Role of phosphatidylinositol 3-OH-kinase activity in the inhibition of apoptosis in haemopoietic cells: phosphatidylinositol 3-OH-kinase inhibitors reveal a difference in signalling between interleukin-3 and granulocyte–macrophage colony stimulating factor

Michael P. SCHEID, Ron W. LAUENER and Vincent DURONIO*

Department of Medicine, University of British Columbia, Jack Bell Research Centre, 2660 Oak St., Vancouver, B.C., Canada V6H 3Z6

Apoptosis of haemopoietic cells can be inhibited by various cytokines, but the specific signalling pathways involved are not well defined. Interleukin (IL)-4 has unique actions since it is unable to activate p21^{ras} or mitogen-activated protein kinase, but can activate PtdIns 3-OH-kinase (PtdIns 3-kinase), the latter effect being shared with most other cytokines. In many cases, IL-4 is able to maintain cell viability by inhibiting apoptosis, but is unable to stimulate continuous proliferation of cells. This led us to investigate the role of PtdIns 3-kinase in inhibition of apoptosis. Two potent inhibitors of PtdIns 3-kinase, wortmannin

and LY294002, rapidly induced apoptosis in cells incubated in the presence of IL-4, at concentrations consistent with their ability to inhibit PtdIns 3-kinase activity in whole cells. Interestingly, the same effect was seen in cells in the presence of IL-3 and Steel factor, while cells incubated in the presence of granulocyte–macrophage colony stimulating factor, and to a lesser extent, IL-5 could bypass the effect of wortmannin or LY294002. Therefore, this study suggests that PtdIns 3-kinase activity provides an important, although not a unique signal, required to inhibit apoptosis in haemopoietic cells.

INTRODUCTION

Gene products involved in prevention or induction of apoptosis have been the subject of intense study over the past few years [1–6]. The importance of apoptosis in the normal turnover of cells, and the problems that can occur when normal apoptosis is disrupted, are becoming increasingly apparent. Haemopoietic cells provide an excellent system in which to study apoptosis, since they are usually dependent upon specific cytokines for their survival. Cells stimulated by cytokines receive signals keeping them alive, thereby inhibiting apoptosis, but the identity of the critical signals for inhibition of apoptosis have not been well defined. Interleukin (IL)-4 is able to keep MC-9 cells alive, but not stimulate their growth, and it can activate PtdIns 3-OH-kinase (PtdIns 3-kinase) [7], but not p21^{ras} [8–10] and mitogen-activated protein kinase (MAP kinase) [11]. Cytokines such as IL-3, IL-5 and granulocyte–macrophage colony stimulating factor (GM-CSF) are able to stimulate continuous proliferation of the cells while activating each of these pathways [7–12].

In this study, we set out to test whether PtdIns 3-kinase was providing an essential signal that was not only required for mitogenesis [13,14], but was also important for inhibition of apoptosis. A potent inhibitor of PtdIns 3-kinase, wortmannin (WM) [15], was capable of inducing apoptosis in the presence of IL-4, as predicted by our hypothesis. Cells incubated in the presence of WM and IL-3 or Steel factor (SLF) also underwent apoptosis. Incubation with GM-CSF and, to a lesser extent, IL-5 could bypass the effect of WM. The same results were also observed with another structurally unrelated inhibitor of PtdIns 3-kinase, LY294002. Therefore, blocking the PtdIns 3-kinase signalling pathway leads to apoptosis of haemopoietic cells in the presence of some, but not all cytokines. These experiments have thus revealed an unexpected difference in the signalling pathways

activated by IL-3 and GM-CSF, which are known to utilize very similar signalling mechanisms.

EXPERIMENTAL

Cells

MC-9 cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (Interger) with 2.5 units/ml penicillin and 2.5 µg/ml streptomycin (Sigma) and 40 µM 2-mercaptoethanol in the presence of 5–10% WEHI-3-conditioned medium. HL-60 cells were grown in the same medium as above, with no added cytokines. FD-MAC 11/4.5 cells were also grown in the same medium in the presence of IL-4 [16].

DNA fragmentation assay

At indicated times of treatment, 2×10^5 cells were pelleted and solubilized in 400 µl of lysis buffer (0.6% SDS and 10 mM EDTA, pH 8.0). Following addition of 100 µl of 5 M NaCl, the samples were mixed by gentle inversion of the tubes. After overnight incubation at 4 °C, samples were pelleted by centrifugation (20 min in an Eppendorf microfuge). Supernatants containing DNA fragments were transferred to clean tubes and the pellets discarded. An aliquot (1 µl) of 1 mg/ml RNase A was added and samples were incubated for 20 min at 37 °C. Next aliquots (500 µl) of Tris-buffered phenol (pH 8.0)/chloroform (1:1, v/v) were added, samples were briefly vortexed and the aqueous layer retained. To this layer were added 55 µl of 3 M sodium acetate and 1.0 ml of ice-cold ethanol. Samples were mixed and incubated for 10 min at –20 °C. DNA was pelleted by centrifugation (10 min at 20000 g) and separated by electrophoresis on a 2% agarose/Tris/borate/EDTA gel.

Abbreviations used: GM-CSF, granulocyte–macrophage colony stimulating factor; IL, interleukin; MAP kinase, mitogen-activated protein kinase; PtdIns 3-kinase, PtdIns 3-OH-kinase; PKC, protein kinase C; SLF, Steel factor; WM, wortmannin.

* To whom correspondence should be addressed.

Visualization of DNA bands was performed by staining with ethidium bromide, destaining in water and viewing on a UV transilluminator. Video images of the stained gels were obtained using a GelPrint 2000 system.

PtdIns 3-kinase assay

For determining the effect of WM on PtdIns 3-kinase in intact cells, cells were washed and incubated in RPMI 1640 medium with 10% (v/v) fetal bovine serum and 10% WEHI-3-conditioned medium in the presence of WM as indicated. Cells were solubilized in buffer containing 1% Triton X-100 and PtdIns 3-kinase was immunoprecipitated using anti-p85 serum (Upstate Biotechnology Inc.) and activity assayed, essentially as described previously [7,17]. Approximately 200 μ g of protein was used per sample; PtdIns 3-kinase activity was normalized per μ g of protein in the original extract. Protein A-Sepharose beads with bound PtdIns 3-kinase were mixed in a 50 μ l reaction mixture containing 30 mM Hepes, pH 7.4, 30 mM MgCl₂, 200 μ M adenosine, and 10 μ g of phosphatidylinositol (Avanti Polar Lipids). The reaction was started by addition of 10 μ Ci of [³²P]ATP (Dupont NEN) and incubated at room temperature for 15 min. The reaction was terminated by addition of 100 μ l of 1 M HCl and lipids were extracted using 200 μ l of chloroform/methanol (1:1, v:v). A 25 μ l sample was spotted and separated on oxalate-treated TLC plates using a solvent system of chloroform/methanol/water/ammonium hydroxide (18:14:3:1, by vol.). Quantification was performed by excising portions of the TLC plate corresponding to PtdIns 3-phosphate, followed by liquid scintillation counting.

RESULTS AND DISCUSSION

The mouse mast cell line, MC-9, was used in this study to compare the ability of various cytokines to inhibit apoptosis. When cells were washed free of cytokine and incubated in medium containing 10% serum only, some fragmentation of DNA was observed within 2 h of cytokine withdrawal, with the maximal level of fragmentation observed at 6 to 8 h (Figure 1). To investigate the role of PtdIns 3-kinase in inhibition of apoptosis, we tested the effect of a potent inhibitor of PtdIns 3-kinase, WM [15], in inducing apoptosis. Incubation of starved cells with WM accelerated the onset of apoptosis (Figure 1), perhaps by inhibition of basal PtdIns 3-kinase activity. However, use of WM presents a problem in experiments spanning more

Figure 1 Apoptosis in MC-9 cells following starvation

Cells were washed three times and incubated in RPMI medium plus 10% fetal bovine serum at 37 °C. At indicated times of starvation, samples were taken and prepared as described. In samples indicated, 200 nM WM was added at time 0.

Figure 2 Dose-response to WM

(a) MC-9 cells were incubated in the presence of twice the indicated concentrations of WM, added at time 0, followed by the indicated concentration (nM) added hourly. Cells were incubated either in RPMI medium plus 10% fetal bovine serum (starved), or in the same medium with 500 ng/ml synthetic IL-3, 500 ng/ml synthetic IL-4, or 60 units/ml of recombinant GM-CSF. After 8 h, cells were prepared as described. (b) MC-9 cells or HL-60 cells were incubated with WM with twice the indicated concentration added at time 0, followed by hourly additions of the indicated concentration. Incubations were for 8 h. CAMPT indicates HL-60 cells incubated in parallel with 1 μ M camptothecin for 6 h to induce DNA fragmentation.

than a few hours because of its relatively short half-life in aqueous solutions at physiological pH and temperature [18]. When we tested the inhibitory activity of WM in an *in vitro* assay of PtdIns 3-kinase activity following incubation of the inhibitor in aqueous solutions, it was found to be decreased by 50% following approximately 1 h at 37 °C in RPMI medium. To overcome the problem of degradation of WM, and to avoid the use of high concentrations of WM that could be affecting other cellular enzymes [19], initial additions of WM in the presence or absence of cytokines were followed by hourly additions of half of the initial concentration. Following such a protocol, cells incubated in the presence of IL-3, IL-4 or GM-CSF were compared. Relatively low concentrations of WM caused apoptosis in the presence of IL-3 or IL-4, with no effect on cells in the presence of GM-CSF, even at 25-fold higher concentrations of WM (Figure 2a). These results suggest that GM-CSF is able to bypass the inhibition of PtdIns 3-kinase by WM, presumably via an alternate signalling pathway.

The specificity of the WM effect in causing apoptosis is further supported by the use of even much lower concentrations in the presence of IL-4, which resulted in at least a partial effect (Figure 2b). On the other hand, HL-60 cells were not affected by WM, even at 10-fold higher concentrations than those shown in Figure 2(b), although a topoisomerase inhibitor, camptothecin, did induce DNA fragmentation in these cells. This suggests that HL-60 cells, which are cytokine-independent, have either bypassed the need for PtdIns 3-kinase by activation of events downstream

Figure 3 Assay of PtdIns 3-kinase activity immunoprecipitated from MC-9 cells

Cells were incubated in the absence or presence of the indicated concentrations of WM for 1, 3 or 6 h. For the 3 h and 6 h time points, hourly additions of 20 nM WM were made as indicated.

of PtdIns 3-kinase, or by activation of the alternative pathway that operates downstream of GM-CSF.

Next, we investigated the effect of WM on the PtdIns 3-kinase activity in whole cells. The activity of PtdIns 3-kinase immunoprecipitated from MC-9 cells pre-incubated with 40 nM WM initially and 20 nM added hourly, was significantly inhibited by 1 h, and after 3 h of incubation, the inhibition was essentially complete, similar to a single treatment with 100 nM WM (Figure 3). The effects observed with low concentrations of WM correlate well with its ability to inhibit PtdIns 3-kinase specifically. The IC_{50} for WM inhibition of PtdIns 3-kinase in an *in vitro* assay was 3–5 nM and complete inhibition required 50–100 nM, while 50% inhibition of myosin light chain kinase was previously shown to require approximately 170 nM [19].

The effect of WM was compared in experiments using a series of cytokines. Cells were incubated with optimal concentrations of either IL-4, GM-CSF, IL-3, IL-5 or SLF, each of which prevented appearance of any DNA fragments (Figure 4a). The effect of WM in inducing apoptosis (at 40 nM initially followed by 20 nM added hourly) was observed with cells incubated in the presence of either IL-3, IL-4 or SLF. However, WM had no effect in cells incubated with GM-CSF and much less effect was observed with cells incubated with IL-5. It should be noted that this partial effect with IL-5 was consistently observed in a number of experiments and was not an artifact of sample recovery. These effects correlated precisely with a bioassay in which an aliquot of the same cells was incubated for a further 48 h (after seven hourly additions of WM) and cell growth and survival was determined (results not shown). The difference between the effects with IL-3, compared with IL-5 and GM-CSF, was consistently observed in a number of experiments, but was quite surprising, given the expectation that the signal transduction pathways downstream of these related receptors are very similar [7,9,20,21]. Furthermore, they cannot easily be explained by differences in sensitivity to WM, since even very high concentrations of the drug cannot induce apoptosis in the

Figure 4 Effect of WM on responses to various cytokines

MC-9 cells were washed and incubated in optimal concentrations of each of the cytokines in the presence or absence of 40 nM WM. Hourly additions of 20 nM WM were made up to 7 h and the cells harvested and treated as described after 8 h.

presence of GM-CSF. It is also interesting that the use of SLF, which activates the tyrosine kinase-encoding *c-kit* receptor but is not as potent in growth stimulation as GM-CSF [22], cannot bypass the effect of WM. The effects observed in MC-9 cells are also not limited to this cell type. Similar results using WM with IL-4 or GM-CSF were also observed in the FD-MAC11/4.5 cells (results not shown), in which IL-4 can stimulate continuous growth but still fails to activate p21^{ras} or MAP kinase [16].

Another inhibitor of PtdIns 3-kinase, LY294002 [23], which is structurally unrelated to WM and acts by a different mechanism [24], was tested in the same assay of apoptosis. The results in Figure 5 show that LY294002, at 10 or 25 μ M, caused apoptosis in cells incubated with IL-3 or IL-4, but not GM-CSF. Similarly, LY294002 also induced apoptosis in the presence of SLF, but had only a partial effect on cells incubated with IL-5 (results not shown).

We have investigated the effect of WM on activation of MAP kinase by IL-3 and GM-CSF to rule out the possibility that the

Figure 5 Apoptosis in MC-9 cells treated with LY294002

Cells were washed and incubated in the presence of optimal concentrations of the indicated cytokines along with a single addition of the indicated concentrations of LY294002. Cells were incubated for 15 h, then harvested and treated as described in the Experimental section.

difference between the two cytokines could be explained by a difference in sensitivity of the p21^{ras} pathway to WM. These experiments revealed that a portion of the MAP kinase activation observed with GM-CSF and IL-3 could be blocked in cells pre-incubated with WM (results not shown). However, the same relative inhibition was observed in each case. The potential role of PtdIns 3-kinase in activating MAP kinase has been suggested by several recent studies [25–28], but it is intriguing that activation of PtdIns 3-kinase by IL-4 does not result in activation of p21^{ras} or MAP kinase. Together these results rule out the possibility that the effect of WM is due to inhibition of a pathway involving p21^{ras} and MAP kinase.

Recently, two other reports have appeared which are directly relevant to our study. Similar conclusions about the importance of PtdIns 3-kinase in blocking apoptosis in PC12 cells were reached by Yao and Cooper [29]. These authors used a dominant negative form of p21^{ras} in PC12 cells to block the p21^{ras} signalling, and were able to show that inhibitors of PtdIns 3-kinase induced apoptosis. In our study, we were able to use a cytokine, IL-4, that has no effect on p21^{ras} or MAP kinase. However, the differences in our results with GM-CSF and IL-5, compared with IL-3, suggest that there is another means by which some cytokines can inhibit apoptosis, independent of p21^{ras} or PtdIns 3-kinase activation. A less likely possibility is that a p21^{ras}-mediated signal is activated by GM-CSF and IL-5, but not IL-3 and SLF. Another study [30] examined the signals downstream of the IL-3 and GM-CSF receptors that inhibited apoptosis, and reached quite a different conclusion, suggesting that p21^{ras} signalling was essential to prevent apoptosis. Clearly, this is not consistent with the ability of IL-4 to prevent apoptosis without activating p21^{ras}. The activation of PtdIns 3-kinase was not investigated in that study. Also, initial experiments examined the effects of murine IL-3 on a murine cell line and subsequent experiments were done using the same cells transfected with human GM-CSF receptors, based on the assumption that the same signals were being transmitted in response to these two cytokines. As our results show, there may be differences in these signals which could alter the interpretation of their results.

Our results demonstrate for the first time that PtdIns 3-kinase plays a major role in inhibiting apoptosis in haemopoietic cells. One prediction is that artificially increasing the level of PtdIns 3-kinase lipid products may keep haemopoietic cells alive in the absence of cytokine. Future experiments designed to deliver these phospholipids into cells will be a direct verification of this hypothesis. Experiments with phorbol esters have suggested that activation of protein kinase C (PKC) can inhibit apoptosis of haemopoietic cells [31,32], which would be consistent with activation of PKC isoforms by 3-phosphorylated inositol phospholipids [33,34]. Therefore, these studies have identified PtdIns 3-kinase and downstream signals as important pathways for inhibition of apoptosis and as such, they may be potential targets for chemotherapeutic drugs designed to kill cells by inducing apoptosis.

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