PML induces a novel caspase-independent death process

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PML nuclear bodies (NBs) are nuclear matrix-associated structures altered by viruses and oncogenes. We show here that *PML* overexpression induces rapid cell death, independent of *de novo* transcription and cell cycling. PML death involves cytoplasmic features of apoptosis in the absence of caspase-3 activation, and caspase inhibitors such as zVAD accelerate PML death. zVAD also accelerates interferon (IFN)-induced death, suggesting that *PML* contributes to IFN-induced apoptosis. The death effector BAX and the cdk inhibitor p27KIP1 are novel NB-associated proteins recruited by PML to these nuclear domains, whereas the acute promyelocytic leukaemia (APL) PML/RARα oncoprotein delocalizes them. Arsenic enhances targeting of PML, BAX and p27KIP1 to NBs and synergizes with PML and IFN to induce cell death. Thus, cell death susceptibility correlates with NB recruitment of NB proteins. These findings reveal a novel cell death pathway that neither requires nor induces caspase-3 activation, and suggest that NBs participate in the control of cell survival.

Introduction

NBs are nuclear matrix-associated structures of unknown function that contain a number of proteins, including PML, SP100, ISG20, PIC1/SUMO-1, LYSP100, PLZF, INT6, CBP, RB1, RFP and ribosomal protein P (refs 1–3; for review, see ref. 4). PML has been shown to exhibit growth-suppressive properties[5–8], a finding recently strengthened by the tumour susceptibility of *Pml*$^{-/-}$ mice[9]. The genes *PML*, *SP100* and *ISG20* are primary targets of interferons (IFN), which suggests that their products have a role in the IFN response[10–12]. Several NB-associated proteins are involved in oncogenesis: RB1, CBP and INT6 are targets of oncoproteins (such as TAX, E1A and SV40T), and RFP, PML and PLZF were isolated as transforming fusion proteins. In APL, recurrent translocations were shown to fuse *PML* or *PLZF* to the gene (*RARA*) encoding retinoic acid receptor α. In t(15;17) APL, PML/RARα fusion disrupts the NB localization of PML (refs 13–16). It has been proposed that APL pathogenesis relies in part on transcriptional silencing of RA target genes through the tethering by PML/RARα of stabilized corepressor-histone acetyl transferase complexes[17–19], and in part on the loss of PML-triggered growth suppression[5,6,20]. Two therapeutic agents, retinoic acid (RA) and arsenic trioxide, that induce clinical remissions through differentiation and apoptosis, respectively, were shown to induce the restoration of the normal NB pattern of PML and associated proteins[14–16,21,22]. One potential explanation for these findings is that PML is involved in programmed cell death, and that alteration in PML function enhances cell survival. PML expression or NB localization is also altered in a number of other situations that involve abnormal cell survival, including virus infections, senescence or other human cancers[6,23].

Programmed cell death is a physiological cell suicide process that is essential in development, maintenance of tissue homeostasis and elimination of genetically altered or infected cells. Conversely, its disregulation is involved in the pathogenesis of several diseases. The most common phenotype of programmed cell death is apoptosis that includes a set of typical cytoplasmic and nuclear features. Execution of apoptosis involves release of mitochondrial proteins through changes in mitochondrial permeability, and is mediated by caspases, a class of cysteine proteases that cleaves key cellular proteins and induces the typical morphological features of nuclear condensation and fragmentation. In a number of instances, specific caspase inhibitors block or delay programmed cell death both *in vitro* and *in vivo*. Several death processes that involve caspase activation, however, are not blocked by the broad caspase inhibitors zVAD-fmk (zVAD) or BD-fmk (BD) (refs 24–27). In particular, the proapoptotic BAX protein, which induces mitochondrial permeability transition and triggers caspase activation, continued to induce cell death in the presence of zVAD (refs 24,26). These findings imply the existence of essential cell death executioners other than the known caspases.

In this report, we demonstrate that PML triggers a very rapid cell death without inducing caspase activation. zVAD upregulates PML protein levels and enhances both PML- and IFN-triggered death, suggesting that PML contributes to IFN-induced apoptosis and that caspase activation may exert a negative feedback on PML-mediated death. We have identified two proapoptotic gene products, BAX and p27KIP1, as NB-associated proteins that are recruited to NBs by PML, which suggests that NBs, as well as PML, have an unsuspected role in the control of cell survival.

Results

PML induces apoptosis in rat embryo fibroblasts

Transfection of a *PML* expression vector sharply diminishes focus formation by oncogenes and growth of established cell lines[5,6]. Indeed, using SV40 large T (SV40T)-transformed rat (or mouse) embryo fibroblasts (REF), we observed a 40-fold

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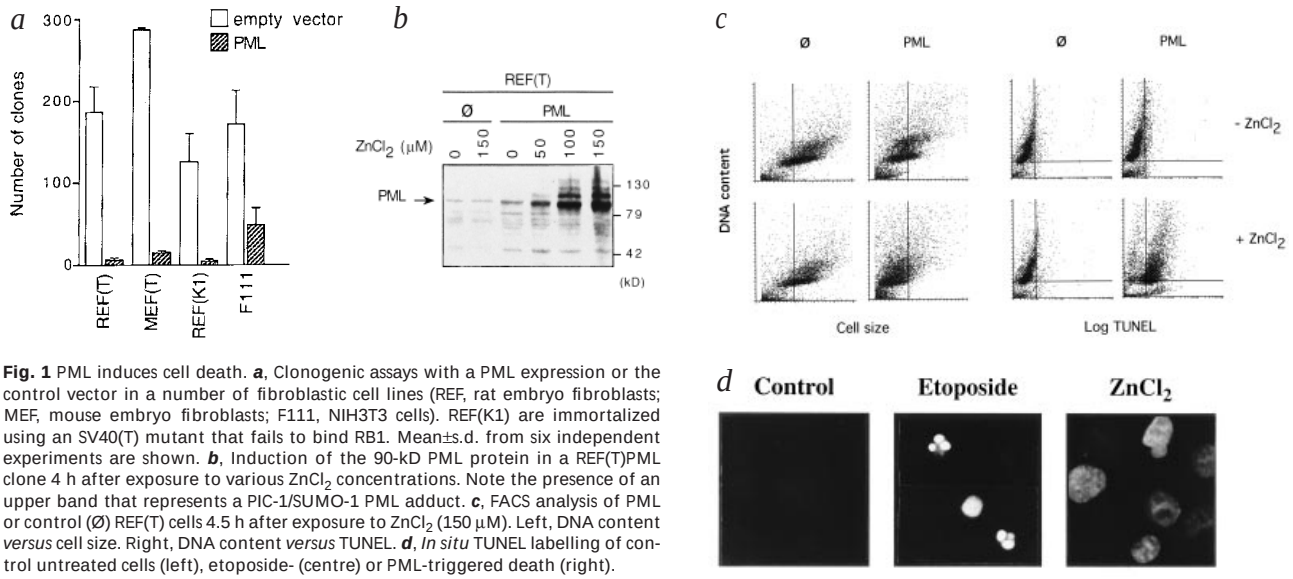


Fig. 1 PML induces cell death. ***a***, Clonogenic assays with a PML expression or the control vector in a number of fibroblastic cell lines (REF, rat embryo fibroblasts; MEF, mouse embryo fibroblasts; F111, NIH3T3 cells). REF(K1) are immortalized using an SV40(T) mutant that fails to bind RB1. Mean±s.d. from six independent experiments are shown. ***b***, Induction of the 90-kD PML protein in a REF(T)PML clone 4 h after exposure to various $ZnCl_2$ concentrations. Note the presence of an upper band that represents a PIC-1/SUMO-1 PML adduct. ***c***, FACS analysis of PML or control (Ø) REF(T) cells 4.5 h after exposure to $ZnCl_2$ (150 μM). Left, DNA content *versus* cell size. Right, DNA content *versus* TUNEL. ***d***, *In situ* TUNEL labelling of control untreated cells (left), etoposide- (centre) or PML-triggered death (right).

reduction in colony formation upon PML expression (Fig. 1*a*). We used vectors encoding *SP100* or a *PML* mutant that lacks the central coiled-coil region as controls (data not shown). To address the mechanism of this effect, we transfected a pool of SV40T-transformed REFs with the zinc-inducible pKSmMT-PML construct, in which a mouse metallothionein-I promoter directs *PML* expression. Zinc exposure lead to dose-dependent *PML* expression (Fig. 1*b*, and data not shown). In the five independent REF(T)PML clones tested, PML expression induced morphological changes suggestive of apoptosis. These changes first appeared at three hours: the cells rounded up, displayed cytoplasmic shrinkage (Fig. 1*c*) and progressively detached from the dish. These morphological changes were associated with a positive TUNEL assay, appearance of sub-G1 DNA (Fig. 1*c*), membrane phosphatidylserine externalization and loss of mitochondrial transmembrane potential (Fig. 2*c*, and data not shown). Nevertheless, cells retained the ability to exclude trypan blue for up to 10 hours. Kinetics of cell death varied according to the concentration of $ZnCl_2$ (48 hours, 50 μM $ZnCl_2$; 6 hours, 150 μM $ZnCl_2$). We saw none of these changes after zinc treatment in any of the three control REF(T)Ø clones (Fig. 1*c*, and data not shown). Despite evidence for DNA cleavage (sub-G1, positive TUNEL, Fig. 1*c*,*d*; loss of DNA viscosity, data not shown), PML-induced cell death was not associated with internucleosomal DNA laddering, consistent with the moderate TUNEL positivity (Fig. 1*c*,*d*).

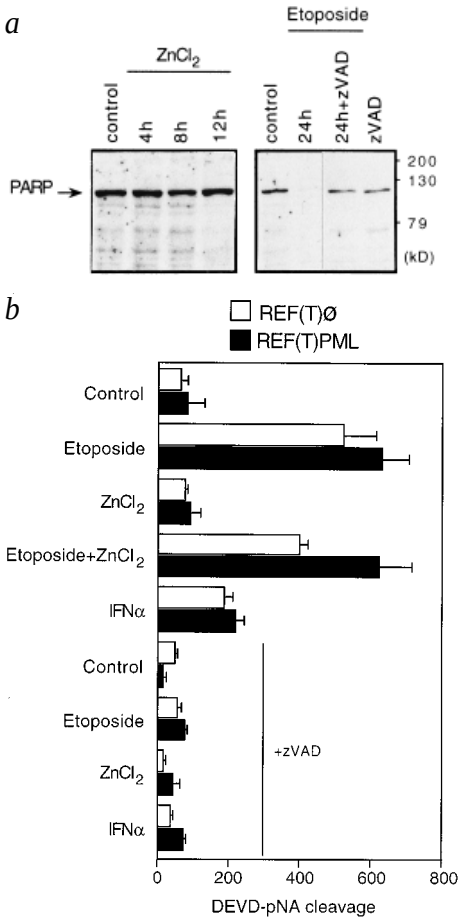
We carried out experiments to exclude a contribution of large T to PML-induced cell death. First, PML expression did not alter SV40T expression or localization in REF(T)PML cells, nor did it degrade P53 or release P53 from large T (data not shown). Second, in HeLa or CHO cells transiently transfected with vectors encoding either a GFP-PML fusion protein or GFP alone, all GFP-PML–positive cells progressively detached from the plate and died within 48 hours, in contrast with control GFP-positive cells. Third, in CHO cells stably transfected with pKSmMT-PML, $ZnCl_2$ induction again led to death only in *PML*-expressing clones. Finally, in REFs expressing an SV40T thermosensitive mutant, SV40T degradation at 39.5 °C induced a baseline level of apoptosis and accelerated PML-triggered cell death.

PML-induced death neither required, nor induced, transition to the S phase of the cell cycle, as PML still triggered death in REF(T)PML, which were blocked at the G1/S boundary by aphidicolin treatment. Moreover, BrdU pulses after $ZnCl_2$ induction showed that DNA replication was unmodified for up to two hours, but abrogated after three hours. Simultaneous TUNEL analysis demonstrated that death was present in all phases of the cell cycle (Fig. 1*c*, and data not shown). Induction of cell death may require *de novo* transcription or may reflect the triggering of pre-existing pathways. To test for such a requirement, we incubated REF(T)PML with $ZnCl_2$ and cycloheximide for two hours, allowing the synthesis of *PML* mRNA, but not its translation. Cells were then rapidly washed and incubated with actinomycin D alone, allowing translation of *PML* mRNA, but not mRNA neosynthesis. In this setting, cell death occurred essentially as in the absence of inhibitors, suggesting that *de novo* transcription was not required. PML-induced cell death could be specifically blocked by some batches of fetal calf sera, and a mixture of protective and non-protective sera (1:1) resulted in protection, indicating the presence of survival factors. Serum protection did not require *de novo* transcription and did not alter PML expression levels. Adding EGF, bFGF, IGF (I or II) or PDGF (A or B) to the medium failed to substitute for the protective effect of serum (data not shown).

Caspases are not activated during PML-triggered death

PML-induced cell death was not associated with typical nuclear morphological features of apoptosis such as chromatin condensation, even late in the process, in contrast with etoposide treatment (Fig. 1*d*). Electron microscopic examination revealed few nuclear defects (in particular, condensation), in contrast with cytoplasmic rounding-up (at 5 hours post-zinc induction) and complete cytoplasmic lysis (at 14 hours).

CPP32 (caspase 3), the major caspase executioner of apoptosis, appeared not to be activated during PML-induced cell death, as two substrates, poly(ADP-ribose)polymerase (PARP) and lamin B, remained uncleaved (Fig. 2*a*, and data not shown). Basal levels of colorimetric caspase substrates YVAD-pNA (caspase 1 class) and DEVD-pNA (caspase 3 class) were not enhanced following PML induction, in contrast with etoposide or IFNα exposure (Fig. 2*b*, and data not shown). Accordingly, the caspase inhibitors zVAD and BD, which significantly delayed etoposide-induced mitochondrial transmembrane potential loss and membrane phosphatidylserine externalization, had no preventive effect on PML-induced changes (Fig. 2*c*). Thus, those protease executioners inhibited by



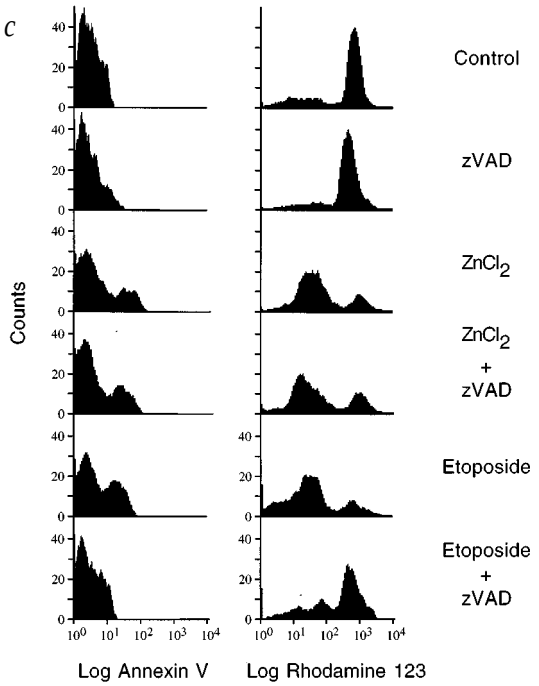


Fig. 2 PML-triggered death is not associated with caspase activation. ***a***, Absence of PARP cleavage during PML-triggered apoptosis. Cells were treated with $ZnCl_2$ (150 μM), etoposide or zVAD for the length of time indicated. ***b***, REF(T)Ø or PML cells were treated as indicated and CPP32 activity determined by DEVD-pNA cleavage. Relative absorbances from three independent determinations are shown. As $ZnCl_2$ was suggested to inhibit CPP32, DEVD-pNA cleavage induced by etoposide was assessed in the continuous presence or absence of $ZnCl_2$. $ZnCl_2$ did not affect DEVD-pNA cleavage. ***c***, Cytometric analysis of REF(T)PML cells treated with $ZnCl_2$, etoposide or the caspase inhibitor zVAD, and untreated cells. Annexin V-FITC (left) or rhodamine 123 (right) labelling.

zVAD are not required for PML-induced cell death. Paradoxically, zVAD (but not BD) accelerated PML-induced cell shrinkage, detachment and DNA fragmentation (71% TUNEL positivity with zVAD plus $ZnCl_2$ *versus* 45% with $ZnCl_2$ alone, whereas zVAD or control were below 8% positivity). In that respect, zVAD consistently increased PML protein levels in stably transfected clones, zinc-treated REF(T)PML or in IFN-treated cells (Fig. 3*a*, and data not shown), which probably accounts for zVAD enhancement of PML death. Other protease inhibitors (anticalpain I/II, E64, leupeptin, TPCK) did not alter the course of apoptosis. Cyclosporin A and aristolochic acid, previously reported to block mitochondrial permeability transition and BAX-triggered death[28], retarded appearance of PML-triggered mitochondrial permeability transition, but not PML death (data not shown).

Arsenic enhances PML-induced cell death

PML partitions between the nucleoplasm and NBs, and on PML overexpression (such as after IFN treatment) its transfer to NBs can be limiting[21,22]. In APL and non-APL cells, arsenic induces the targeting of nucleoplasmic PML to NBs, coupled to its covalent post-translational modification by PIC1/SUMO-1 (ref. 22; V. Lallemand, manuscript in preparation) and PML degradation[21]. When REF(T)PML cells were treated with $ZnCl_2$ and arsenic (10^{-6} M), we found a sharp acceleration in the morphological changes associated with cell death. At three hours, DNA cleavage, determined by TUNEL assays, was similarly augmented (77% positive cells for cotreatment with $ZnCl_2$ *versus* 45% for $ZnCl_2$ alone and less than 5% for arsenic or control). Arsenic increase in death induction paralleled PML localization on larger NBs, despite a decrease in the total PML content[21]. Thus, NB-associated, rather than total amount of PML protein correlates with induction of apoptosis.

Arsenic and zVAD enhance PML- and IFN-induced apoptosis

PML is a primary target gene of IFNα, β and γ (ref. 10). In REF(T), IFNα induced both *PML* expression (Fig. 3*a*) and caspase activation (Fig. 2*b*), leading to death by apoptosis. PML-induced death triggered by IFNα was enhanced by both zVAD and arsenic (42% TUNEL positivity for IFNα alone, compared with 60% and 63% with IFN/zVAD and IFN/arsenic, respectively). In this setting, zVAD again increased PML expression levels (Fig. 3*a*) and arsenic enhanced its NB localization, although decreasing the total amount of PML (Fig. 3*b*). The similarity of zVAD and arsenic synergy with PML- and IFN-triggered death suggests that, although IFNα induces caspase activation, PML has a role in IFN-triggered death.

Synergism between IFN and arsenic was also observed in different cellular systems, such as HeLa and U373 cell lines. Moreover, when primary monocytes were exposed to IFNα, they underwent a progressive apoptosis leading to the complete death of the culture in seven days (Fig. 3*c*). GM-CSF, G-CSF and IL3 rescued monocytes from IFNα-triggered death. The caspase inhibitor BD had no preventive effect, whereas zVAD accelerated IFNα-induced death, which now occurred in 12 hours in the whole population (Fig. 3*c*). Little or no death was encountered with zVAD alone for up to 20 days in most (8/11) primary cultures, although some death was apparent after 7 days for the 3 others.

PML recruits BAX and p27KIP1 to nuclear bodies

As BAX was also shown to induce caspase-independent death, we examined whether PML influenced the expression of BAX. Unexpectedly, double labelling of HeLa or Cos-6 cells with anti-PML and anti-BAX antibodies showed a cytoplasmic localization of BAX and the colocalization of endogenous nuclear BAX and

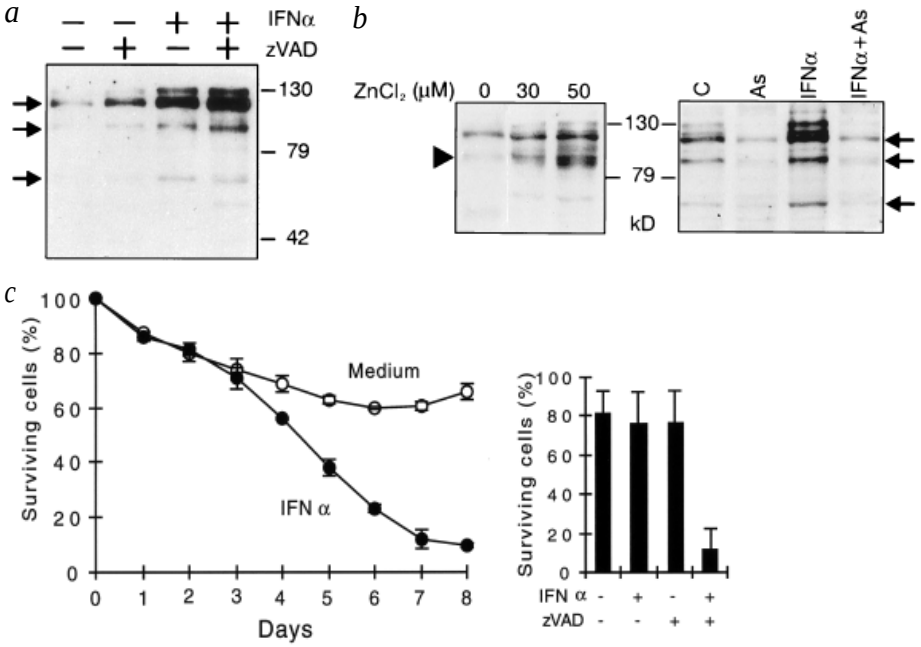


Fig. 3 IFN induces zVAD-accelerated cell death. ***a***, zVAD stabilizes PML in REF(T) cells. ***b***, IFNα (1000 U/ml) induces rat PML in REF(T)PML cells. Arsenic leads to the complete degradation of PML isoforms (arrows), indicating their specificity. Similar levels of PML expression are found following induction with 50 μM $ZnCl_2$ (assuming a similar affinity of the antibody for human and endogenous rat PML). ***c***, Left, survival of monocytes treated with 1000 U/ml IFNα. One experiment of every five is shown. TUNEL assays demonstrate that the decrease in cell numbers is due to apoptosis. Right, histograms indicate the effect of zVAD (100 μM) addition at 24 h. Mean±s.d. of 11 experiments are shown.

PML on NBs (Fig. 4*a*,*b*). Immunoelectron microscopy demonstrated a preferential association of BAX to the periphery of NBs (data not shown). Transfection of *PML* or *PML/RARA* in HeLa cells induced the recruitment of endogenous BAX to PML-labelled structures, arguing for a contact between BAX and PML (Fig. 4*a*). Similarly, zinc treatment of REF(T)PML cells induced NB targeting of BAX (data not shown). In APL cells, BAX, similar to PML and all NB antigens tested to date, displayed a microspeckled fluorescence which shifted to a normal one on retinoic acid or arsenic treatment (data not shown).

To ensure that recruitment of BAX also occurs in untransfected cells, we treated HeLa cells with IFN and/or arsenic to induce both PML expression and its NB targeting. IFN alone enhanced BAX localization to NBs (Fig. 4*b*). Combined with IFN, arsenic treatment enhanced BAX recruitment (Fig. 4*b*). Arsenic alone only slightly enhanced NB association of BAX. As the total amount of BAX did not vary (data not shown), these modifications result from BAX recruitment to NBs. However, no evidence for a direct interaction between PML and BAX could be demonstrated by GST-pulldown experiments or co-immunoprecipitations. Similar observations were made for the cdk inhibitor p27KIP1, which is partially NB associated, recruited by IFN/arsenic treatment (data not shown), delocalized in APL cells and retargeted by RA or arsenic treatment (Fig. 4*c*). Altogether, BAX and p27KIP1 are identified as two novel NB antigens whose NB targeting parallels induction of apoptosis.

BAX was recently shown to be a cytoplasmic protein that is targeted to the mitochondria after induction of apoptosis[29]. Fractionation experiments of HeLa cell lysates into cytoplasm, mitochondria and nuclei confirmed that a small fraction of BAX was in the nucleus, whereas no BAX was detectable in mitochondria (Fig. 4*d*). Recent studies have demonstrated the shift of BAX

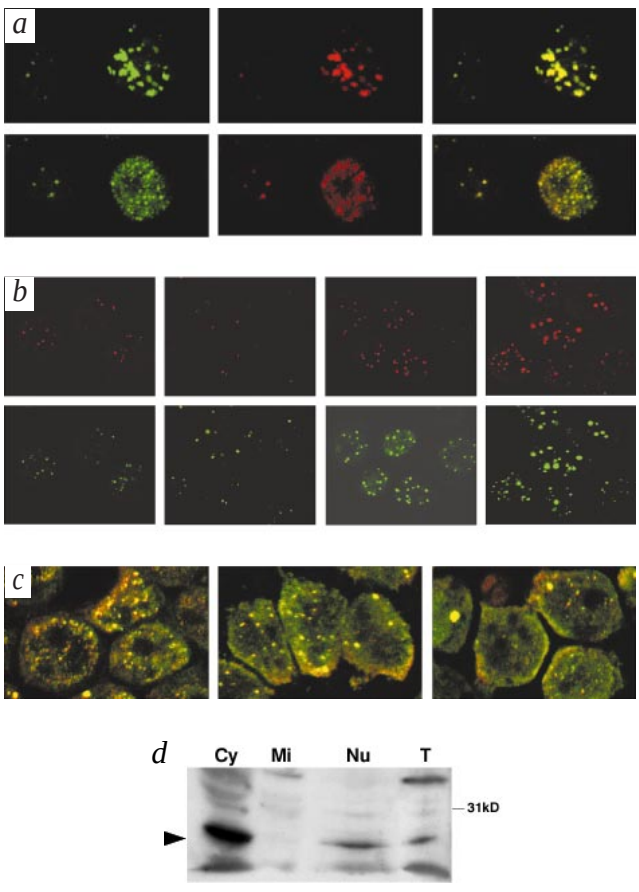


Fig. 4 BAX and p27KIP are NB antigens. ***a***, HeLa cells were transfected with a *PML* (top) or a *PML/RARA* (bottom) expression vector, double-stained with anti-PML (green, left) and anti-BAX (red, middle) or both (right), and analysed by confocal microscopy. Although as expected, cytoplasmic staining of BAX is found (although hardly visible at these settings of the confocal microscope), colocalization between endogenous PML and endogenous nucleoplasmic BAX is observed in the untransfected cell on the left. In transfected cells, overexpressed PML or PML/RARA recruit BAX. ***b***, Endogenous PML recruits BAX to NBs. HeLa cells were treated with IFN and/or arsenic for 24 h and double-stained with BAX (red, top) or PML (green, bottom). From left to right: untreated, arsenic, interferon, arsenic and interferon. Note the appearance of a diffuse nucleoplasmic PML staining after interferon treatment that disappears on addition of arsenic. ***c***, NB4 cells were treated with vehicle (left), retinoic acid 10^{-6} M (centre) or arsenic (right). Cells were double stained with anti-p27KIP1 (green) or PML (red) and analysed by confocal microscopy. Note the shift from the microspeckled pattern (right) to standard PML NBs (centre) or single, large, typical arsenic-exposed PML bodies (right). ***d***, A fraction of BAX protein is located in the nucleus. HeLa cells were lysed and fractionated into cytoplasm/light membranes (Cy), mitochondria/lysosomes (Mi) and nuclei (Nu). The total extract of HeLa cells (T) is used as a positive control.

from cytoplasm to nucleus during two distinct apoptotic processes[30,31], consistent with our localization studies.

Discussion

PML triggers caspase-independent cell death. Our results demonstrate that PML expression triggers caspase-independent death in the absence of *de novo* transcription. The absence of chromatin condensation, nuclear fragmentation and internucleosomal DNA cleavage is consistent with the lack of caspase 3 activation, known to be required for apoptosis-related nuclear changes[32]. Several cell death inducers, including BAX and MYC expression, ceramides, inhibition of the ubiquitin degradation pathway and adenovirus E4orf4, have been shown to induce apoptosis in the presence of zVAD and BD, suggesting that caspase-independent executors of cell death exist[25,26,33,34]. The selective target of zVAD involved in enhancement of PML- or IFN-triggered apoptosis remains to be identified. Inhibition of PML degradation by a caspase or non-caspase protease is a potential mechanism, because etoposide-induced death led to the appearance of distinct, shorter PML fragments, which disappeared upon zVAD exposure (data not shown). Although BAX, similar to PML, has the ability to induce cell death in the presence of caspase inhibitors, its overexpression, in contrast with PML, triggers caspase (including caspase-3) activation. Our findings represent the first identification, to our knowledge, of a death pathway mediated by an endogenous gene product that does not trigger caspase activation. Therefore, it is possible that PML reveals the existence of a central, yet unsuspected, executionary pathway that neither requires nor involves caspase activation.

Is the death signal recruitment to NBs? Despite the fact that PML was reported to rapidly shuttle between the nucleus and the cytoplasm[35], it is unexpected that a nuclear protein would principally trigger cytoplasmic signs of apoptosis. When PML expression is high, the majority of the protein is in the nucleoplasm rather than in the nuclear matrix[21,22]. The fact that arsenic, which accelerates PML targeting to NBs and degrades PML, accelerates PML-triggered death suggests the NB-associated PML protein is critical for death induction. Accordingly, arsenic enhancement of PML death was only evident with high PML levels, when its NB-targeting becomes limiting. PML traffic to NBs *per se* could be the critical determinant for death, for example, by recruiting other NB proteins. Previously, PML was suggested to recruit SP100 and PLZF to NBs (refs 21,36). Here, we demonstrate that it is also the case for BAX and p27KIP1. Recruitment of proapoptotic proteins to NBs, or alternatively, titration of survival factors from the cytoplasm or nucleoplasm, may constitute the death signal. PIC-1, whose binding to PML is greatly enhanced by arsenic, could represent an attractive target as its overexpression was proposed to modulate apoptosis[37]. Following submission of this article, arsenic was proposed to induce apoptosis in a PML-independent manner[38]. The arsenic trioxide concentrations that lead to significant death in their experimental system were 10-fold higher than the maximal concentration that we used, and it is possible that in such conditions arsenic triggers a PML-independent death.

Multiple roles of the PML death pathway. Apart from interferon stimulation[10], exposure to genotoxic agents was recently suggested to induce *PML* expression[39] (data not shown). Resistance of $Pml^{-/-}$ mice and cells, not only to IFNs, but also to a variety of apoptosis inducers[40], suggests a broad involvement of the PML death pathway. Studies on immunostained human carcinomas demonstrated a correlation between loss of PML expression and tumour invasion, consistent with loss of apoptotic control[6]. Many DNA viruses have developed specific strategies to block apoptosis[41]. Alterations in PML NB localization have been found in many viral infections, for example, the immediate-early HSV1 gene *ICPO* was shown to specifically degrade PML (refs 42,43).

Implications for APL pathogenesis. In APL, PML/RARA delocalizes PML and all other known NB antigens[13–16]. PML/RARA expression has been shown to antagonize apoptosis[44] induced by serum deprivation or TNFα (ref. 45). Conversely, arsenic induces apoptosis, as it retargets NB antigens to their normal localization[21,46]. We suggest that the enhanced targeting of NB antigens by arsenic is, at least in part, responsible for triggering PML-mediated death in APL cells. In that respect, arsenic-induced death of APL cells is not blocked by zVAD (M. Gianni, manuscript in preparation), and RA-induced degradation of PML/RARA sharply sensitizes NB4 cells to arsenic-triggered apoptosis[47], consistent with a traffic/recruitment model.

Clinical implications. Our findings argue for a cautious approach to therapies aimed at blocking apoptosis through the broad inhibition of caspases, as agents such as zVAD may exacerbate PML- and IFN-induced death. Findings of zVAD-mediated enhancement of TNF-induced death have been very recently reported, supporting the view that caspase may, in some instances, be involved in protection against cell death[48]. Our data suggest that PML participates in IFN-induced cell death. zVAD or arsenic potentiation of IFN-induced death may have important therapeutic implications in IFNα-responsive malignancies. For example, we have recently observed a dramatic

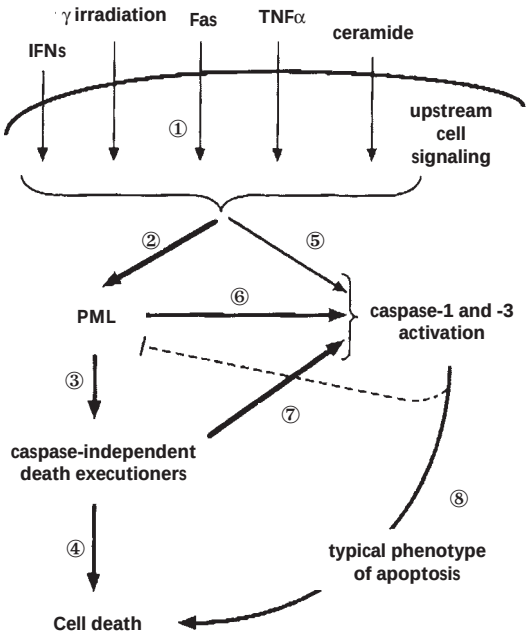


Fig. 5 A model for a pivotal role of PML in cell death (ref. 40 and this paper). 1–4, PML as an inducer of caspase-independent death pathway. Various exogenous cell signals (1) that may or not trigger upstream signalling caspases all converge towards PML (2). PML then activates caspase-independent cell death executioners (3), leading to cytoplasmic features of apoptosis. 5–8, PML as a checkpoint for caspase-1 and -3 activation. Caspase-1 and -3 activation occurs downstream of PML and requires both exogenous cell signal transduction (1 and 5) and PML-induced signalling (2), either upstream (6) or downstream (7) of PML-induced activation of caspase-independent executioners. Caspase-3 cleavage (8) then induces the typical nuclear phenotype of apoptosis and may cleave PML (hatched line), exerting a negative feedback that delays the PML-induced cell death process.

potentiation of arsenic and IFNα in cells of acute T cell leukaemia, an IFN-sensitive malignancy[49].

A novel and central cell death pathway? After submission of this manuscript, we became aware of results[40] showing that mouse PML (which is dispensable for normal embryonic development[9]) is required after birth both for caspase-1 and -3 activation, and for cell death induced by several exogenous death signals, including IFNs, Fas ligation, TNFα and ceramides. This suggests that this role of PML in programmed cell death induction may be related to its capacity to activate caspases. Our results, however, indicate that PML induces cell death in the absence of caspase activation. Can these findings be reconciled? One obvious possible explanation is the use, in both studies, of different cell populations. We propose another, unifying, interpretation (Fig. 5). Accordingly, the apparent divergence between the two studies could reveal the existence of a central and yet unsuspected cell death pathway in which PML acts as a pivotal checkpoint. On one hand, PML expression directly triggers the activation of caspase-independent cell death executioners that induce most cytoplasmic features of apoptosis. On the other hand, caspase-1 and -3 activation, in response to several exogenous death stimuli, requires both PML and upstream transduction of these exogenous cell signals to lead to the typical nuclear features of apoptosis.

Such a model provides an explanation for the paradoxical report that several death processes associated with caspase activation are not blocked by caspase inhibitors (the sole effect of the inhibitors being the blockade of the nuclear phenotype of apoptosis). An extreme implication of this model is that in most instances in which caspase inhibitors block the induction of cell death (as exemplified by Fas-mediated death), such effect is not due to a blockade of the executioners of cell death, but of the upstream caspase-mediated cell signalling (that is, the activation of caspase-8 by the death domain associated to the cytoplasmic portion of the Fas receptor). The possible existence of an ancient and evolutionarily conserved caspase-independent cell death pathway[50] is further suggested by the findings that human proapoptotic genes such as *BAX* and *BAK* (and the *Caenorhabditis elegans* gene *CED4*) can induce cell death in yeast, despite the fact that yeast appear to lack caspase[51].

Methods

Plasmid construction. A *Sac*I-*Bgl*II fragment (–69, +55 bp) from the mouse metallothionein promoter inserted in a pKS background was fused to the *Bgl*II-*Bam*HI fragment of a human *PML* cDNA (ref. 52), yielding pKSmMT-PML. For the GFP-PML fusion, the same PML fragment was inserted in the *Bgl*II site of pEGFP-1 vector (Clontech). A retroviral vector expressing PML was constructed by inserting a full-length human *PML* cDNA (ref. 52) at the *Eco*RI site in SRαtkneo.

Cell culture. REF(T) are pooled rat embryo fibroblasts immortalized by an SV40T expression vector. For clonogenicity assays, cells were transfected with SRatkneo-PML (10 μg) or SRαtkneo in 100 mm culture dishes and G418 selected (500 μg/ml). To obtain inducible clones, REF(T) were cotransfected with pKSmMT-PML and a hygromycin resistance vector (DSP-Hygro). Resistant colonies were examined for PML expression after 4 h $ZnCl_2$ treatment (150 μM) and subjected to a second round of cloning by limiting dilution. CHO inducible clones were similarly constructed. Monocytes were prepared as described[53]. Etoposide (Biomol Research Laboratories) was used at 100 μM for 16–24 h. zVAD (benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone, Bachem) was used at 250 μg/ml. Rat IFNα was from Access BioMedical. Human IFNα was kindly provided by Schering-Plough. Antibodies against human PML were as described[13]. Western-blot analysis of endogenous rat PML was performed with the 5E10 monoclonal, which detects both human and rat PML. BAX was detected using an affinity purified rabbit polyclonal directed against aa 80–98 (Santa Cruz). Two other antibodies directed against other peptides (11–30, Santa Cruz; 12–24, Zymed) gave similar results, although much weaker. p27KIP1 was detected using a monoclonal antibody (Transduction Laboratories).

Assays for apoptosis. Cells were treated for 2 h with $ZnCl_2$ (150 μM, unless otherwise indicated) in the presence or absence of heat-inactivated fetal calf serum, washed and incubated in $ZnCl_2$-free medium. The TUNEL assay was performed according to the manufacturer's instructions (Boehringer; *in situ* cell death detection kit) except for the fixation step (4% formaldehyde in PBS for 10 min). Cellular DNA content was assessed by a 10-min incubation in propidium iodide (50 μg/ml) and RNase A (100 μg/ml) at 4 °C. Analysis of phosphatidylserine expression on the outer leaflet of cell-membranes was performed by using Annexin-V-Fluos (Boehringer) and loss of mitochondrial polarity by rhodamine 123 (Molecular Probes), according to the manufacturer's instructions. Samples were analysed on a FACScan analyser (LYSIS II software, Becton Dickinson). For caspase substrate cleavage, 5×10^6 cells were washed in PBS and incubated for 1 h at 4 °C in lysis buffer (200 μl; 10 mM Hepes pH 7.4, 2 mM EDTA, 2 mM DTT, 0.1% CHAPS). After centrifugation, supernatant (20 μl) and reaction buffer (180 μl; 100 mM Hepes pH 7.4, 2% glycerol, 5 mM DTT, 0.5 mM EDTA, 50 μM DEVD-pNA, Biomol Research Laboratories) were mixed and absorbance at 405 nm was measured after 4 h of incubation at 37 °C. The SA-252 anti-PARP polyclonal antibody was purchased (Biomol Research Laboratories).

Cell fractionation. After washing once in cold PBS and dounce homogenization of cells (in 250 mM sucrose, 10 mM Tris pH 7.4), the fractions were separated by differential centrifugation sedimentation[54]. Sub-cellular fractions were resuspended in sample buffer and aliquots of each fraction corresponding to 5×10^6 cells were subjected to western-blot analysis.

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