutic agents Par-4-overexpressing lymphocytes exhibited enhanced caspase-3 activity (Fig. 5, B and C). After 24 h cells treated with 1-β-D-arabinofuranosylcytosine experienced a decline in caspase-3 activity, although caspase-3 activity remained still higher in Par-4-positive cells. In doxorubicin-treated cells caspase-3 activity experienced an additional increase between 10 and 24 h. Once more the increase was more pronounced in Par-4-overexpressing cell clones (Fig. 5C). The lack of caspase-3 activity in Par-4-expressing clones in absence of chemotherapeutic agents excludes caspase-3 as the protein responsible for the observed spontaneous PARP cleavage. Furthermore, these results provide evidence that the enhanced PARP cleavage in Par-4-overexpressing cells observed on incubation with 1-β-D-arabinofuranosylcytosine and doxorubicin is at least partially caused by elevated caspase-3 activity.

The Proapoptotic Function of Par-4 Is Suppressed by Inhibition of Caspases. The obtained results outlined a mechanism of Par-4-promoted apoptosis dependent on caspase activation, we additionally evaluated the role of caspases by incubating mock- and Par-4-expressing clones with the chemotherapeutic agents with and without the presence of the broad-spectrum caspase-inhibitor zVAD-fmk, a peptide considered to block all of the known caspases. As shown in Fig. 6, zVAD-fmk completely blocked apoptosis up to 48 h in all of the clones independent of the Par-4 expression level and the chemotherapeutic agent applied. After 72 h there was a slight increase in the number of apoptotic cells despite the presence of zVAD-fmk. This might be because of a mitochondrial dysfunction leading to cell death even without caspase activity, or there might be a decreased PARP-cleaving activity, i.e., PARP-cleaving activity without a proapoptotic agent and secondly, that PARP-cleaving activity is augmented over time in Par-4-overexpressing Jurkat clones on incubation with different chemotherapeutic agents.

To evaluate the enhanced loss of MMP on induction of apoptosis by chemotherapeutic agents, whereas ectopic Par-4 expression without a proapoptotic stimulus remains without influence on the MMP. **Par-4 Overexpression Promotes Cleavage of PARP.** To evaluate if the enhanced loss of MMP in Par-4-overexpressing cells translates into altered caspase activation, we first assessed cleavage of PARP, an enzyme cleaved by activated caspases, among them caspase-3. In contrast with the control clones, Par-4-overexpressing clones exhibited PARP-cleaving activity already in absence of chemotherapy-induced apoptosis (Fig. 4, A and C, control). On treatment with 1-β-D-arabinofuranosylcytosine and doxorubicin PARP-cleavage was enhanced in Par-4-overexpressing cells as compared with mock-transfected clones (Fig. 4, A–D, Ara-C, Doxo). This demonstrates that overexpression of Par-4 is accompanied by “spontaneous” PARP-cleaving activity, i.e., PARP-cleaving activity without a proapoptotic agent and secondly, that PARP-cleaving activity is augmented over time in Par-4-overexpressing Jurkat clones on incubation with different chemotherapeutic agents.

**On Chemotherapeutic Treatment Par-4 Promotes Activation of Caspase-3.** PARP cleavage is attributed to caspase activation, in particular to activation of caspase-3. Caspase-3 is not only known to cleave PARP but is also considered to be the central executioner caspase during apoptosis (17). To test the hypothesis that the above-described PARP-cleavage is attributable to an activation of caspase-3, we measured quantitative changes in caspase-3 activity by flow-cytometric analysis. Fig. 5A demonstrates that Par-4-overexpressing and control cells exhibited the same level of caspase-3 activity in the absence of a proapoptotic stimulus. After incubation with the different chemotherapeutic agents Par-4-overexpressing lymphocytes exhibited enhanced caspase-3 activity (Fig. 5, B and C). After 24 h cells treated with 1-β-D-arabinofuranosylcytosine experienced a decline in caspase-3 activity, although caspase-3 activity remained still higher in Par-4-positive cells. In doxorubicin-treated cells caspase-3 activity experienced an additional increase between 10 and 24 h. Once more the increase was more pronounced in Par-4-overexpressing cell clones (Fig. 5C). The lack of caspase-3 activity in Par-4-expressing clones in absence of chemotherapeutic agents excludes caspase-3 as the protein responsible for the observed spontaneous PARP cleavage. Furthermore, these results provide evidence that the enhanced PARP cleavage in Par-4-overexpressing cells observed on incubation with 1-β-D-arabinofuranosylcytosine and doxorubicin is at least partially caused by elevated caspase-3 activity.

**The Proapoptotic Function of Par-4 Is Suppressed by Inhibition of Caspases.** Because the obtained results outlined a mechanism of Par-4-promoted apoptosis dependent on caspase activation, we additionally evaluated the role of caspases by incubating mock- and Par-4-expressing clones with the chemotherapeutic agents with and without the presence of the broad-spectrum caspase-inhibitor zVAD-fmk, a peptide considered to block all of the known caspases. As shown in Fig. 6, zVAD-fmk completely blocked apoptosis up to 48 h in all of the clones independent of the Par-4 expression level and the chemotherapeutic agent applied. After 72 h there was a slight increase in the number of apoptotic cells despite the presence of zVAD-fmk. This might be because of a mitochondrial dysfunction leading to cell death even without caspase activity, or there might be a decreased PARP-cleaving activity, i.e., PARP-cleaving activity without a proapoptotic agent and secondly, that PARP-cleaving activity is augmented over time in Par-4-overexpressing Jurkat clones on incubation with different chemotherapeutic agents.

**On Chemotherapeutic Treatment Par-4 Promotes Activation of Caspase-3.** PARP cleavage is attributed to caspase activation, in particular to activation of caspase-3. Caspase-3 is not only known to cleave PARP but is also considered to be the central executioner caspase during apoptosis (17). To test the hypothesis that the above-described PARP-cleavage is attributable to an activation of caspase-3, we measured quantitative changes in caspase-3 activity by flow-cytometric analysis. Fig. 5A demonstrates that Par-4-overexpressing and control cells exhibited the same level of caspase-3 activity in the absence of a proapoptotic stimulus. After incubation with the different chemotherapeutic agents Par-4-overexpressing lymphocytes exhibited enhanced caspase-3 activity (Fig. 5, B and C). After 24 h cells treated with 1-β-D-arabinofuranosylcytosine experienced a decline in caspase-3 activity, although caspase-3 activity remained still higher in Par-4-positive cells. In doxorubicin-treated cells caspase-3 activity experienced an additional increase between 10 and 24 h. Once more the increase was more pronounced in Par-4-overexpressing cell clones (Fig. 5C). The lack of caspase-3 activity in Par-4-expressing clones in absence of chemotherapeutic agents excludes caspase-3 as the protein responsible for the observed spontaneous PARP cleavage. Furthermore, these results provide evidence that the enhanced PARP cleavage in Par-4-overexpressing cells observed on incubation with 1-β-D-arabinofuranosylcytosine and doxorubicin is at least partially caused by elevated caspase-3 activity.

**The Proapoptotic Function of Par-4 Is Suppressed by Inhibition of Caspases.** Because the obtained results outlined a mechanism of Par-4-promoted apoptosis dependent on caspase activation, we additionally evaluated the role of caspases by incubating mock- and Par-4-expressing clones with the chemotherapeutic agents with and without the presence of the broad-spectrum caspase-inhibitor zVAD-fmk, a peptide considered to block all of the known caspases. As shown in Fig. 6, zVAD-fmk completely blocked apoptosis up to 48 h in all of the clones independent of the Par-4 expression level and the chemotherapeutic agent applied. After 72 h there was a slight increase in the number of apoptotic cells despite the presence of zVAD-fmk. This might be because of a mitochondrial dysfunction leading to cell death even without caspase activity, or there might be a decreased PARP-cleaving activity, i.e., PARP-cleaving activity without a proapoptotic agent and secondly, that PARP-cleaving activity is augmented over time in Par-4-overexpressing Jurkat clones on incubation with different chemotherapeutic agents.
inhibitory effect of zVAD-fmk over time. Taken together these data demonstrate that the proapoptotic function of Par-4 critically depends on caspase activation.

Par-4-promoted PARP Cleavage Is Completely Abrogated by General Caspase Inhibition But Only Partially Blocked by Inhibition of Caspase-3. From our results described above emerges the picture that the proapoptotic function of Par-4 in lymphatic cells critically depends on general caspase-activation and, after stimulation with chemotherapeutic agents, enhanced caspase-3 activity. To corroborate our data on the role of caspase activation in Par-4-promoted apoptosis, alterations in PARP-cleaving activity were determined after incubation of Jurkat clones with doxorubicin without and with inhibition of general caspase-activation as well as specific inhibition of caspase-3 activity. As demonstrated in Fig. 7A under incubation of cells with doxorubicin broad-spectrum caspase, inhibition by zVAD-fmk completely abrogated PARP-cleaving activity in Par-4-overexpressing cells as well as in negative control clones. In contrast, selective inhibition of caspase-3 by DEVD-CHO abolished PARP-cleaving activity in Par-4-negative mock clones but was unable to completely abrogate PARP-cleavage in Par-4-overexpressing clones (Fig. 7B). The assessment of PARP-cleaving activity without the apoptotic stimulus induced by doxorubicin reveals that PARP-cleavage in Par-4 positive cells is completely abrogated in presence of DEVD-CHO and zVAD-fmk (Fig. 7C). Abrogation of PARP cleavage in Par-4-overexpressing clones by broad-spectrum caspase inhibition additionally proves caspase-dependency of Par-4-promoted apoptosis. Moreover, these results also stress the contribution of increased caspase-3 activity in chemotherapy-induced PARP cleavage in Par-4 expressing cells.

Overexpression of Par-4 Enables Jurkat T Cells to Circumvent Inhibition of Caspase-3 by Activation of Caspase-9, -7, and -6. To additionally investigate the mechanisms responsible for the PARP-cleavage observed in Par-4-overexpressing cell clones in the absence of a proapoptotic stimulus, as well as their persistent PARP-cleaving activity on inhibition of caspase-3, expression levels of the pro-caspases-9, -7, and -6 were assessed. Whereas procaspase-9 levels remained unaltered by Par-4 expression alone, incubation with doxorubicin led to a decrease in the expression of procaspase-9 in the Par-4-positive clones, consistent with an increased caspase-9 activation in Par-4-positive clones as compared with Par-4-negative clones (Fig. 8A, Doxo). Noteworthy, incubation with the caspase-3 inhibitor reestablished the procaspase-9 expression level in the Par-4-negative clones.
clones to an extent comparable with the expression in absence of a proapoptotic stimulus. In contrast, Par-4-expressing cells retained the ability to activate caspase-9 despite the presence of the caspase-3 inhibitor (Fig. 8A, DEVD). Because previous experiments using Jurkat cell extracts demonstrated PARP-cleavage by caspase-7 after activation of caspase-9 (18), we next determined changes in the caspase-7 expression levels. Incubation with doxorubicin activated caspase-7 in both Par-4-negative as well as positive cell clones (Fig. 8B, Doxo). On inhibition of caspase-3, cleavage of caspase-7 was hindered in Par-4-negative cells, whereas Par-4 expression enabled Jurkat cell clones to additionally augment caspase-7 activation (Fig. 8B, DEVD). Caspase-6 is considered to be the third effector caspase besides caspase-3 and -7 (19, 20). As demonstrated in Fig. 8C by decreased expression levels of the procaspase, caspase-6 is activated on doxorubicin exposure exclusively in Par-4-overexpressing Jurkat clones in the presence of the caspase-3 inhibitor. These results provide evidence that overexpression of Par-4 not only promotes quantitative changes in caspase activation but moreover enables lymphatic cells to circumvent inhibition of caspase-3 by alternatively activating caspases.

Fig. 5. Expression of Par-4 enhances activation of caspase-3 induced by different chemotherapeutic agents. Activation of caspase-3 over time (2, 6, 10, and 24 h) was assessed in the two mock-transfected clones and the three stably Par-4-transfected Jurkat clones. Results are means of triplicates; bars, ± SE; similar results were obtained in three separate experiments. A, activation of caspase-3 without incubation of a chemotherapeutic agent (control). B, activation of caspase-3 after incubation with 1-/H9252-D-arabinofuranosylcytosine (Ara-C). C, activation of caspase-3 after incubation with doxorubicin (Doxo).

Fig. 6. Broad-spectrum caspase-inhibition abrogates Par-4-promoted apoptosis irrespective of the applied chemotherapeutic agent. For clearness of representation figures depict changes in the rate of apoptosis in one Par-4-transfected clone (MTPARB8). Data are representative of three independent experiments carried out with both mock-transfected clones and the three Par-4-transfected clones. Rates of apoptosis in mock-transfected clones were comparable with the results depicted in Fig. 2 and were not influenced by the presence of zVAD-fmk. Results are means of triplicates; bars, ± SE. The figure shows the percentage of apoptotic cells after incubation with 1-/H9252-D-arabinofuranosylcytosine (Ara-C) or doxorubicin (Doxo) in absence and presence of zVAD-fmk (ZVAD).

Fig. 7. Under a proapoptotic stimulus Par-4-induced PARP cleavage is abrogated by broad-spectrum caspase inhibition but only partially blocked by inhibition of caspase-3. For clearness of presentation, blots depict expression levels in one mock-transfected Jurkat clone and one Par-4-transfected clone after 24 h of incubation with doxorubicin. Expression levels and cleavage patterns are representative of results obtained for both mock-transfected clones and three Par-4-transfected clones. A, cleavage of PARP in one mock-transfected Jurkat clone (MT-A24) as compared with the Par-4-transfected Jurkat clone (MTPARB8) without and with presence of the broad-spectrum caspase-inhibitor zVAD-fmk (ZVAD). B, cleavage of PARP in one mock-transfected Jurkat clone (MT-A24) as compared with the Par-4-transfected Jurkat clone (MTPARB8) without and with presence of the caspase-3 inhibitor DEVD-CHO (DEVD). C, cleavage pattern of PARP in the absence of doxorubicin after incubation with the broad-spectrum caspase-inhibitor (ZVAD) and the caspase-3 inhibitor (DEVD).
Par-4 Induces Down-Regulation of cIAP1 and XIAP. Because it is known that activation of downstream caspases is hindered by IAPs (21), we hypothesized that the Par-4-associated alterations of caspase activation might be because of changes in the expression levels of IAPs. It has been shown previously that XIAP, cIAP1, and cIAP2 inhibit caspase-9, thereby blocking proteolytic cleavage of caspase-3, -6, and -7 (22). Fig. 9 demonstrates that on induction of apoptosis with doxorubicin, Par-4-expressing cells experienced a down-regulation of the protein expression of cIAP1 and XIAP (Fig. 9, A and C, Doxo), whereas the levels of cIAP2 remained unaltered independently of the Par-4 expression level and the presence of a caspase-3 inhibitor (Fig. 9B). Of note is the persisting decrease in the levels of cIAP1 as compared with all of the other conditions tested (Fig. 9A). These results provide evidence that overexpression of Par-4 in neoplastic lymphocytes promotes activation of caspases on induction of apoptosis by down-regulating cIAP1 and XIAP, and that Par-4 enables lymphatic cells to maintain that down-regulation despite inhibition of caspase-3 activation.

DISCUSSION

The aim of this study was to evaluate functional consequences of ectopic expression of Par-4 on apoptosis of lymphatic cells after demonstrating previously a deregulated expression of Par-4 in different populations of ex vivo neoplastic lymphocytes (14).

Originally Par-4 was isolated by differential screening for genes up-regulated after induction of programmed cell death in prostate cancer cells (23, 24). Subsequent experiments in nonhematopoetic cells proved that down-regulation of Par-4 is a prerequisite for survival of malignant cells and tumor progression (25), and that Par-4 exerts a proapoptotic influence by down-regulating the expression of antiapoptotic Bcl-2 (8). Bcl-2 is a well-described proto-oncogene in normal and malignant hematopoetic cells conferring longevity (11).

To assess the impact of Par-4 expression on apoptosis of lymphatic cells, we ectopically expressed Par-4 in the T-lymphoblastic leukemia/lymphoma cell line Jurkat. The fact that stable expression of Par-4 did not interfere with generation of viable cell clones is in accordance with results in nonhematopoetic cells demonstrating that Par-4 exerts its proapoptotic effect only after a concomitant proapoptotic stimulus. To more accurately define a potential proapoptotic influence of Par-4 expression in lymphatic cells the percentage of apoptotic cells without and with incubation of differently acting cytotoxic drugs was measured. The obtained data confirm that ectopic Par-4 alone does not alter the rate of apoptosis but increases programmed cell death on incubation with chemotherapeutic agents.

Taking into account our finding that Par-4-overexpressing Jurkat cells exhibit decreased levels of Bcl-2, we conclude that the reduction of antiapoptotic Bcl-2 shifts the rheostat of pro- and antiapoptotic Bcl-2 family members, thus favoring induction of apoptosis on incubation of cells with a proapoptotic stimulus. The fact that Bcl-2 levels are diminished before treatment with chemotherapeutic agents corresponds to the notion that preexisting Bcl-2 levels determine chemosensitivity of lymphatic cells (26, 27).

We additionally demonstrate that Par-4 expression and simultaneous Bcl-2 down-regulation results in enhanced disruption of MMP on
stimulation with chemotherapeutic agents. Compared with our observations on induction of apoptosis, Par-4 expression alone, i.e., in the absence of a proapoptotic stimulus, did not disturb MMP.

When evaluating whether the enhanced disruption of MMP in Par-4-overexpressing clones translates into premature or amplified activation of caspases, clones overexpressing Par-4 already exhibited PARP-cleaving activity in absence of a proapoptotic stimulus. The concept on caspase activation postulates that caspases are synthesized as relatively inactive precursors requiring proteolytic cleavage after receipt of divergent proapoptotic stimuli for activation (20, 28, 29). A study published recently on the ordering of caspases and their cleavage-patterns in Jurkat whole cell extracts confirmed the central role of caspase-3 among the executioner caspases, whereas caspases-6 and -7 are considered to play inferior but highly specialized roles during programmed cell death (18).

From the demonstration that Par-4-overexpressing clones exhibit PARP-cleaving activity without undergoing programmed cell death, one might hypothesize that Par-4 overexpression already activates one or several caspases, which then cleave(s) PARP, a signal unable to execute programmed cell death by itself but able to enhance and accelerate the response to proapoptotic stimuli. Actually, a “spontaneous activation” of caspases was observed by Fearnhead et al. (30) in oncogene-transfected cells sensitizing cells to drug-induced apoptosis by increased levels of Apaf-1 interacting with caspase-9. The fact that PARP cleavage in all clones independent of Par-4 expression is completely abrogated by incubation with a broad-spectrum caspase-inhibitor proves that the observed PARP cleavage is actually caused by activated caspases. As demonstrated by quantitative measurements of caspase-3 activity this central executioner caspase was not activated by Par-4 expression alone but increased its activity exclusively after incubation with chemotherapeutic agents. Nevertheless, after incubation with proapoptotic stimuli caspase-3 activity was additionally augmented in Par-4-overexpressing clones. Noteworthily, whereas inhibition of caspase-3 completely abrogated PARP-cleaving activity in Par-4-negative Jurkat clones, inhibition of caspase-3 was not sufficient to prevent PARP cleavage in Par-4-overexpressing clones in presence of a proapoptotic stimulus.

Investigating the effects of Par-4 expression in lymphatic cells the results presented in this study provide evidence that overexpression of Par-4 enhances activation of caspase-9, caspase-7, and caspase-6. As demonstrated, Par-4-overexpressing cells thus possess an increased capability to activate caspase-6, -7, and -9 despite the presence of a caspase-3 inhibitor. Taking into account previous findings proving that in Jurkat cells only caspase-7 and caspase-9 possess PARP-cleaving activity whereas caspase-9 and caspase-6 do not (18), the persistent PARP-cleavage observed here in presence of the caspase-3 inhibitor is most likely caused by activation of caspase-7. Moreover, there is evidence that caspase-9 is able to activate caspase-3 as well as caspase-7. Caspase-3 then transfers the signal farther down-stream by activating caspase-6 (18). Our results in Par-4-negative Jurkat cell lines support this model, but the presented data additional show an alteration of caspase activation on Par-4 overexpression. Besides quantitative changes, Par-4 overexpression also elicits qualitative changes as demonstrated by the differences in the activation of the caspases mentioned above on inhibition of caspase-3 activity. Of note is the ability of Par-4-positive cells to activate caspase-6 despite inhibition of caspase-3, because thus far caspase-3 is considered to act mainly upstream of caspase-6 (18).

Activation of caspases is negatively regulated by IAPs (22, 31). In particular, studies evaluating the role of XIAP, cIAP1, and cIAP2 demonstrated their potential to directly inhibit caspase activation by blocking caspase-9, thereby preventing proteolytic cleavage of caspase-3, -6, and -7 (22). Among the IAP-related proteins XIAP is considered to be the most potent (31).

Consequently, we tested the hypothesis that the changes in caspase activation observed on Par-4 expression are accompanied by altered expression levels of IAPs. The data presented here provide evidence that cIAP1 and XIAP levels are down-regulated in Par-4-expressing cell clones. On inhibition of caspase-3 activity, this down-regulation persists exclusively in Par-4-overexpressing cell clones, potentially being responsible for the increased activation of caspases-7 and -9.

Although it is acknowledged that many questions concerning the functional role of Par-4 in lymphatic cells remain to be answered, this is the first study outlining the impact of overexpression of Par-4 on major components of the apoptotic cascade of lymphocytes.

REFERENCES

activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. 


Alnemri, E. S. Molecular determinants of the caspase-promoting activity of Smac/ 
DIABLO and its role in the death receptor pathway. J. Biol. Chem., 275: 36152– 

and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J., 16: 6914– 
6925, 1997.

23. Sells, S. F., Wood, D. P., Jr., Joshi-Barve, S. S., Muthukumar, S., Jacob, R. J., Crist, 
S. A., Humphreys, S., and Rangnekar, V. M. Commonality of the gene programs 
induced by effectors of apoptosis in androgen-dependent and -independent prostate 

24. Sells, S. F., Han, S. S., Muthukumar, S., Maddiwar, N., Johnstone, R., Boghaert, E., 
Gillis, D., Liu, G., Nair, P., Monnig, S., Collini, P., Mattson, M. P., Sukhatme, V. P., 
Expression and function of the leucine zipper protein Par-4 in apoptosis. Mol. Cell. 

downregulation of the pro-apoptotic protein Par-4 is critical for Ras-induced survival 


27. Pepper, C., Hoy, T., and Benley, D. P. Bcl-2/Bax ratios in chronic lymphocytic 
leukaemia and their correlation with in vitro apoptosis and clinical resistance. Br. J. 


29. Sells, S. F., Wood, D. P., Jr., Joshi-Barve, S. S., Muthukumar, S., Jacob, R. J., Crist, 
S. A., Humphreys, S., and Rangnekar, V. M. Commonality of the gene programs 
induced by effectors of apoptosis in androgen-dependent and -independent prostate 

30. Sells, S. F., Han, S. S., Muthukumar, S., Maddiwar, N., Johnstone, R., Boghaert, E., 
Gillis, D., Liu, G., Nair, P., Monnig, S., Collini, P., Mattson, M. P., Sukhatme, V. P., 
Expression and function of the leucine zipper protein Par-4 in apoptosis. Mol. Cell. 

31. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. X-linked IAP is a 
In Lymphatic Cells Par-4 Sensitizes to Apoptosis by Down-Regulating Bcl-2 and Promoting Disruption of Mitochondrial Membrane Potential and Caspase Activation


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/6/1768

Cited articles
This article cites 29 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/6/1768.full.html#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/6/1768.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.