

Distinct effects of thrombopoietin depending on a threshold level of activated Mpl in BaF-3 cells

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Summary

Thrombopoietin (TPO) plays a critical role in megakaryopoiesis through binding to its receptor Mpl. This involves activation of various intracellular signaling pathways, including phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways. Their precise role in TPO-mediated proliferation, survival and differentiation is not fully understood. In the present study, we show that TPO induces different biological responses in Mpl-transduced BaF-3 cells, depending on the cell surface density of Mpl and the resulting activation level of signaling pathways. TPO mediates cell proliferation in cells expressing high levels of Mpl but only mediates survival without proliferation in cells expressing low levels of the receptor. By using the kinase inhibitors PD98059 and LY294002, we further showed that the activation level of

the PI3K and MAPK p42/44 pathways is a determining factor for the proliferative effect. In cells expressing low levels of Mpl, the survival effect was strongly dependent on the activation level of the PI3K/AKT, but not the MAPK p42/44 pathway. Moreover, this effect was correlated with the phosphorylation level of BAD but not with the expression level of Bcl-X_L. However, PI3K pathway inhibition did not increase apoptosis when BaF-3 cells proliferated in response to TPO, indicating a compensating mechanism from other Mpl signaling pathways in this case.

Key words: Mpl, Threshold activation level, Proliferation, Survival, Signaling pathways

Introduction

TPO is a ligand for the Mpl receptor (de Sauvage et al., 1994; Lok et al., 1994; Wendling et al., 1994), a member of the hematopoietic growth factor receptor superfamily. This family of receptors is characterized by conserved cysteine residues, a common amino acid motif (WSXWS) in the extracellular domain, and the absence of intrinsic tyrosine kinase activity in the intracellular domain. A high level of cell surface expression of Mpl appears to be limited to hematopoietic cells belonging to the megakaryocytic platelet lineage, from colony-forming unit-megakaryocyte (CFU-MK) progenitors to mature platelets (Debili et al., 1995a). Cell surface expression of Mpl is also detected on murine Lin^{lo}Sca⁺c-kit⁺ and human CD34⁺CD38⁻ primitive cell populations (Solar et al., 1998).

The function of the Mpl/TPO system is well characterized. Numerous experiments have shown that TPO stimulates proliferation and differentiation of megakaryocytic progenitors in vitro and in vivo (Debili et al., 1995b; Kaushansky et al., 1995). More recently, Mpl- and TPO-deficient mice were reported to exhibit a drastic decrease in the number of platelets and cells of the megakaryocytic lineage (Gurney et al., 1994), demonstrating a major role for Mpl activation in megakaryopoiesis and platelet production. The Mpl/TPO system also acts on more primitive cells. Mpl- and TPO-deficient mice showed a 50% reduction in the absolute number of all myeloid-committed progenitors (Alexander et al., 1996). In vitro, TPO alone promotes survival of early hematopoietic progenitors (Jacobsen et al., 1996; Borge et al., 1997; Matsunaga et al., 1998) and in combination with early-acting

cytokines such as stem cell factor (SCF), FLT3 ligand or interleukin 3 (IL-3) it greatly increases the production of committed progenitors (Ku et al., 1996; Ramsfjell et al., 1996; Ramsfjell et al., 1997). These data indicate that, in addition to its essential function for megakaryopoiesis, Mpl activation is required to maintain and/or expand early and committed myeloid progenitor cells.

Binding of TPO to the Mpl receptor leads to the phosphorylation and activation of numerous signaling molecules, including those in the JAK/STAT pathway, the MAPK pathway and the PI3K pathway (Drachman et al., 1995; Sattler et al., 1997; Matsumura et al., 1998). Their involvement in cellular events such as survival, proliferation and differentiation has been described but their precise role in TPO-mediated biological responses is not fully understood.

In the present study, we show that signaling pathways activated by TPO produce distinct effects in Mpl-transduced BaF-3 cells, depending on the level of expression of Mpl on the cell surface. TPO mediates cell proliferation in cells expressing high levels of cell surface Mpl, whereas it mediates cell survival alone (without proliferation) in cells expressing low levels of the Mpl receptor. In addition, we defined the roles of Mpl signaling pathways in these different effects.

Materials and Methods

Cytokines, antibodies and reagents

WEHI-3B conditioned medium was used as a source of murine IL-3. Human recombinant TPO was a generous gift from Kirin (Tokyo,

Japan). Mouse anti-Flag M1 monoclonal antibody was purchased from Sigma (Saint-Quentin Fallavier, France) and R-Phycoerythrin (R-PE)-conjugated goat F(ab')₂ anti-mouse antibody from Caltag (Burlingame, CA). Mouse anti-phospho-ERK and rabbit anti-phospho-STAT5, -phospho-AKT and -AKT antibodies were provided by New England Biolabs (Beverly, MA); rabbit anti-STAT5, -ERK1, -ERK2 and BAD were from SCB (Santa Cruz, CA); and mouse anti-Bcl-x were from BD Transduction Lab (Lexington, KY). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were from Jackson Immunoresearch (West Grove, PA). PD98059 MEK inhibitor was purchased from Calbiochem (San Diego, CA), and LY294002 PI3K inhibitor, AG490 JAK2 inhibitor and propidium iodide (PI) were from Sigma.

Cell lines

The murine cell line BaF-3 (Palacios and Steinmetz, 1985) and BaF3-Mpl clones were maintained in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco) and 3% WEHI-3B-conditioned medium. NIH-3T3 and 293 EBNA (Invitrogen, Groningen, The Netherlands) cell lines were cultured in DMEM medium (Gibco) with 10% FCS.

Plasmid constructs

Full-length human c-DNA from pZen-MplP-SVNeo (Goncalves et al., 1997) was used for constructions. To introduce the Flag epitope tag, a 115 bp oligonucleotide duplex 5'-cgttaacatgttccatgtttctttatagatatctttggaaattccactgatcctgtctgtcctgtcactagtctgattacaagatgacgatgacaa-gccgtctct-3' / 5'-ctagagaagacggctgtcatcgtcattctgtaacagaactagtgac-aggcagcagaacaaggatcagtgagggaattccaagatatctaaaagaacatggacatg-ttaacgagct-3' encoding the IL-7 leader (Park et al., 1990), the Flag sequences (Brizzard et al., 1994) and *Bbs*I restriction site were inserted into the *Sac*I/*Xba*I sites of the vector pBluescript II (Stratagene, La Jolla, CA) to obtain the plasmid pSKFlag. To introduce the Mpl coding sequence in frame with the IL7 and Flag sequences, a part of the extracellular domain of Mpl was amplified with a 0.1 µg of pZen-MplP-SVNeo with a sense primer (5'-cctagaagacccaagatgtctctctg-3'), corresponding to the *Bbs*I restriction site, cohesive end on the pSKFlag after cutting with *Bbs*I, and Mpl sequence starting immediately after the leader coding sequence, and an antisense primer (5'-gttccaccctctgctgcag-3') located downstream of *Hind*III site in the Mpl cDNA. After cutting with *Bbs*I and *Hind*III, the PCR product was ligated into the corresponding sites of the pSKFlag vector. A *Hind*III/*Xho*I fragment from pZen-Mpl-SVNeo was inserted into the corresponding sites of this plasmid in order to obtain the full-length Flag-Mpl cDNA. The fidelity of inserts was confirmed by sequencing. Flag-Mpl cDNA was then subcloned into the *Hpa*I/*Xho*I sites of the murine stem cell virus-based bicistronic retroviral vector MIGR-IRES-GFP (generous gift of J. P. Miller, University of Pennsylvania, Philadelphia, PA) in which the original polylinker (*Bgl*II, *Xho*I, *Hpa*I and *Eco*RI) was replaced by the sequence 5'-gatctagaattctgtcagacttaactgcggccgactcgag-3' and the *Sa*II site downstream of GFP was mutated by *Sa*II cutting, blunting by Klenow and religation.

Retroviral infection

The Flag-Mpl receptor construct cloned into the retroviral vector MIGR-IRES-GFP enables the simultaneous expression of the receptor protein and the green fluorescent protein (GFP), and allows the selection of infected cells based on GFP expression using a fluorescence-activated cell sorter (FACS). Transient MIGR-Flag-Mpl supernatant production was accomplished by transfecting the 293 EBNA cell line that has an amphotropic host range. To improve the stability of the retrovirus particles, we chose a vesicular stomatitis virus G (VSV-G) pseudotyping technique. Briefly, 0.5 µg of each

MIGR-Flag-Mpl, VSV-G and gag-pol plasmids were co-transfected using EXGEN reagent according to the manufacturer's recommendations (Euromedex, Souffelweyersheim, France). Cells were washed at 24 hours and supernatants were collected at 48 hours, filtered and frozen at -80°C. This strategy regularly gives a transfection efficiency of 50% and a viral titer of 10⁶ CFU/ml by monitoring the number of GFP-positive cells in the NIH-3T3 cell line (data not shown).

Supernatants were used to infect BaF-3 cells. Cells were washed once and 10⁵ cells were cultured for 48 hours in 6-well tissue culture plates (Falcon Grenoble, France) containing 2 ml of RPMI, 10% FCS, 6% WEHI-3B, 50% retroviral supernatant and 4 µg/ml of polybrene. After infection, cells were maintained in RPMI, 10% FCS, 3% WEHI-3B for 2 days. Highly GFP-positive cells were then cloned at one cell per well into 96-well tissue culture plates using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). These BaF3-Mpl clones were named Clone A, B, C... and were used as clones expressing high levels of Mpl receptor on their cell surface. To obtain clones expressing low levels of Mpl on their cell surface, a pool of faintly GFP-positive cells was also sorted and maintained in RPMI, 10% FCS, 3% WEHI-3B for 7 days. Cells were then labeled with anti-Flag and PE-goat anti-mouse antibodies as described below, and Flag-positive cells were cloned at one cell per well into 96-well tissue culture plates using the FACS Vantage flow cytometer. Thirty clones were randomly selected and named clone 1, 2, 3... 30. To increase Mpl expression in the low-level-expressing clones, clones 8, 16 and 28 were retransduced with the Mpl retroviral construct as described above for BaF-3 parental cells, and a pool of highly GFP- and Flag-positive cells were sorted. These three retransduced pools derived from clones 8, 16 and 28 were named clone 8+, 16+ and 28+. To avoid any alteration in the Mpl cell surface expression, aliquots of each clone were thawed every 3 weeks and during this lapse of time, receptor expression was regularly monitored by flow cytometric analysis.

Proliferation assays

Cells were washed three times and 2500 cells per well were plated into 96-well tissue culture plates (Falcon) containing RPMI, 10% FCS and the indicated concentration of TPO or 5% WEHI-3B as a control. After incubation at 37°C for the indicated time, 10 µCi of [³H]thymidine (specific activity 185 GBq/mmol; Amersham, Courtabœuf, France) was added to each well and the cells further incubated for 4 hours at 37°C prior to scintillation counting.

Cell stimulation

Cells were washed three times and resuspended at 4×10⁶ cells/ml in RPMI alone. After a starvation of 3 hours at 37°C, cells were left unstimulated or stimulated with 50 ng/ml of TPO for 15 minutes at 37°C. When used, inhibitors were added at the indicated concentration 30 minutes before TPO stimulation. For long-term stimulation, cells were washed three times and directly resuspended at 2×10⁶ cells/ml (for western blots analysis) or 4×10⁵ cells/ml (for apoptosis analysis) in RPMI, 10% FCS with 10 ng/ml of TPO. When used, inhibitors were added at the indicated concentration together with TPO. DMSO (Sigma) was used as a control for the inhibitor studies at 1.4 µl/ml, which corresponded to the maximum concentration of DMSO present during these experiments.

Flow cytometric analysis

A saturating concentration of antibodies was used for all labeling. Because binding of M1 anti-Flag antibody is dependent on calcium, 1 mM of CaCl₂ was added to the buffer during all incubations and washes. Cells (5×10⁵/sample) were washed and incubated in PBS and 0.5% FCS for 10 minutes at 4°C with 8.4 µg/ml of mouse M1 anti-

Flag antibody or left unlabelled. After two washes, cells were labeled with 0.4 $\mu\text{g/ml}$ of PE-conjugated goat anti-mouse antibody for 20 minutes at 4°C. Cells were then washed once and analysed for fluorescence with a FACScan flow cytometer (Becton Dickinson). For apoptosis analysis, 4×10^5 cells were collected from stimulated cultures (see above), washed once and labeled overnight in 400 μl of citrate buffer containing 25 $\mu\text{g/ml}$ PI, 50 $\mu\text{g/ml}$ RNase (Merck, Darmstadt, Germany) and 0.1% Nonidet P40 (Sigma), in the dark at 4°C. Percentages of apoptotic cells, corresponding to cells with sub-cell cycle PI incorporation, were determined by flow cytometry using a FACSsort (Becton Dickinson).

Western blot analysis

Cell stimulation was blocked by washing cells with 50 ml of cold $1 \times$ PBS. Total cell lysates were obtained by incubating 10^8 cells/ml in a hypertonic buffer containing 1/100 Triton X-100, 20 mM Hepes pH 7.9, 350 mM NaCl, 10 mM KCl, 1 mM EDTA pH 8, 20% glycerol, 1/25 Complete Protease Inhibitors (Boehringer Mannheim, Germany) and 1 mM Na_2SO_4 . Lysates were incubated for 30 minutes at 4°C, centrifuged at 13,000 g for 6 minutes at 4°C, then supernatants were stored at -80°C until used. Protein concentration was determined using the Bio-Rad DC Protein colorimetric assay (Hercules, CA). Unless specified, 60 μg of protein per sample was used and further analysed as described (Rosa Santos et al., 2000).

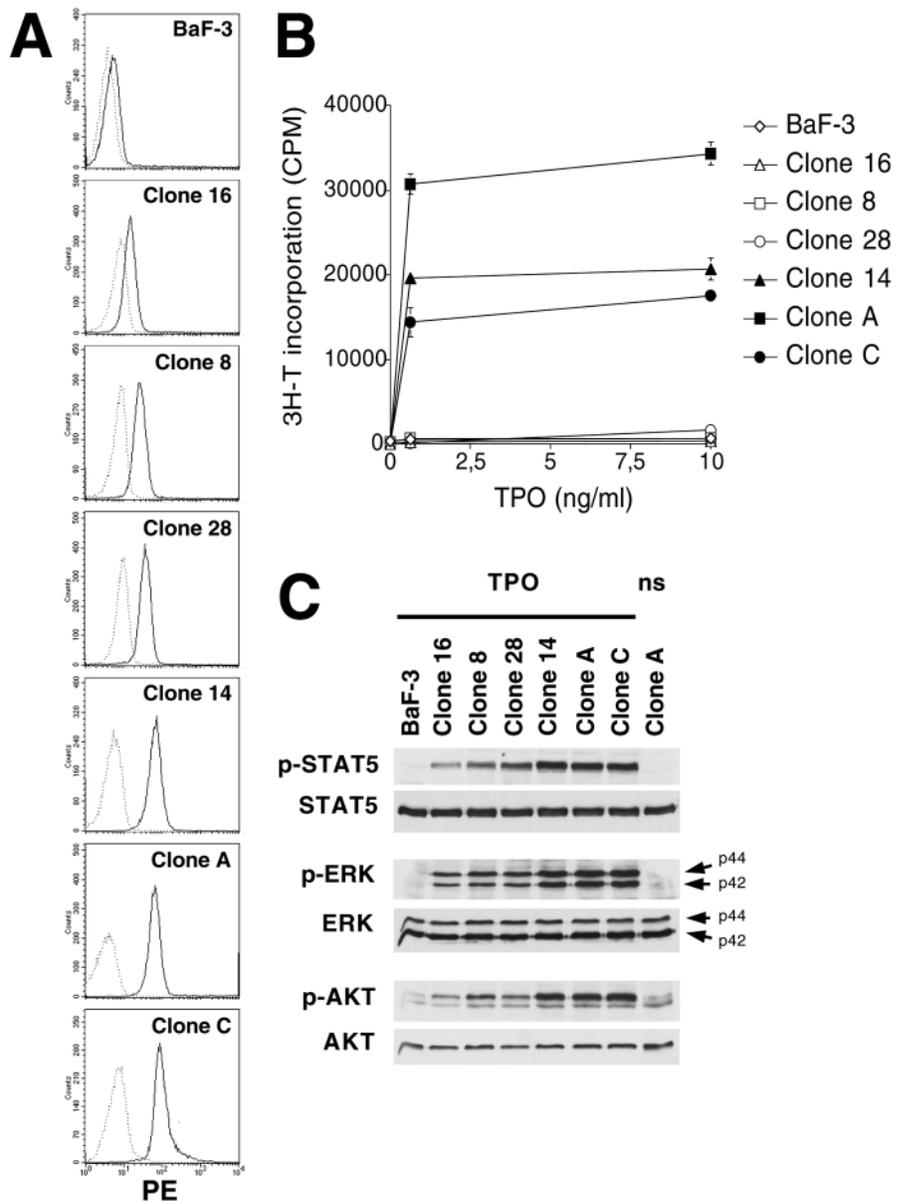
Results

Activation of Mpl signaling pathways but absence of TPO-mediated proliferation in clones with low levels of cell surface expression of Mpl

The BaF-3 cell line is strictly dependent on IL-3 for its survival and does not respond to TPO. Human Mpl, tagged with the Flag epitope sequence at the N-terminus of the

receptor, was introduced into BaF-3 cells by retroviral infection and clones expressing low levels of the retroviral vector were derived. Four clones, named clone 16, 8, 28 and 14 were then selected for their increasing levels of Mpl cell surface expression (Fig. 1A), and were compared with clones A and C, which were derived from cells expressing high levels of retroviral vector.

Clones A and C exhibited a proliferative response to TPO (Fig. 1B) but only clone 14, which expressed an equivalent level of Mpl on the cell surface, was able to give a similar result. Although expressing significant levels of Mpl on their cell surface, ^3H thymidine incorporation by clones 16, 8 and 28 after TPO treatment was low and not significantly different from that of BaF-3 parental cells, even when the TPO concentration exceeded 100 ng/ml (data not shown). This result indicated that a threshold level of activated Mpl was necessary for TPO-mediated proliferation of BaF-3 cells. When signaling pathway activities were studied, phosphorylation levels of



receptor, was introduced into BaF-3 cells by retroviral infection and clones expressing low levels of the retroviral vector were derived. Four clones, named clone 16, 8, 28 and 14 were then selected for their increasing levels of Mpl cell surface expression (Fig. 1A), and were compared with clones A and C, which were derived from cells expressing high levels of retroviral vector.

Table 1. Flag antigen expression on cell surface of clones expressing and overexpressing Mpl

	Flag-PE MFI
BaF-3	5
Clone 16	15
Clone 8	26
Clone 28	37
Clone 16+	42
Clone 8+	61
Clone 28+	70
Clone 14	68
Clone C	89

Means fluorescence intensity (MFI) of Flag-PE-labeled cells were determined by flow cytometric analysis. Results are representative of two independent experiments.

STAT5, ERK and AKT were found to be very similar for clones 14, A and C after 15 minutes of TPO stimulation (Fig. 1C). These signaling pathways were also activated in clones 16, 8 and 28, but at a lower level than that observed for clones 14, A and C. These results therefore suggested that proliferation of Mpl-transduced BaF-3 cells requires a threshold activation level of Mpl signaling pathways.

Overexpression of Mpl in low-Mpl-expressing clones restores TPO-induced cell proliferation

To confirm that insufficient Mpl cell surface expression was responsible for the absence of TPO-mediated proliferation, clones 16, 8 and 28 were retransduced with the Mpl retroviral construct and, for each clone, the most Flag-positive cells were sorted. These three Mpl-overexpressing clones were named clone 16+, 8+ and 28+ (Table 1). After 48 hours of TPO stimulation, a proliferative response similar to that of clone 14 was observed for clones 16+, 8+ and 28+, in contrast to the absence of [³H]thymidine incorporation by parental clones 16, 8 and 28 (Fig. 2A; Fig. 1B). This indicated that the low Mpl expression on the cell surface was responsible for the absence of TPO-mediated proliferation of clone 16, 8 and 28. Study of signaling pathways demonstrated that the phosphorylation levels of STAT5, ERK and AKT were significantly increased after 15 minutes of TPO stimulation for clones 16+, 8+ and 28+ compared with parental clones 16, 8 and 28, and in fact were similar to the phosphorylation levels observed in clone 14 (Fig. 2B; Fig. 1C). This suggested that a threshold activation level of Mpl signaling pathways was necessary for TPO-mediated proliferation of Mpl-transduced BaF-3 cells.

TPO induces a survival effect in low-Mpl-expressing clones

The anti-apoptotic effect of TPO was examined by quantifying the sub-G1 peak after propidium iodide incorporation. As expected, parental BaF-3 cells exhibited an identical pattern of time-dependent appearance of apoptotic cells when either cytokine-deprived or stimulated with TPO (Fig. 3). In contrast, 10 ng/ml of TPO fully inhibited apoptosis of clone 14 (Fig. 3) and clone A (data not shown), even after 34 hours of stimulation, whereas more than 80% of the cells were in the sub-G1 peak after 34 hours of cytokine

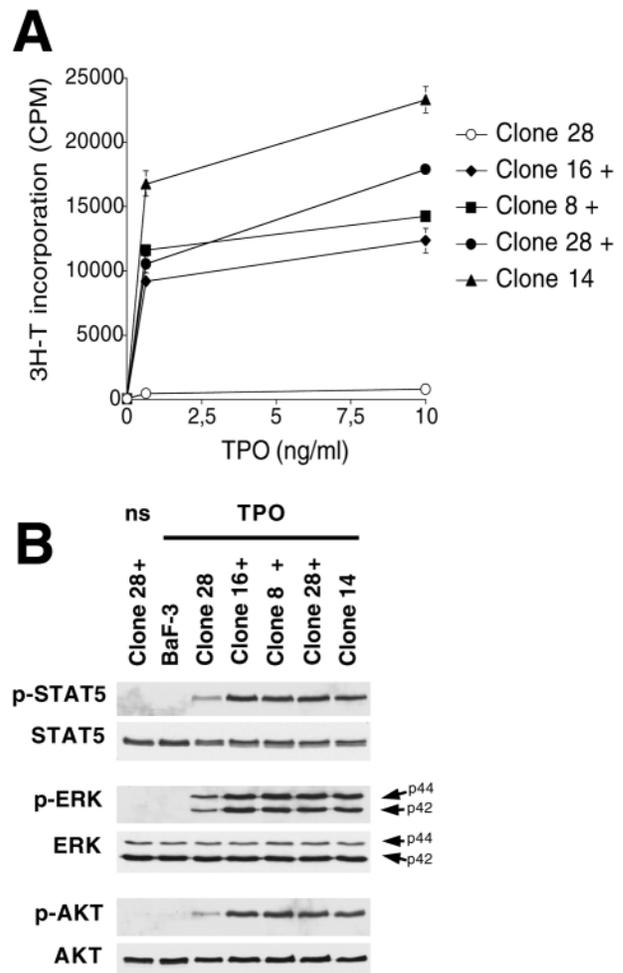


Fig. 2. Proliferation and high activation of signaling pathways are restored in previously underexpressing clones that were retransduced to increase expression of Mpl. (A) Proliferation assay. Cells were incubated with the indicated concentration of TPO for 48 hours and cell proliferation was quantitated by [³H]thymidine incorporation. The results shown are the means \pm s.d. of triplicate experiments. (B) Western blots analysis of signaling pathways activated in clones stimulated by TPO. Cells were washed three times, deprived of cytokines for 3 hours, and then stimulated with 50 ng/ml of TPO for 15 minutes. Activation of STAT-5, ERK and AKT were determined as described in Fig. 1. ns, no stimulation.

deprivation. At this concentration, a clear anti-apoptotic effect of TPO was also observed in clones with low levels of Mpl expression. Therefore, although insufficient for TPO-induced cell proliferation, the level of Mpl cell surface expression of clones 16, 8 and 28 was sufficient to provide TPO-mediated cell survival. However, this effect was transient, as it did not permanently protect cells from apoptosis but delayed its appearance. Indeed, compared with cytokine-deprived cells, the TPO-induced survival effect was maximal at 15 hours for clone 16 (73% versus 36% of apoptotic cells, respectively, a decrease of 37%), at 21 hours for clone 8 (a decrease of 40%) and at 24 hours for clone 28 (a decrease of 72%).

The influence of TPO concentration was also studied (Fig. 4). A TPO concentration of between 10 ng/ml and 1 ng/ml was

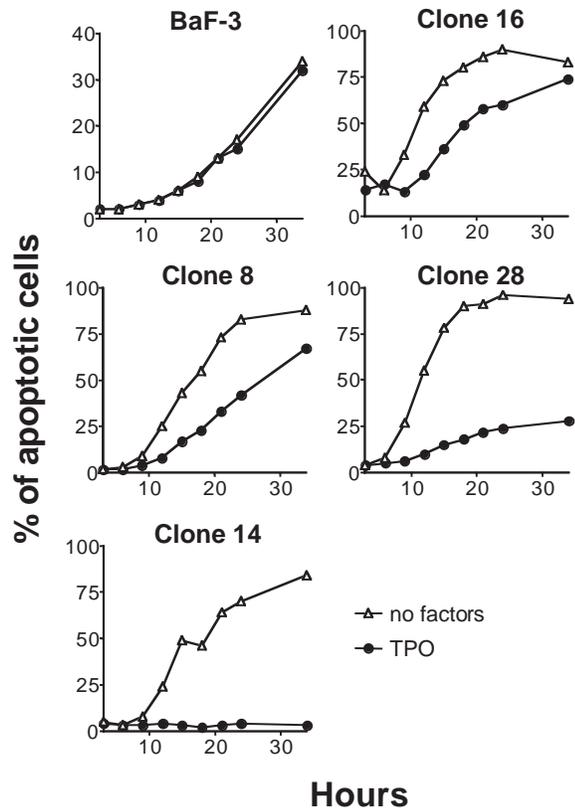


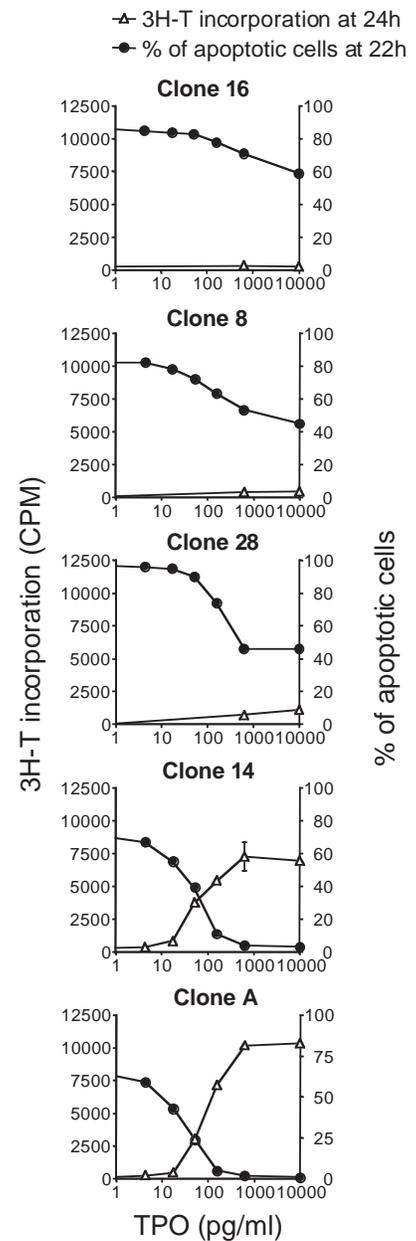
Fig. 3. Effect of TPO on cell survival in clones with low levels of Mpl expression on their cell surface. Cells were washed three times and left deprived of cytokines or stimulated with 10 ng/ml of TPO. At the indicated time, cells were collected and labeled with propidium iodide. Percentages of apoptotic cells in the sub-G1 peak were determined by flow cytometry. Results are representative of two independent experiments.

required for both proliferation and survival events in clones 14 and A. In contrast, clones 16, 8 and 28 responded to increasing TPO concentrations by yielding reduced numbers of apoptotic cells, but without a significant change in [^3H]thymidine incorporation. Therefore, TPO mediated distinct effects in Mpl-transduced BaF-3 cells, depending on a threshold level of activated Mpl: TPO induced proliferation at high levels of cell surface expression of Mpl, but survival alone without a proliferative effect at low levels of Mpl expression. The survival effect remained transient in low-Mpl-expressing clones, even when TPO concentrations exceeded 100 ng/ml (data not shown).

Study of Mpl signaling pathways indicated that ERK and AKT were still activated after 9 hours of TPO stimulation in clones 16, 8 and 28, but to a lesser extent than in clone 14 and A (Fig. 5A). However, phosphorylation of STAT5 was clearly observed in clones 14 and A, but was at the limit of detection in clone 28 and not detectable in clones 8 and 16.

Cytokines suppress apoptosis by regulating expression of bcl-2 family members. STAT5 regulates the expression of the anti-apoptotic protein Bcl-X_L and the pro-apoptotic signaling protein BAD is inactivated after phosphorylation by AKT (del Peso et al., 1997; Dumon et al., 1999). In our clones, the

Fig. 4. TPO-dependent survival and proliferation of clones expressing Mpl. (A) Cells were washed three times and stimulated with various concentrations of TPO. At the indicated times, cells were either labeled with PI and percentages of apoptotic cells determined by flow cytometry (right axis) or cell proliferation was quantitated by [^3H]thymidine incorporation (left axis). The results shown for [^3H]thymidine incorporation are means \pm s.d. of triplicate experiments. Results are representative of two independent experiments.



expression level of Bcl-X_L did not correlate with the phosphorylation level of Mpl signaling pathways. After 3 hours of TPO stimulation, the expression level was slightly decreased in clone 14 and A, compared with clone 16, 8 and 28, and was similar between clones after 9 hours of TPO stimulation (Fig. 5B). This suggests that the TPO-mediated survival effect observed in Mpl-expressing clones did not depend on the expression level of Bcl-X_L. In contrast, TPO-induced phosphorylation of BAD in clones 16, 8 and 28 after 3 hours of stimulation, compared with unstimulated cells (Fig. 5B). This result indicated that the activation level of signaling pathways in clones with low levels of Mpl expression, was sufficient to inactivate BAD. Moreover, the phosphorylation level of BAD decreased markedly in clones 8 and 28 after 9 hours of TPO stimulation, and was at the limit of detection in clone 16, whereas it was still observed in clones 14 and A. This result indicated that BAD was

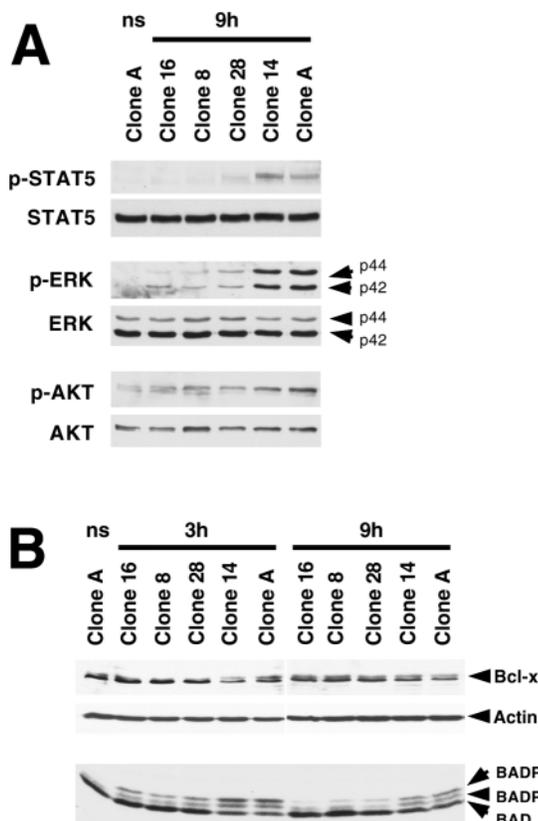


Fig. 5. Western blot analysis of signaling pathway intermediates activated in Mpl-expressing clones after long-term TPO stimulation. Cells were washed three times and stimulated with 10 ng/ml of TPO for 3 or 9 hours. Cells were lysed and 60 μ g of protein per lane was fractionated by SDS-PAGE. Lysate of clone A deprived of cytokines for 3 hours was used as a control. (A) Activation of STAT-5, ERK and AKT after 9 hours of TPO stimulation was analyzed as described in Fig. 1. (B) Expression level of Bcl-x_L and BAD phosphorylation analyzed by immunoblotting with anti-Bcl-x and anti-BAD antibodies. As a control, blots labeled with the anti-Bcl-x antibody were stripped and relabeled with a mouse anti-actin antibody. ns, no stimulation. Positions of unphosphorylated (BAD), singly (BADP1) and doubly phosphorylated BAD (BADP2) are indicated.

transiently inactivated by TPO in clones with low levels of Mpl expression and this phenomenon correlated with the transient nature of the alternative TPO-mediated survival effect.

Analyses of Mpl signaling pathways involved in the TPO-mediated proliferative and survival effect

Inhibition of ERK activation with 50 μ M of the MEK inhibitor PD98059 resulted in a more than twofold decrease of TPO-mediated proliferation in clone A (3642 \pm 359 cpm of [³H]thymidine incorporation, versus 10,213 \pm 453 cpm with TPO alone; Fig. 6A) and clone 14 (3765 \pm 388 cpm versus 7981 \pm 243 cpm, data not shown). A similar effect was obtained with 10 μ M of the PI3K inhibitor LY294002 (3423 \pm 117 cpm for clone A, Fig. 6A; and 2639 \pm 163 for clone 14, data not shown). These results indicated that the activation level of both MAPK p42/44 and PI3K pathways was a determining factor

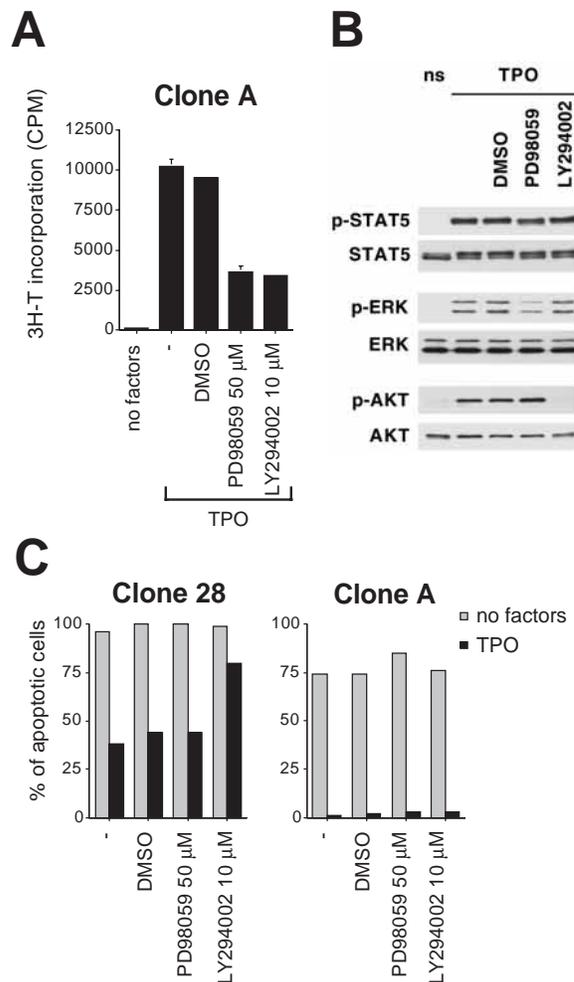


Fig. 6. The PI3K/AKT pathway is involved in the TPO-mediated survival effect observed in clones with low levels of Mpl expression. (A) Proliferation assays. Cells of clone A were washed three times and left cytokine-deprived or incubated for 24 hours with 10 ng/ml of TPO, solvent alone (0.14% DMSO), or the indicated concentration of inhibitors. Cell proliferation was quantitated by [³H]thymidine incorporation. The results shown are means \pm s.d. of triplicate experiments. (B) Western blot analysis of STAT-5, ERK and AKT activation. Cells from clone A were washed three times, deprived of cytokines for 3 hours and stimulated with 50 ng/ml of TPO for 15 minutes, DMSO (inhibitor solvent) alone, 50 μ M of the MEK inhibitor PD98059 or 10 μ M of the PI3K inhibitor LY294002. Activation of STAT5, ERK and AKT was analyzed as described in Fig. 1. ns, no stimulation. (C) Apoptosis analysis. Cells were washed three times and left cytokine-deprived or stimulated for 22 hours with 10 ng/ml of TPO, solvent alone (0.14% DMSO), or the indicated concentration of inhibitors. Cells were then labeled with PI and percentages of apoptotic cells were determined by flow cytometry. Results are representative of two independent experiments.

for the TPO-mediated proliferation of Mpl-transduced BaF-3 cells.

The PI3K inhibitor LY294002 at a concentration of 10 μ M fully abolished phosphorylation of AKT after TPO stimulation but did not abolish ERK or STAT5 activity (Fig. 6B). At this concentration, the transient survival effect of clone 28 after 22 hours of TPO stimulation (Fig. 6C) was decreased twofold with

80% apoptotic cells, versus 44% with solvent alone (DMSO). This result indicated that the PI3K inhibitor strongly inhibited the TPO-mediated survival effect, since the percentage of dead cells increased to approximately the same level as that observed without TPO stimulation (99%). The same result was obtained with clone 8 (69% apoptotic cells with LY294002, 35% with DMSO alone and 89% without TPO stimulation; data not shown). In combination, these results indicated that the activation level of the PI3K pathway was a determining factor for the alternative TPO-mediated survival effect. However, 10 μM of LY294002 did not affect the TPO-mediated survival of clones A and 14 (Fig. 6C, and data not shown), even though activity was totally abolished in clone A (Fig. 6B) and although TPO-mediated proliferation was partially inhibited by LY294002 in clone A (Fig. 6A) and clone 14 (data not shown). These results therefore indicated that activation of the PI3K pathway was not essential for survival when Mpl-transduced BaF-3 cells proliferate in response to TPO.

Inhibition of ERK activation by 50 μM of the MEK inhibitor PD98059 (Fig. 6B) did not affect the TPO-mediated survival of clone 28 (44% apoptotic cells; Fig. 6C) and clone 8 (36%; data not shown). Also, it did not inhibit the TPO-mediated survival of clone A and 14 (Fig. 6C, and data not shown), despite a level of inhibition of TPO-mediated proliferation similar to that obtained with 10 μM of LY294002. This result indicated that the MAPK p42/44 pathway was not involved in the TPO-mediated survival of Mpl-transduced BaF-3 cells, despite long-term activation of ERK in clones with low levels of Mpl expression.

Discussion

Previous studies have reported that the Mpl/TPO system is involved in both cell proliferation and survival effects. In the present study, we demonstrate that TPO induces different biological responses in Mpl-transduced BaF-3 cells, depending on the cell surface density of Mpl and the resulting level of activation of different signaling pathways. TPO mediates cell proliferation in cells expressing high levels of Mpl but mediates only survival without proliferation in cells expressing low levels of the receptor. We further demonstrated that the activation level of the PI3K and MAPK p42/44 pathways is a determining factor for the proliferative effect. The cell survival effect was strongly dependent on the activation level of the PI3K/AKT, but not the MAPK p42/44 pathway. However, PI3K pathway inhibition did not induce apoptosis when BaF-3 cells with high levels of Mpl proliferated in response to TPO.

The PI3K and MAPK p42/44 pathways have been reported to be involved in TPO-mediated proliferation of BaF-3 cells (Dorsch et al., 1997; Rojnuckarin et al., 1999; Geddis et al., 2001). In the present study, we show that the activation level of MAPK p42/44 and PI3K was higher in the proliferative effect than in the cell survival effect. Moreover, the inhibitors LY294002 and PD98059 impaired TPO-mediated proliferation of BaF-3 cells. Together, our results suggest that the TPO-mediated proliferation of BaF-3 cells requires a threshold stimulation level of both PI3K and MAPK p42/44 pathways that depends on the cell surface density of Mpl. Several studies in other systems reported the cooperation of these pathways in the proliferation event (Craddock et al., 2001; Pozios et al.,

2001; Zubilewicz et al., 2001). It has also been reported that constitutively active forms of the p110 catalytic subunit of PI3K leads to oncogenic transformation without significant activation of ERK, indicating that activation of the MAPK p42/44 pathway is not essential for cell growth (Aoki et al., 2000). Conversely, constitutively active forms of MEK lead to factor-independent proliferation, suggesting that activation of MAPK p42/44 is sufficient for triggering cell growth (Mansour et al., 1994; Seger et al., 1994). These findings suggest that cell-cycle entry could be governed by stimulation of signaling pathways involved in proliferation, but that the nature of the activated signaling pathway is not a determining factor. Additionally, we cannot exclude that the activation level of STAT5 is involved in the triggering of the TPO-mediated proliferative effect in our system, which would require further detailed studies.

Our results also indicate that the cell survival effect is associated with weak but sustained activation of the PI3K pathway, and inactivation of BAD. Use of the LY294002 inhibitor demonstrated a major contribution of PI3K activation in this effect. Majka et al. have recently reported that the PI3K/AKT pathway is involved in the TPO-mediated inhibition of apoptosis in megakaryoblasts (Majka et al., 2000), a finding that is consistent with our results. However, our results demonstrate that inhibition of the PI3K/AKT pathway does not affect cell survival when cells are actively proliferating in response to TPO. This result suggests that, in the proliferative effect, the activation level of other Mpl signaling pathways is sufficient to compensate for the inhibition of AKT and to prevent apoptosis. Such an effect has already been reported in BaF-3 cells, whereby the simultaneous inhibition of the PI3K/AKT pathway by LY294002 and of STAT5 by a dominant-negative isoform, is necessary for reducing IL-3-dependent survival. LY294002 inhibited the phosphorylation of BAD and the dominant-negative isoform of STAT5 affected the Bcl-X_L expression (Rosa Santos et al., 2000). Here we show that the Bcl-X_L expression is not increased when BaF-3 cells proliferate in response to TPO. This suggests that, in this case, another mechanism compensates for the inhibition of AKT and for survival of proliferating cells. The JAK2 inhibitor AG490 at a concentration of 25 μM strongly affected the proliferation of clone A and decreased the transient survival effect of clone 8 and 28 after TPO stimulation (data not shown). However, neither phosphorylation of STAT5, nor phosphorylation of ERK and AKT after TPO stimulation was affected by AG490 at this concentration in clones A and 28 (data not shown). Therefore, we were not able to conclude on the involvement of STAT5 in the TPO-mediated proliferative and survival effect. This would require further analysis. We also demonstrate a weak but sustained activation of ERKs in the cellular survival effect in the absence of proliferation. However, the PD98059 inhibitor did not affect the anti-apoptotic effect mediated by TPO in BaF-3 cells. Similar results were reported in megakaryoblast (Fichelson et al., 1999; Majka et al., 2000), indicating that MAPK p42/44 pathway activation is not essential for the TPO-mediated survival event.

Taken together, our results underscore the importance of quantitative differences in receptor activation in the generation of qualitatively different biological responses (reviewed by Zandstra et al., 2000). The biological responses mediated by

the Mpl receptor are dependent on the activity of downstream signaling pathways being sustained over a threshold level: PI3K and MAPK p42/44 activity for the proliferative effect, and PI3K activity for the cell survival effect in the absence of proliferation, although a different mechanism is involved in cell survival when cells are actively proliferating. In our system, the cell survival effect in the absence of proliferation is transient. We were unable to maintain permanent survival of BaF-3 cells expressing low levels of Mpl in the absence of a proliferative response to TPO. This result suggests that proliferation and permanent survival are linked effects in our system.

A threshold level of activity of signaling pathways has already been reported in the case of TPO-mediated differentiation involving the MAPK p42/44 pathway (Rouyez et al., 1997; Matsumura et al., 1998; Rojnuckarin et al., 1999). This effect of MAPK p42/44 could not be evaluated in our system because BaF-3 cells do not differentiate in response to TPO, even in the presence of an overactive form of ERK2 (Rojnuckarin et al., 1999). Thus, it is possible that our results do not accurately reflect the proliferative and cell survival mechanisms that operate in primary megakaryocytic cells. Nevertheless, our system could highlight the way by which TPO alone acts as a survival factor of early hematopoietic progenitors and, in synergy with other cytokines, as a proliferative factor of these cells. Further studies using primary hematopoietic cells will be required to validate this hypothesis.

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