



Pim-1 kinase protects hematopoietic FDC cells from genotoxin-induced death

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The hematopoietic cell S/T kinase Pim-1 was originally discovered as a target of murine leukemia provirus integration, and when expressed at increased levels is predisposing to lymphomagenesis. Recently, Pim-1 has been shown to enhance the activities of p100, c-Myb and cdc25a, and in part this might explain reported effects on mitogenesis. In the context of cytokine withdrawal, Pim-1 also can attenuate programmed cell death (PCD). Cytokine withdrawal, however, alters signaling pathways and can complicate the dissection of mitogenic vs apoptotic responses. To better study possible effects of Pim-1 on PCD, a hematopoietic cell model was developed in which proliferation was supported efficiently by SCF plus EPO in the absence of endogenous Pim-1 gene expression. This was provided by factor-dependent FDCW2 cells that express endogenous and functional c-Kit, and were transfected stably with truncated Epo receptor form mutated at a Y343 STAT5 binding site. In proliferating cells, exogenously expressed Pim-1 was observed to efficiently inhibit PCD as induced by either Co⁶⁰ or adriamycin, and the dose-dependent nature of this effect was established in several independent clones. By comparison, effects of exogenous Pim-1 on mitogenesis were nominal. In addition, in cell fractionation studies an estimated 25% of M_r 34 000 Pim-1 (but not M_r 44 000 Pim-1) was present in nuclear extracts. Thus, Pim-1 efficiently buffers hematopoietic progenitor cells against death as induced by several clinically important apoptotic agents, and may directly target nuclear effectors. *Oncogene* (2000) 19, 3684–3692.

Keywords: Pim-1 kinase; genotoxin-induced apoptosis

Introduction

Pim-1 is a S/T kinase that is structurally related most closely in its catalytic domain to cGMP-dependent and phosphorylase kinases (Selten *et al.*, 1986). Pim-1 expression is relatively high in cells of hemato-lymphoid origin, and is induced primarily in response to cytokines which act via a subset of Janus kinase-linked type 1 receptors (including those for IL-2, IL-3, IL-6, IL-9, Epo, C-CSF, GM-CSF, Tpo and prolactin)

(Buckley *et al.*, 1995; Dautry *et al.*, 1988; Demoulin *et al.*, 1999; Lilly *et al.*, 1992; Miura *et al.*, 1994; Nagata and Todokoro, 1995). Transcript expression has also been characterized in testes (Wingett *et al.*, 1992), carcinoma-derived keratinocytes (Stewart and Rice, 1995) and in neuronal cells of the hippocampus (together with pim-2 and -3) (Konietzko *et al.*, 1999). Originally, Pim-1 was discovered as a common site for murine leukemia provirus integration wherein integration typically within a 3' non-coding exon increases Pim-1 expression (Selten *et al.*, 1986), and predisposes T cells to transformation (van Lohuizen *et al.*, 1989). In mice nullizygous for Pim-1, normal hematopoiesis is largely unaffected with the exception of a detectable erythroid cell microcytosis (Laird *et al.*, 1993). In primary cultures of marrow from Pim-1^{-/-} mice, however, diminutions in IL-7-dependent B cell colony formation (Domen *et al.*, 1993a) and IL-3-dependent mast cell proliferation are apparent (Domen *et al.*, 1993b). More recently, through proviral tagging of Pim-1^{-/-} Eμ-myc mice, a related Pim-2 proto-oncogene has also been cloned which likewise is expressed in hematopoietic cells (van der Lugt *et al.*, 1995). In addition, transcripts from Pim-1 and Pim-2 genes can be translated not only from a canonical AUG codon to yield a M_r 34 000 kinases but also from upstream CUG codons to yield a M_r 44 000 species (Saris *et al.*, 1991; van der Lugt *et al.*, 1995).

Despite these advances in understanding Pim kinase structure and expression, the nature of Pim-1's physiological substrates and the pathways they affect have remained relatively obscure. One well-defined property is the ability of Pim-1 to act in strong synergy with c-Myc and N-Myc in promoting lymphomagenesis, especially as studied in c-Myc mice expressing Pim-1 from an immunoglobulin heavy chain enhancer cassette (van Lohuizen *et al.*, 1989). This has led to one model wherein Pim-1 might directly enforce c-Myc pathways, and in transformed Rat1 fibroblasts Pim-1 has been reported to phosphorylate and activate cdc25a (Mochizuki *et al.*, 1999), a putative c-Myc target gene. Recently, Pim-1 has also been shown to enhance the activity of the hematopoietic transcription factor, c-Myb (Levenson *et al.*, 1998), and this appears to involve an indirect mechanism whereby Pim-1 phosphorylates the transcriptional co-activator p100. Given the established role of c-Myb as a hematopoietic oncogene (Weston, 1999) this further broadens the oncogenic significance of Pim-1.

A second set of pathways that Pim-1 has been demonstrated to affect are those leading to pro-

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grammed cell death (PCD). Early studies in EμPim-1 mice documented a survival advantage for CD4⁺/CD8⁺ thymocytes exposed to dexamethasone (Moroy *et al.*, 1993). In addition, recent studies in IL-3-dependent FDCP1 cell lines have demonstrated a Pim-1-dependent inhibition from the apoptosis which occurs following the withdrawal of IL-3 (Lilly and Kraft, 1997). This includes the inhibition, due to Pim-1 expression, of reactive oxygen species production and of membrane potential decay in mitochondria (Lilly *et al.*, 1999). Cytokine withdrawal, however, disrupts numerous events which are ongoing in proliferating progenitor cells including cytokine-dependent gene transcription events and mitogenesis *per se*. In addition, proliferative status can significantly affect sensitivity to clinically applied apoptotic agents (Siu *et al.*, 1999). Therefore, in the present investigation an FDC cell line model has first been developed in which growth and survival are supported at normal levels in the presence of SCF (via endogenous c-Kit) plus Epo (via an ectopically expressed Epo receptor truncation mutant), but in which endogenous Pim-1 gene expression is not activated. In these FDCER-HY343F cells, the ability of exogenously expressed Pim-1 kinase to inhibit Co⁶⁰ and adriamycin induced PCD in proliferating cells was investigated. Pim-1 proved to selectively protect against PCD due to each agent. By comparison, effects of exogenous Pim-1 on mitogenesis were nominal. Finally, M_r 34 000 Pim-1 (but not M_r 44 000 Pim-1) was also shown to reside in nuclei, and this at least in part may explain Pim-1's ability to modulate several nuclear effectors (Koike *et al.*, 2000; Leverson *et al.*, 1998; Mochizuki *et al.*, 1999; Weston, 1999).

Results

FDCER-HY343F cells as a model system for studies of Pim-1 effects on genotoxin-induced PCD in proliferating progenitor cells

Towards choosing a cell line model for the present investigations of Pim-1 effects on PCD, the expression of endogenous pim transcripts in several IL-3-dependent hematopoietic progenitor cell lines (FDCW2ER-H, 32D, BaF/3) first was assayed (Figure 1, upper left panel). Pim-1 was expressed at comparable levels in each line. 32D and BaF/3 cells, however, proved to express significantly higher levels of Pim-2 transcripts. To avoid potential complications associated with predicted overlapping activities of Pim-1 and Pim-2 (van der Lugt *et al.*, 1995), FDCW2 cell lines were used in subsequent studies. In addition, the ectopic expression of Pim-1 in BaF/3 cells somewhat unexpectedly has been observed to promote factor-independent growth (Nosaka *et al.*, 1999) (Joneja and Wojchowski, unpublished result). Since Pim-1 itself is not transforming (van Lohuizen *et al.*, 1989) this outcome may depend upon a pre-existing mutation or otherwise altered growth regulatory pathway in BaF/3 cells (and in fact has been reported for high-passage FDCP1 cells) (Lilly and Kraft, 1997). In the FDCW2 cell line model chosen for further investigations, the regulated transcription of the endogenous Pim-1 gene via a stably transfected minimal Epo receptor H form was

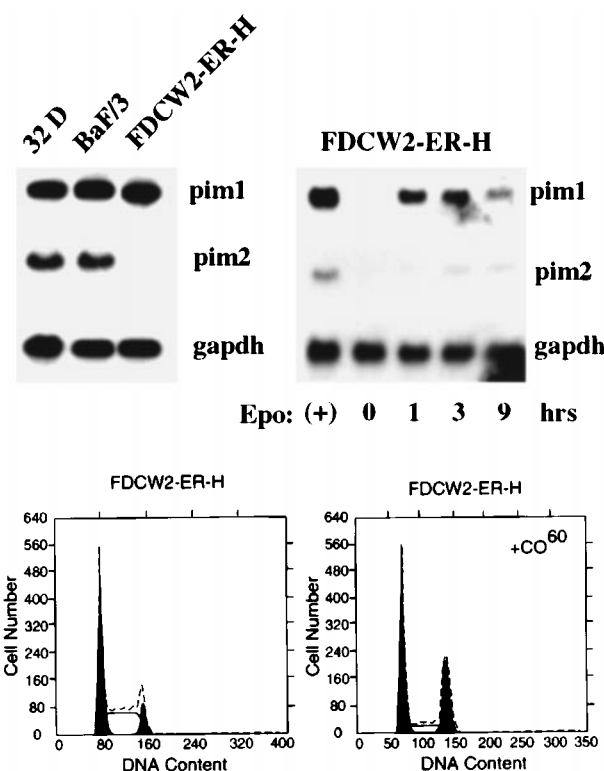


Figure 1 Expression of Pim-1 and 2 transcripts in FDCW2-ERH, 32D, and BaF/3 cells, and wt p53-mediated cell cycle arrest of FDCW2-ER-H cells in response to Co⁶⁰. Upper panel: In FDCW2-ERH, 32D and BaF/3 cells cultured in IL-3 (left panel) Pim-1 transcripts were observed to be expressed at comparable levels while Pim-2 transcript levels were higher in 32D and BaF/3 cells. In FDCW2-ERH cells Pim-1 gene transcription was activated efficiently by Epo following cytokine withdrawal (right panel). 'Plus' (+) indicates growing cells. Otherwise, cells were deprived of cytokines for 10 h, and then exposed to Epo (10 U/ml) for the intervals indicated. Lower panel: Exposure of FDCW2-ERH cells to Co⁶⁰ efficiently arrested cell cycle progression at G1/S and G2/M phases (left panel, exponentially growing cells; right panel, exponentially growing cells 6 h post-irradiation, Co⁶⁰)

also confirmed (Figure 1, upper right panel). In addition, exposure to ionizing radiation was shown to induce an accumulation of FDCW2 cells at G1/S and G2/M phases of the cell cycle (Figure 1, lower panels) indicative of wt p53 activity.

Next, in FDCW2 cells stably expressing a minimal H Epo receptor form (i.e., FDCER-HY343 cells), exogenous Pim-1 was expressed (via stable transfection with pEF-Neo-Pim-1) and its ability to inhibit PCD as induced by cytokine withdrawal was assayed. As shown in Figure 2a (upper panel), this activity was confirmed, and Pim-1 proved to attenuate this pathway to PCD 3–4-fold as compared to parental FDCER-HY343 cells. Also investigated were the combined effects of exposure to either Co⁶⁰ or adriamycin and subsequent cytokine withdrawal. In these latter treatment groups, PCD as induced in quiescent cells via these routes likewise was attenuated significantly by ectopically expressed Pim-1 (Figure 2a, center and lower panels). To confirm the programmed nature of death events, PCD due to cytokine withdrawal also was accessed by flow cytometric TUNEL assays (Figure 2b, upper panel). Finally, in these primary experiments the sustained expression of exogenous

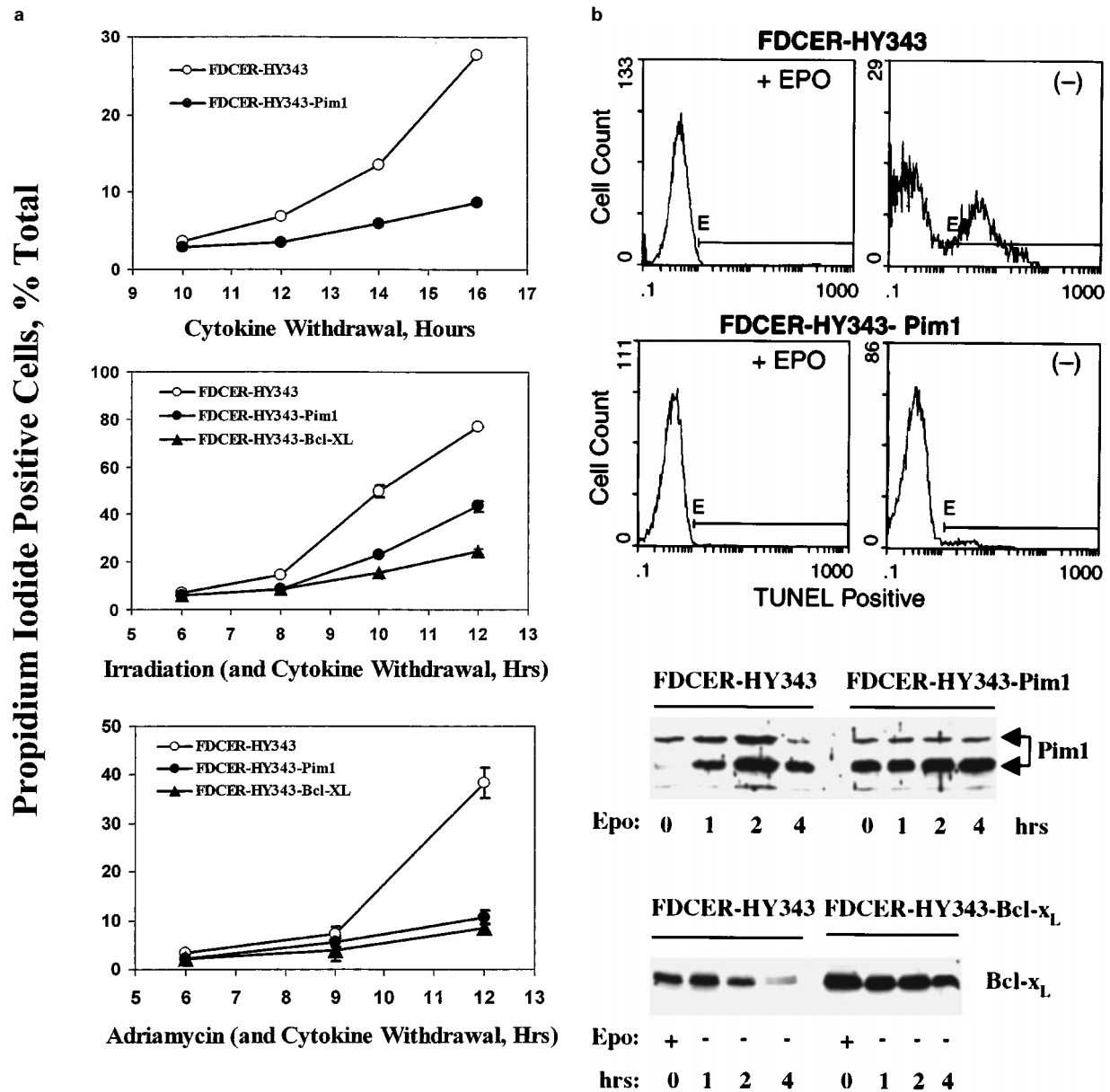


Figure 2 Ectopic expression of Pim-1 in FDCW2-ERH cells inhibits PCD due to cytokine withdrawal alone, and in combination with Co^{60} or adriamycin. (a) Pim-1 was expressed in FDCW2-ERH cells using a dicistronic vector, and effects on apoptosis in several contexts of cytokine withdrawal were assayed, including withdrawal of IL-3 (upper panel), exposure to Co^{60} followed by cytokine withdrawal (center panel) and exposure to adriamycin followed by cytokine withdrawal (lower panel). For combined treatments, protection against PCD due to Pim-1 expression (closed circles) was compared to that afforded by ectopically expressed Bcl-xL (closed triangles). Here, cell death was assayed based on staining with PI. (b) Pim-1-dependent protection from PCD due to cytokine withdrawal was also assayed by flow TUNEL (upper panels). Levels of Pim-1 and Bcl-xL expression in FDCER-HY343 cells (i.e., FDCER-HY343F, Pim-1 and FDCER-HY343-Bcl-xL cells) were assayed by Western blotting following IL-3 withdrawal and subsequent stimulation with Epo for the indicated intervals (lower panels)

Pim-1 and Bcl-x_L in the above FDCER-HY343 derived cell lines was confirmed by Western blotting (Figure 2b, lower panel).

Cytokine withdrawal alters the expression of many cytokine response genes (including Pim-1), and cells senesce in G_1/G_0 . In such cells it is uncertain whether observed effects of ectopically expressed Pim-1 might reflect those exerted in growing cells. Also, sensitivities of growing vs quiescent cells to anti-neoplastic agents can differ markedly. In subsequent experiments therefore, conditions were sought under which factor-dependent FDCW2 cells could be propagated at normal rates via a route which circumvents endogenous Pim-1 expression. Previously our laboratory has

shown that FDCW2 cells express c-Kit, and proliferate at moderate rates in response to SCF (Jones et al., 1997). In the present study, c-Kit was discovered to act in mitogenic synergy with an Epo receptor form which retains no cytoplasmic (P)Y residues, and proved to be essentially inactive in growth and survival signaling, i.e., ER-HY343F. This receptor form and its related parent truncation mutant, ER-HY343, are diagrammed in Figure 3 (upper panel). In FDCER-HY343F cells, IL-3 but not Epo plus SCF supported Pim-1 gene expression (Figure 3, center panel). However, as indicated above, Epo plus SCF did support the growth of FDCER-HY343F cells at normal rates (Figure 3, lower panel). This system (which relies on signals

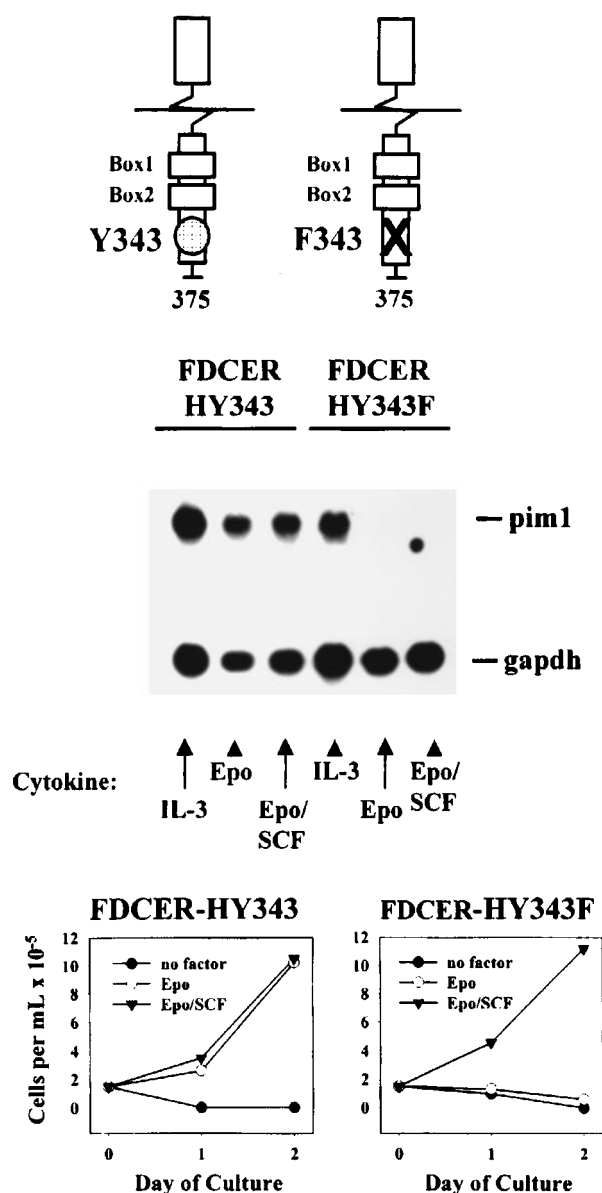


Figure 3 Growth, but not Pim-1 expression, is supported efficiently by Epo plus SCF in FDCER-HY343F cells. Upper panel: To provide a model system for tests of effects of exogenous Pim-1 expression in proliferating hematopoietic cells, FDC(W2) cells were transfected stably with the diagrammed truncated Epo receptor form mutated at Y343 (a site required for STAT5 binding and for induced Pim-1 gene transcription) to phenylalanine (Y343F). Center panel: In these cells IL-3, but not SCF plus Epo, supported Pim-1 gene transcription (as shown here by Northern blotting). Lower panel: Nonetheless, SCF plus Epo efficiently supported the growth of FDCER-HY343F cells at normal rates in the absence of Pim-1 expression (as determined by viable cell counts)

normally co-propagated by endogenous c-Kit and Epo receptors) (Wu *et al.*, 1995) therefore provided the opportunity to next selectively test in a proliferating, yet essentially Pim-1 null background, possible effects of exogenously expressed Pim-1 on PCD as induced by genotoxic agents, and on mitogenesis.

Pim-1 inhibits Co⁶⁰ or adriamycin induced PCD in proliferating FDCER-HY343F cells

Towards testing effects of Pim-1 on genotoxin-induced PCD, FDCER-HY343F cells were electrotransfected

with a pEF-Neo-Pim-1 expression vector, and clones which stably expressed exogenous Pim-1 at appreciable levels were identified initially by Northern blotting (see Figure 5 below). Next, the induction of PCD by Co⁶⁰ or adriamycin in the representative clonal lines FDCER-HY343F-Pim-1 -c7 and -c11 (vs control Pim-1-deficient parental cells, each propagated in SCF plus Epo) was assayed. As illustrated in Figure 4, Pim-1 proved to buffer against PCD as induced by each of these agents. For Co⁶⁰ (Figure 4, left panels), induced death events were inhibited on average 2.76-fold (± 0.55) as compared to parental Pim-1-deficient controls. For adriamycin, protection against PCD due to Pim-1 was 2.37-fold (± 0.61) (Figure 4, right panels). Pim-1 expression levels in clones studied in these experiments were assayed in advance by Northern and Western blotting, and inhibition of PCD due to cytokine withdrawal was also confirmed in all clones studied. The dose-dependent relationship between levels of Pim-1 expression and ability to inhibit apoptosis in response to adriamycin or Co⁶⁰ is illustrated in Figures 5 and 6. In addition, in the course of Western blotting experiments whether Pim-1 occurred only within the cytoplasm was also assessed. Interestingly, an estimated 25% of M_r 34 000 Pim-1 (but not M_r 44 000 Pim-1) was discovered within nuclear extracts (Figure 6, lower panel). Integrity of nuclear (vs cytoplasmic) extracts also was confirmed by Western blotting with a monoclonal antibody to nucleoporin (data not shown). FDCW2 cell lines also express Fas, and are sensitive to Fas ligand-induced PCD (Itoh *et al.*, 1993). Since Fas-mediated pathways to caspase activation and death are comparably well understood (Konopleva *et al.*, 1999), Pim-1 also was tested as a possible modulator of this more direct PCD pathway but proved ineffective in repeated experiments (Pircher, Zhao, unpublished results).

Pim-1 does not substantially enhance cytokine-dependent FDCW2 cell growth, or c-myc transcript expression

As introduced above, the ectopic expression of Pim-1 in at least certain hematopoietic cell lines can affect growth (Nosaka *et al.*, 1999). It was therefore important to test whether any such effects were exerted by exogenously expressed Pim-1 in FDCER-HY343F-Pim-1 cells. As illustrated by [³H]dT incorporation assays (Figure 7a, upper panel) and as determined by cell count assays (Pircher, unpublished) for four representative clones studied, exogenous Pim-1 expression did not effectively promote proliferation in the absence or presence of cytokines. An examination of [³H]dT incorporation in the absence of cytokines revealed that exogenous Pim-1 increased background [³H]dT incorporation (Figure 7a, lower panel). However, this increase was only 1–3% of cytokine-induced rates and as mentioned above, mitogenesis (as assayed by direct cell counts) was not supported. For the Epo receptor form HY343F, signaling activity was confirmed in experiments which demonstrated its ability to support Epo-stimulated *c-myc* transcription (Figure 7b). Thus, in contrast to effects on PCD, overall effects of Pim-1 on proliferation in this model system (and in 32D cells as well, Joneja, data not shown) were nominal. Finally, because Pim-1 has recently been reported to activate Cdc25a and therefore to possibly

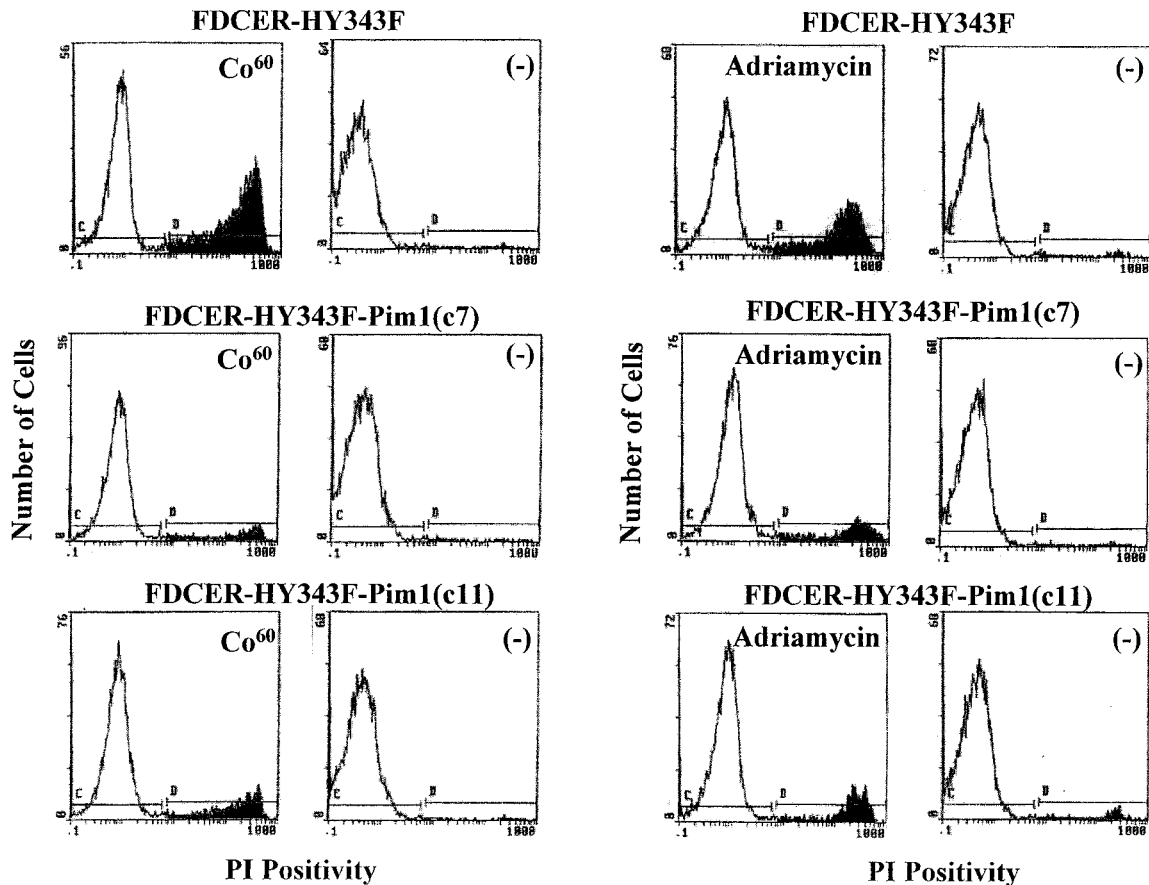


Figure 4 Ectopic expression of Pim-1 in proliferating FDCER-HY343F cells protects against PCD due to Co^{60} and adriamycin. FDCER-HY343F cells or derived transfected lines ectopically expressing Pim-1 (FDCER-HY343F-Pim-1 (c7) and (c11)) were expanded in SCF plus Epo. Apoptotic sensitivities of growing cells to Co^{60} (left panels) or adriamycin (right panels) then were assayed (PI positivity). Frequencies of PCD due to Co^{60} were inhibited by Pim-1 on average 2.76-fold (± 0.55) ($n = \text{four experiments}$), while PCD due to adriamycin was inhibited 2.37-fold (± 0.61) ($n = \text{four experiments}$)

stimulate *c-myc* gene expression (Mochizuki *et al.*, 1999), levels of *c-myc* transcripts in Pim-1 expressing clones were also assayed by Northern blotting and phosphorimaging. However, the results of repeated independent experiments reproducibly showed *c-myc* transcript levels in these lines to be 40–50% lower than levels in parental FDCER-HY343F cells (Pircher, unpublished).

Discussion

Based on reports that Pim-1 can attenuate PCD as induced in hematopoietic progenitor cells by cytokine withdrawal (Lilly and Kraft, 1997; Lilly *et al.*, 1999) or in B cells by dexamethasone (Moroy *et al.*, 1993), whether Pim-1 might also buffer PCD in growing progenitor cells as induced by Co^{60} or adriamycin was investigated. Specifically, an approach was developed by which factor dependent FDCW2 cells could be propagated at normal rates in the absence of endogenously expressed Pim-1. Here, endogenous *c-Kit* plus an exogenously expressed Epo receptor form lacking all cytoplasmic (P)Y sites proved to provide signals necessary and sufficient for survival and mitogenesis, but not for Pim-1 gene transcription. This result reveals that the mitogenic synergy known to be exerted by SCF and Epo (Broudy, 1997) does not depend strictly either upon events associated with the

recently described tyrosine phosphorylation of the Epo receptor by *c-Kit* (Wu *et al.*, 1995), or upon signals propagated via Epo phosphotyrosine sites *per se*. Mechanisms which support this novel route of synergy between *c-Kit* and ER-HY343F are under investigation. In addition, this result is consistent with the suggestion that STAT5 acts as an important activator of Pim-1 gene transcription (Mui *et al.*, 1996; Rui *et al.*, 1998), and with the previous notion that Pim-1 transcription is not stimulated efficiently by various RTKs, including *c-Kit* (Domen *et al.*, 1993b).

With specific regards to PCD, recent studies by Lilly *et al.* (1999) have documented that certain apoptotic events as induced by cytokine withdrawal are inhibited by the exogenous expression of Pim-1 in IL-3-dependent FDCP1 cells. This included the inhibition of PCD-associated decays in mitochondrial membrane potential, and of the production of reactive oxygen species. By comparison, studies by Mochizuki *et al.* (1997, 1999) of the effects of the forced co-expression of Pim-1 with either *Cdc25a* or *c-Myc* in Rat-1 fibroblasts have yielded somewhat disparate results. In this model, Pim-1 increased rates of *Cdc25a*-dependent transformation to anchorage-independent growth but also at the same time somewhat increased rates of PCD beyond those induced by the over-expression of these additional oncogenic factors. In the absence of over-expressed *cdc25a* or *c-Myc*, however, exogenous Pim-1 had little if any effect on either PCD or transforma-

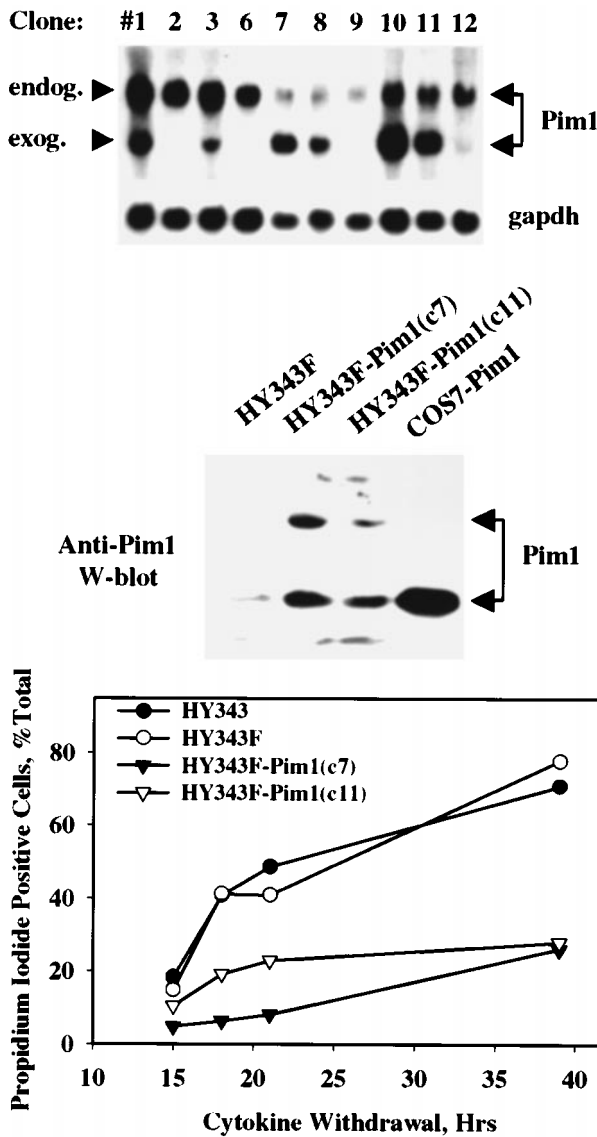


Figure 5 Ectopic expression of Pim-1 in stably transfected FDCER-HY343F cells. Upper panel: Expression of exogenous Pim-1 transcripts in clonal lines stably co-transfected with pEF-Pim-1 and pAPuro. In this Northern blot screen for positive clones, cells were expanded in IL-3. Transcripts from pEF-Pim-1 resolve from larger endogenous Pim-1 transcripts. Center panel: Pim-1 protein expression in FDCER-HY343F-Pim-1 cells (clones 7 and 11) vs control parental FDCER-HY343F cells. Cells were expanded in the presence of SCF plus Epo and total cell lysates (50 ug per lane) were analysed by Western blotting. As a control a lysate from Cos 7 cells were transfected with pEF-Pim-1 was co-analysed. Lower panel: Also confirmed the ability of Pim-1 as expressed in FDCER-Hy343 cells to inhibit PCD due to cytokine withdrawal

tion (and Pim-1 is not normally expressed in this cell line model). Results of the present tests of effects of Pim-1 on PCD due to cytokine withdrawal in myeloid FDCW2 cells (as well as 32D cell lines) are in line with those by Lilly *et al.* (1999) as summarized above. In addition they illustrate the ability of Pim-1 to inhibit apoptosis as activated further by prior exposure to either Co⁶⁰, or adriamycin (see Figure 2), and provide the important novel result that as expressed in proliferating cells, Pim-1 also significantly inhibits PCD due to either ionizing radiation or adriamycin (see Figures 4–6). As mentioned above, ectopic expression of Pim-1 in lymphoid cells in Eμ-Pim-1

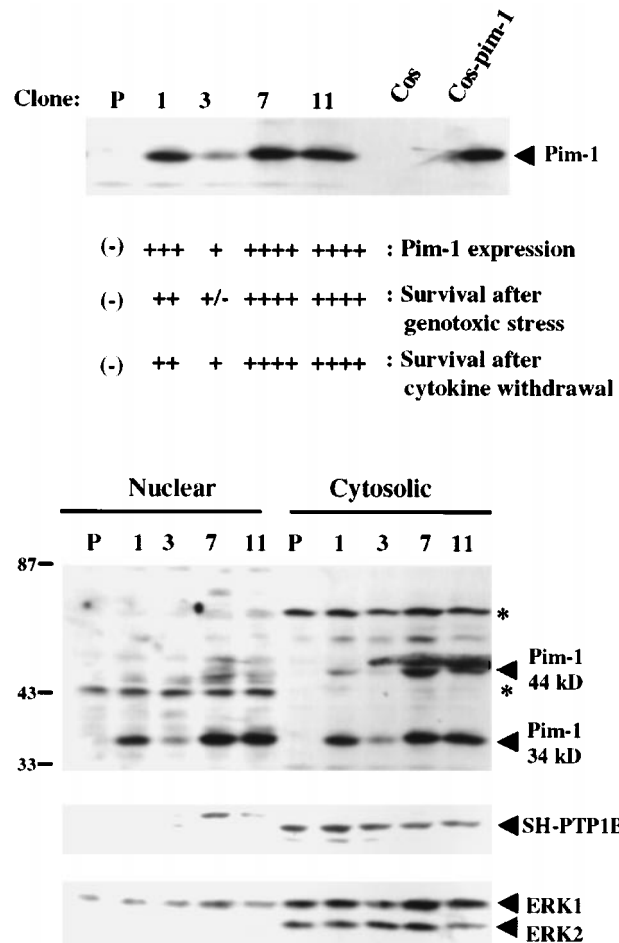


Figure 6 Levels of exogenous Pim-1 expression correlate with protection against Co⁶⁰, adriamycin or cytokine withdrawal induced apoptosis. Upper panel: Shown (by Western blotting) are levels of Pim-1 expression in FDCER-HY343F-Pim-1 clones 1, 3, 7 and 11 vs parental (P) FDCER-HY343F cells. As a positive control, Pim-1 expressed in Cos cells was co-analysed. Indexed are relative levels of protection against apoptosis (survival) observed. Lower panel: In Western blot experiments *M_r* 34 000 Pim-1 (but not *M_r* 44 000 Pim-1) was discovered to occur in nuclear extracts. As markers, blots were stripped and probed for SH-PTP1B (cytoplasmic) and ERK1 and 2. Indexed by asterisks (*) are non-specific stained proteins, which also verify clear fractionation of nuclear vs cytoplasmic components

mice also previously has been shown to inhibit apoptosis as induced by dexamethasone (Moroy *et al.*, 1993). However, relatively little is understood concerning mechanisms of dexamethasone-induced death, and this effect of Pim-1 was observed only in a FAS-dependent *lpr/lpr* background.

The present findings also raise certain additional questions regarding any mitogenic effects which Pim-1 might exert. In Pim-1^{-/-} mice, no obvious defects in hematopoiesis are detected (van der Lugt *et al.*, 1995). This might be a consequence of compensation, yet in Eμ-Pim-1 transgenic mice numbers of B and T cells are likewise normal unless (as in approximately 10% of these mice) leukemic disease develops (van Lohuizen *et al.*, 1989). Presently, in FDCW2 cell lines no significant effects on mitogenesis or cell cycle distributions were observed. This is in contrast to studies by Nosaka *et al.* (1999) in which Pim-1 expression in BaF/3 cells led to factor-independent growth. However, this was indicated to perhaps be a consequence of retroviral over-

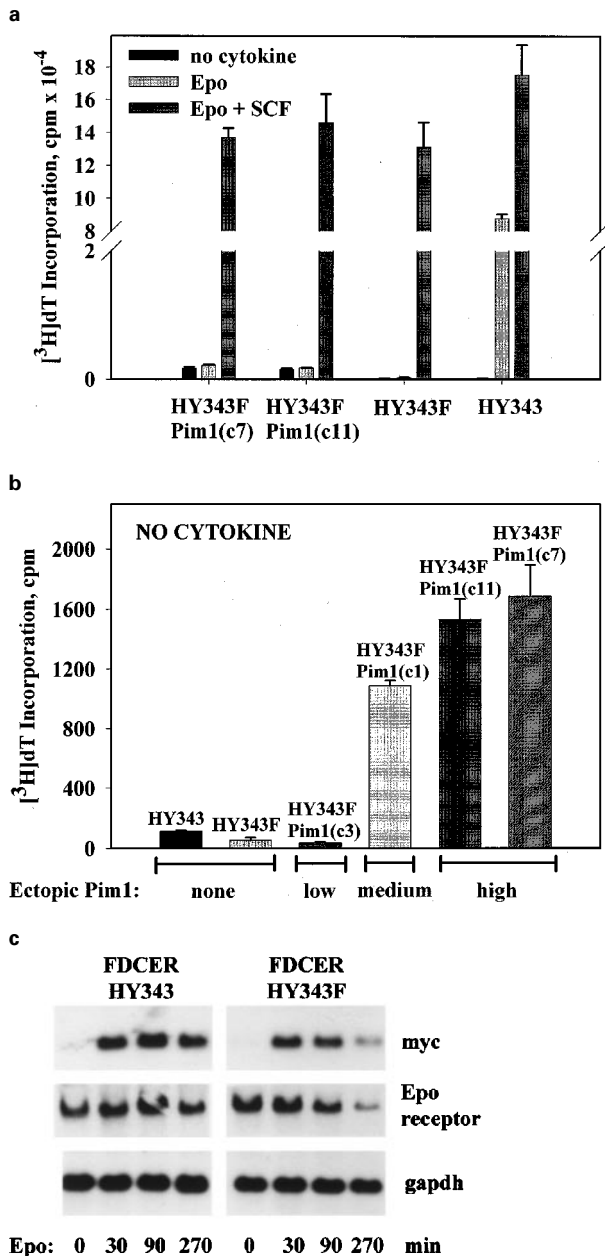


Figure 7 Pim-1 expression does not support proliferation in FDCER-HY343F cells, and only nominally affects levels of [³H]dTh incorporation. (a) Shown are maximal levels of [³H]dTh incorporation in parental FDCER-HY343F cells and derived FDCER-HY343F-Pim-1 cells (clones 7 and 11) exposed to Epo, Epo plus SCF, or no cytokine. (b) Illustrated on an expanded ordinate scale are increases in background rates of [³H]dTh incorporation in FDCER-HY343F-Pim-1 cells. Data shown are for one lower level expressing clone (c3), a medium level expressing clone (c1) and for two higher level expressing clones (c7 and c11). (c) Illustrated is the activity of the Epo receptor form ER-HY343F in mediating *c-myc* gene transcription. FDCW2-HY343F cells (or -HY343 cells as a control) were deprived of IL-3 for 10 h, and were then exposed to Epo (20 U/ml). At the indicated intervals, RNA was isolated and *c-myc* transcript levels were assayed by Northern blotting. Also assayed were transcripts for endogenous Pim-1, exogenous Epo receptor forms, and *gapdh*

expression and/or multiple integrations. Overall, these reports and the present findings suggest that Pim-1 directly does not markedly affect mitogenesis.

In contrast, Pim-1 acts in proliferating hematopoietic progenitor cells to efficiently inhibit PCD due to Co⁶⁰, or

adriamycin (see Figure 4). The significance of these results is underlined by the common use of these agents to treat cancers (Klein *et al.*, 1985; Siu *et al.*, 1999) and by the speculation that their effective doses against hematopoietic targets might be lessened if either Pim-1 or its co-acting factors could be inhibited. Despite intensive research, relatively little is understood as to how either ionizing radiation or anthracyclines link to apoptotic machinery (e.g., caspases). DNA damage by ionizing radiation is sensed by p53, PCNA, ATM, PARP, JNK, PKC, ceramides, ROS and possibly p73- α and *c-Abl* (Agami *et al.*, 1999; Watters, 1999; Xu and Morris, 1999). Adriamycin forms DNA adducts (Zeman *et al.*, 1998), inhibits several key enzymes (TopoII, RNA PolII), and likewise activates p53 and ROS production (Chuang and Chuang, 1979; Tewey *et al.*, 1984). Where Pim-1 might exert effects in these complex response pathways is unknown. In this context, Lilly *et al.* (1999) interestingly have reported that Pim-1 expression in FDCP1 cells leads to increases in Bcl-2 expression. In FDCW2 cells ectopically expressing Pim-1, however, no such effects were observed for Bcl-2 or Bcl-xL at either the transcript or protein level (Pircher, unpublished). Finally, the present observation that *M_r* 34 000 Pim-1 apparently translocates to the nucleus is notable for two reasons: Firstly, the prospect is provided that *M_r* 34 000 Pim-1 might interact directly with several recently reported nuclear targets (Koike *et al.*, 2000; Levenson *et al.*, 1998; Mochizuki *et al.*, 1999; Weston, 1999). Secondly, no such nuclear compartmentalization was detected for *M_r* 44 000 Pim-1 and this might at least provide an initial clue as to possible non-overlapping roles for these forms as produced from alternate CUG or ATG translation sites (Saris *et al.*, 1991).

Materials and methods

Epo receptor, Pim-1 and Bcl-xL expression vectors

The Epo receptor form 'H' is a previously described truncation mutant lacking residues 376–483 (including seven of eight cytoplasmic (P)Y residues) (Quelle *et al.*, 1996). This receptor form was prepared by PCR using a *Bgl*II to *Cla*I Epo receptor cDNA fragment and the following primers: 5'-TGGTCCT-CATCTCGCTGTTGCTGA-3' and 5'-GCTCTAGACTAAG-CCTTCATCCATAGTCACAGGGTCCAC-3'. This PCR product was cloned stepwise to pCRScript, to pSL1180wtER (*Bgl*II to *Xba*I), and to a dicistronic pMK10₅₉ vector (Koyayashi) (*Spe*I to *Xba*I). In the related Epo receptor form ERH-Y343F, a Y343 site was mutated to F343 using the above template and the following primers: 5'-GATCGGGCCCT-TACTGGGAGCCGGTGGGCAGTGAGCATGCCAGG-ACACCTTCTTGGTATTGGATAAGTGG-3' and 5'-GCTC-TAGA CTAAGCTTCATC CATA GTCAC AGGT CCAC-3'. The resulting PCR product was cloned to pSP72 as an *Apa*I to *Xba*I fragment, to pSL1180wtER (*Bgl*II to *Xba*I) and to pMK10₅₉ (*Spe*I to *Xba*I). All products were confirmed by sequencing. The vector pEFNeo-Pim-1 was prepared by cloning a full-length murine Pim-1 cDNA to pEFNeo (Tsang *et al.*, 1997) as a 1200 bp *Eco*RI to *Not*I fragment. pMK-Bcl-xL was prepared by cloning a 800 bp murine *bcl-xL* cDNA to pSL1180 at *Xba*I and *Xho*I sites, and pMC10₅₉ as a 800 bp *Spe*I to *Xba*I fragment.

Cell lines

pMK1059-ERY343 and -ERY343F constructs were electro-transfected into FDCW2 cells as described (Seshasayee *et al.*,

1998), and stable lines expressing each receptor form (i.e. FDCERHY343 and FDCERHY343F cells) were selected in G418 (0.9 mg/ml). For Pim-1 expression, pEFNeo-Pim-1 was linearized with *XmnI* and was co-transfected with pAPuro (5 μ g linearized with *ScaI*) into FDCER-HY343F cells. Cells then were selected in puromycin (0.4 μ g/ml), cloned by limiting dilution, and screened by Northern and Western blotting. For Bcl-xL expression, pEFNeo-Bcl-xL and was co-transfected with pAPuro into FDCER-HY343F cells. Clones expressing Bcl-xL were isolated as above. FDCW2, FDCER-HY343, FDCER-HY343F, FDCER-HY343F-Pim-1, FDCER-HY343F-Bcl-xL, BAF/3 and 32D cell lines were maintained in Opti-MEM medium, 7% fetal bovine serum (FBS), 4% conditioned medium from WEHI-3 cells (as a source of IL-3) (Ihle *et al.*, 1982), penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin B (0.25 μ g/ml). Alternatively, FDC cell sublines were expanded in the presence of SCF (50 ng/ml) (Peprotech) plus rh-Epo (25 U/ml).

Northern and Western blotting

RNA was isolated using 1 ml of Trizol reagent (Life Technologies) per 10^7 cells (Chomczynski and Sacchi, 1987). Electrophoresis was in 1.2% agarose gels containing formaldehyde (6% in gels, 3% in electrophoresis buffer). RNA was blotted to Nytran membranes (Schleicher and Schuell) and fixed by UV irradiation (312 nm for 3 min) and heating (1 h at 80°C under vacuum). 32 P-labeled probes were prepared by random priming (Prime-a-Gene system, Promega) of 25 ng of template cDNA (1.3 kb *EcoRI*, *XbaI* fragment of pCMP2Δ3-Pim-1 or 0.8 kb *KpnI*, *XhoI* fragment of pSP-GAPDH). Probes were purified using Sephadex G-50 microcolumns (Pharmacia Biotech) and were used at 2×10^6 c.p.m. per ml. Hybridizations were for 2 h at 68°C in QuickHyb solution (Stratagene). Membranes were washed at 50°C in 0.1% SDS in $0.2 \times$ SSC (30 mM NaCl, 3 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$) and exposed to X-Omat film (Kodak). For reprobing, membranes were stripped in 50% formamide in $0.1 \times$ SSC for 1 h at 65°C. For Western blotting, cells were collected, washed in Opti-MEM medium and lysed directly in either RIPA buffer (1% NP-40, 0.05% NaDOC, 0.1% SDS in 138 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 pH 7.4) with 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, 50 μ g/ml phenylmethylsulfonyl fluoride or M-per lysis buffer (Pierce). Samples were denatured at 100°C for 5 min, electrophoresed in SDS polyacrylamide gels (10%), and blotted to nitrocellulose membranes (Micron Separations Inc.). Antisera used were to Pim-1 (see Acknowledgments), Bcl-xL (Transduction Labs), MAPK (UBI, catalog #06-182) and SH-PTP1 (UBI, catalog #06-117). Antibody-antigen complexes were detected using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (Amersham Life Science).

Assays of mitogenesis, and PCD

Mitogenesis was assayed based on cytokine-stimulated rates of incorporation of [*methyl*- ^3H] thymidine (^3H]dT). Cells ($50 \mu\text{l}$ at 3×10^5 cells per ml in Opti-MEM medium, 7% FBS) were exposed to cytokines ($50 \mu\text{l}$) for 48 h. ^3H]dT was then added (1 μCi per assay) and at 2 h of incubation incorporated levels were determined (1205 Betaplate counter, KBL Pharmacia). Cell counts were performed in 0.1% Trypan blue in PBS (138 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 pH 7.4). Alternatively, non-viable/apoptotic cells were assayed by staining with propi-

dium iodide (PI) (5 $\mu\text{g/ml}$ for 2 min) and flow cytometry (Coulter XL-MCL), or by TUNEL (TdT-mediated X-dUTP nick end labeling). In these assays, exponentially growing cells were adjusted to 3×10^5 cells per ml, expanded to 8×10^5 cells per ml, washed twice in Opti-MEM medium, and for cytokine withdrawal were then cultured at 5×10^5 cells per ml in Opti-MEM medium, 1% FBS. Alternatively, cells at 4×10^5 cells per ml were exposed to Co^{60} (300 Rads) or adriamycin (0.025 ng/ml) and were cultured for 6 h prior to cytokine withdrawal. In TUNEL assays, fragmented DNA was labeled with biotin dNTPs (Trevigen FlowTACSTM Apoptosis Detection Kit) and was detected using FITC-streptavidin and flow cytometry. For assays of PCD in proliferating FDCER-HY343F-Pim-1 cells, cultures were initiated at 3×10^5 cells per ml in Opti-MEM medium, 5% FBS, Epo (5 U/ml) and SCF (25 ng/ml). Cells then were exposed to Co^{60} (600 Rads) or adriamycin (0.125 ng/ml) and at subsequent intervals were assayed for PCD.

Preparation of nuclear, and non-nuclear extract

Cells were washed twice at 4°C in 10 mM NaCl, 6 mM MgCl_2 , 10 mM Tris, pH 7.4 and incubated on ice for 20 min in 10 mM NaCl, 6 mM MgCl_2 , 1 mM dithiothreitol, 0.1% Triton X-100, 0.1 mM Na_3VO_4 , 10 mM Tris, pH 7.4, containing 0.4 mM PMSF, 3 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, 1 $\mu\text{g/ml}$ leupeptin. Nuclei then were collected by microcentrifugation, and the 0.1% Triton-X soluble supernatant was recovered. Nuclei then were washed, and extracted for 30 min at 4°C in 3 volumes of 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA 1 mM dithiothreitol, 0.1 mM Na_3VO_4 , 20% glycerol, 20 mM HEPES, pH 7.9, containing 0.4 mM PMSF, 3 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, and 1 $\mu\text{g/ml}$ leupeptin. Samples then were centrifuged for 10 min at 8000 g and protein concentrations were adjusted to equivalence (Bradford reagent, Sigma) and stored at -80°C .

Abbreviations

PCD, programmed cell death; S/T, serine/threonine; SCF, stem cell factor; EPO, erythropoietin; IL, interleukin; GM-CSF, granulocyte macrophage colony stimulating factor; Tpo, thrombopoietin; M_r , molecular weight (relative mass); ER, erythropoietin receptor; TUNEL, TdT-mediated X-dUTP nick end labeling; ^3H]dT, [*methyl*- ^3H]thymidine; RTK, receptor tyrosine kinase; PCNA, proliferating cell nuclear antigen; ATM, ataxia-telangiectasia mutated; PARP, poly (ADP ribose) polymerase; JNK, *c-jun* N-terminal kinase; PKC, protein kinase C; ROS, reactive oxygen species; PKC, protein kinase C; PCR, polymerase chain reaction; FBS, fetal bovine serum; PBS, phosphate buffer saline; PI, propidium iodide; FITC, fluorescein isothiocyanate.

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