Hematopoietic Growth Factors

research paper

haematologica 2002; 87:1242-1247
http://www.haematologica.org/2002_12/1242.htm

Lyn kinase is activated following thrombopoietin stimulation of the megakaryocytic cell line B1647

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Background and Objectives. B1647 is a cell line derived from bone marrow cells of a patient with acute myeloid leukemia (M2) with a complete erythro-megakaryocytic phenotype and bears both k and p isoforms of c-mpl. Interestingly, spontaneous B1647 cell proliferation is significantly potentiated by thrombopoietin (TPO).

Design and Methods. We aimed to evaluate the proliferative signal transduction events following the activation of c-mpl and we stimulated B1647 cells with TPO 40 ng/mL for 3, 7, 15 and 30 minutes; cells were then lysed and whole lysates were immunoprecipitated with anti-phosphotyrosine antibodies.

Results. In our hands, TPO stimulation induced phosphorylation of several substrate proteins in B1647 cells. The increase in tyrosine phosphorylation from background spontaneous activation was transient, maximal after 10 minutes and declined to reach constitutive levels after 30 minutes. In particular, protein substrates between 50 and 140 kDa appeared to be selectively phosphorylated by TPO. We demonstrated that Jak2, Stat3 and Shc were activated in B1647 cells after TPO, as already shown for different cell lines by other authors. Moreover, Lyn kinase activation was detected. Grb2 co-immunoprecipitated with phosphorylated proteins. The phosphorylation of Syk kinase was not demonstrated, whereas Vav was activated by TPO.

Interpretation and Conclusions. The pattern of protein phosphorylation determined in B1647 cells by TPO testifies the role of this cytokine in sustaining cell growth and indicates Lyn tyrosine kinase as a possible target protein in transduction of the TPO proliferative signal.

Key words: thrombopoietin, acute leukemia, Lyn, signal transduction.

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Thrombopoietin (TPO) plays a determinant role in the physiologic regulation of megakaryocytopoiesis.[1,2] Although erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-11 (IL-11) and interleukin-3 (IL-3) have a role in inducing platelet and megakaryocyte production and function,[3] studies performed with TPO and c-mpl knock-out mice have definitively revealed the prominence of TPO.[4] c-Mpl/TPO-receptor is a member of the hematopoietin receptor superfamily and is most probably activated through homodimerization.[5] TPO plural activity is promoted by different cytoplasmic functional regions of the c-mpl/TPO-receptor.[6] The intracellular domain of receptors belonging to the hematopoietin receptor superfamily shares two short membrane proximal regions named box 1 and box 2, highly conserved and critical for many receptor functions.[6] c-Mpl does not contain the other C-terminal homology motif, designated box 3, essential in G-CSF-receptor and gp130 lineage specific signaling.[7] It has been shown that mutation or loss of either box 1 or box 2 of c-Mpl results in arrest of cell proliferation and lack of Janus kinase activation.[6] c-Mpl, like the other receptors of the superfamily, does not possess intrinsic tyrosine-kinase activity. Nevertheless tyrosine phosphorylation plays an important role in its intracellular signaling, by recruiting non-receptor tyrosine kinases to mediate the phosphorylation of target proteins.[8] In c-Mpl, box 1 mediates TPO proliferative signaling through a Jak-Stat cascade, whereas box 2 transduces cell viability messages and mediates feedback signals to box 1. The COOH-terminus is essential for Shc and Ship phosphorylation.[3,8] Although all this information is at present available, it does not fully reveal the pattern of different signals transduced during induction of maturation or proliferation by TPO, particularly the involvement of kinases. Among the kinases essential in intracellular signaling, Src kinase p53/p56 Lyn is involved in message transducing via the B-cell antigen receptor complex,[9] via Fc type I recep-

tor[10] and via G-CSF-R.[11,12] Employing the B1647 erythro-megakaryocytic human cell line as a model,[13] we demonstrated that Src-family kinase Lyn is involved in TPO signaling. The pattern and timing of tyrosine phosphorylation of other known protein substrates associated or not associated with Lyn in TPO-stimulated B1647 cells was also analyzed.

Design and Methods

Cell line

The B1647 megakaryocytic cell line was routinely maintained in IDMEM medium in the presence of 5% pooled human sera. Cells expressed both k and p isoforms of c-Mpl, as demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) at every passage in culture.

Proliferation assays

B1647 cells were cultured as mentioned above. After one day-wash out in the absence of sera, cells were plated on 96 micro-wells plates at the concentration of 0.1×10^6/mL in serum-free medium in the presence of the following cytokines: EPO 2 U/mL and TPO (10 pg/mL to 50 ng/mL) (Stem Cell Technologies, Vancouver, CA, USA), but also IL-1β 1 ng/mL, IL-3 10 ng/mL, IL-6 100 U/mL, IL-7 10 U/mL, IL-11 100 ng/mL, IL-12 25 ng/mL, G-CSF 10 ng/mL, GM-CSF 50 ng/mL, SCF 20 ng/mL, LIF 100 ng/mL (Stem Cell Technologies), IL-9 10 U/mL, b-FGF 100 ng/mL, and PDGF 100 ng/mL (R&D Systems, Minneapolis, MN, USA). Control cultures were carried out in the absence of any factor in serum-free medium. After 72 hours of culture in these conditions, cells were exposed for 4 hours to tritiated thymidine ([3H]-TdR, 1 μCi/well, specific activity 42 Ci/mmol, Amersham, UK). Pulsed cells were harvested by an automated cell harvester (Skatron, Norway) and incorporation of [3H]-TdR was evaluated by β scintillation counting. Alternatively, cells were counted after staining with trypan blue. The results were expressed as the absolute numbers of viable cells (trypan blue exclusion test).

Preparation of cell lysates

B1647 cells were incubated in IDMEM medium in the absence of serum and growth factors for 20 hours. After starvation, cells were stimulated with TPO 40 ng/mL for 3, 7,10, 15 and 30 minutes at 37°C. Stimulation was stopped by cooling the cell suspension in ice; cells were lysed with 50 mM Tris HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate and protease inhibitors. Insoluble material was removed by centrifugation for 30 minutes at 12,000 g at 4°C.

Immunoprecipitation and Western blotting

Cell lysates were incubated with antiphosphotyrosine (pTyr) antibody clone PY20 or anti-Lyn monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 90 minutes at 4°C, then adsorbed on Protein A Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) for 30 minutes at 4°C. Immune complexes were washed 5 times with lysis buffer (*see above*), eluted and denatured at 95°C for 5 minutes in Laemmli buffer. Lysates were loaded onto 8% and 10% SDS-PAGE gels, subjected to electrophoresis and transferred onto nitrocellulose filters. Filters were then blocked and probed with monoclonal antibodies anti-pTyr, anti-Lyn, anti-Stat3, anti-Stat5, and anti-Grb2 (Transduction Laboratories, Exington, NY, USA), polyclonal antibodies anti-Vav, and anti-SH-PTP1 (Transduction Laboratories, Exington, NY, USA), polyclonal antibodies anti-Jak2 (Pharmingen, San Diego, CA, USA) and anti-Syk (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-Shc (kindly provided by Prof. Pelicci, Perugia, Italy). Immune complexes were finally detected with horseradish conjugated specie-specific secondary antibodies (HPR-conjugated anti-mouse IgG, Roche or HPR-conjugated anti-rabbit IgG, Chemicon, Temecula, CA, USA) followed by enhanced chemoluminescence reaction (Super Signal Ultra Chemoluminescent Substrate, Pierce, USA) To reprobe with another primary antibody, filters were treated at 50°C for 30 minutes with stripping buffer containing 100 mM 2 mercaptoethanol, 62.5 mM TrisHCl pH 6.7 and 2% SDS, and then used for further study.

Results

Proliferation assays

B1647 cells were cultured in serum-free medium for a maximum of 72 hours, a period after which no active cell cycling was maintained if human or fetal calf serum was not added. The presence of optimal concentrations of several different growth factors (i.e. IL-3, GM-CSF, IL-6, EPO; *see Design and Methods*) did not affect B1647 cell proliferation *in vitro* (*data not shown*). Tritiated thymidine uptake and cell count (employed as measures of active cell growth) were significantly enhanced (with respect to control non-stimulated cells) only in the presence of TPO (Figure 1).

Time course of protein tyrosine phosphorylation in B1647 cells

After 20 hours of serum and growth factor starvation, B1647 cells were stimulated for 3, 7, 10, 15 and 30 minutes with supra-optimal doses of

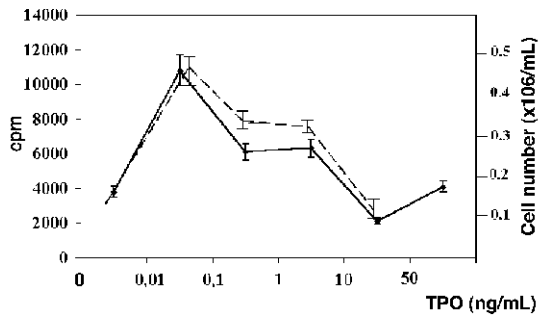

Figure 1. B1647 cell proliferation is enhanced by TPO. Cell growth in the presence of increasing concentrations of TPO was evaluated after 72 hours of culture by tritiated thymidine uptake (solid line) and cell count (coarse broken line). Results are the mean of triplicate experiments.

recombinant human TPO 40 ng/mL to investigate the time course of protein activation. There was a rapid and transient tyrosine phosphorylation of protein substrates, which reached its maximum after 10-15 minutes (Figure 2A). Background phosphorylated tyrosines were present in control unstimulated cells, consistently with the spontaneous proliferative activity demonstrated by B1647 cells. After 3 minutes of TPO stimulation phosphorylated proteins with approximate molecular weight 50-60 KDa and 90-100 KDa were detected in the immnunoprecipitates. These proteins seemed absent in non-stimulated cells: their phosphorylation was maximal at 10-15 minutes of TPO exposure, and they were dephosphorylated concomitantly to all the other activated substrates (in particular p130). Return to control background level was observed after 30 minutes of TPO stimulation.

Lyn-kinase activation by TPO

Anti-phosphotyrosine immunoprecipitates of TPO-stimulated cells indicate the presence of p60-p50 substrates (Figure 2A). Probing with specific anti-Lyn antibodies demonstrated the activation of this kinase by TPO (Figure 2B). Lyn co-immunoprecipitated with phosphorylated proteins also in the absence of TPO stimulation. To confirm the involvement of Lyn kinase in c-Mpl signal transduction, we performed anti-Lyn immunoprecipitations. Lyn was transiently phosphorylated by TPO stimulation (Figures 3 A,B). Anti-Lyn antibodies clearly co-immunoprecipitated other protein substrates of supposed molecular weight 70 and 50 KDa. The latter was demonstrated to be activated Shc. p70 was not defined as Syk, which is frequently associated with Lyn after growth factor stimulation in other growth factor receptor signaling, nor SH-PTP1, previously demonstrated to be involved in TPO/c-Mpl signaling. It is possible that this phosphorylated substrate corresponds to Tec kinase, indicated as one of the kinases recruited by c-Mpl.

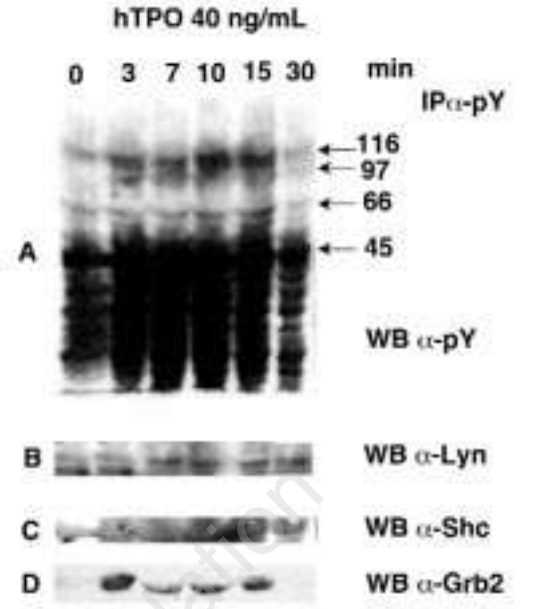

Figure 2. Tyrosine phosphorylation after TPO stimulation. Anti-phosphotyrosine immunoprecipitates of B1647 cells treated with TPO 40 ng/mL for different times were run on SDS-PAGE 10%. Western blot anti-phosphotyrosine (A), anti-Lyn (B), anti-Shc (C), and anti-Grb2 (D) show the presence of activated protein substrates.

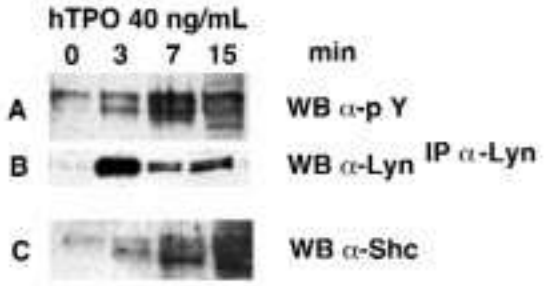

Figure 3. Lyn kinase is activated in B1647 cells by TPO. Anti-Lyn immunoprecipitates of B1647 cells were run on SDS-PAGE 10% and blotted with anti-phosphotyrosine antibodies (A), anti-Lyn antibodies (B) and anti-Shc antibodies (C). Anti-Lyn immunoprecipitation was co-immunoprecipitating three proteins, Lyn, Shc and a third one of apparent molecular weight 70, which was excluded from being Syk.

TPO induces the tyrosine phosphorylation of Shc

TPO stimulation activated Shc in B1647 cells (Figure 2C). Maximal phosphorylation was present at 7-10 minutes, and had declined by 30 minutes. Grb2 docking protein was co-immunoprecipitated with phosphorylated proteins, (Figure 2D) apparently at constant levels after 3 to 15 minutes, decreasing by 30 minutes after stimulation, but with slightly different kinetics from that of Shc. Moreover, Shc was demonstrated to be directly associated with Lyn (Figure 3C).

TPO selectively activates Jak2

The high molecular weight protein phosphorylated in B1647 in response to TPO, with maximal activation 10-15 minutes after stimulation, was demonstrated to be Jak2 (Figures 2A and 4A), whereas anti-Jak1 immunoblotting did not show that Jak1 was involved.

Stat activation after TPO

Anti-phosphotyrosine immunoprecipitates were blotted with anti-Stats antibodies, and the presence of activated Stat3 and Stat5 was maximal 7 minutes after TPO stimulation (Figure 4B and 4C). Although after 15 minutes the activation of 90 KDa tyrosine phosphorylated proteins was decreasing (Figure 2A), the presence of Stat3 and Stat5 was still detectable by specific antibodies.

TPO is able to activate Vav

In anti-phosphotyrosine immunoprecipitates of TPO-stimulated B1647, Vav was present and thus functionally activated, with maximal phosphorylation after 15 minutes (Figures 4D and 2A).

Discussion

We demonstrate here for the first time that Src-kinase Lyn is involved in the TPO/c-Mpl signaling pathway, using as an *in vitro* model the B1647 human megakaryocytic cell line, which proliferates after TPO stimulation.

The B1647 erythro-megakaryocytic human cell line was established from the bone marrow of a 14-year old boy with acute myeloid leukemia (FAB M2). It expresses both isoforms of c-Mpl (κ and p) and EPO-R on its surface. B1647 cells are CD34 negative, CD33, CD38 and HLA DR positive, and CD41 and von Willebrand factor positive.[13] B1647 cells, given their phenotype, can be considered prototypes of common erythroid and megakaryocyte progenitors. Moreover, although not growth factor dependent, B1647 spontaneous proliferation is increased exclusively by TPO and in our opinion this property renders it an ideal, naturally occurring model to analyze, perhaps enabling TPO proliferative signal transduction to be dissected.

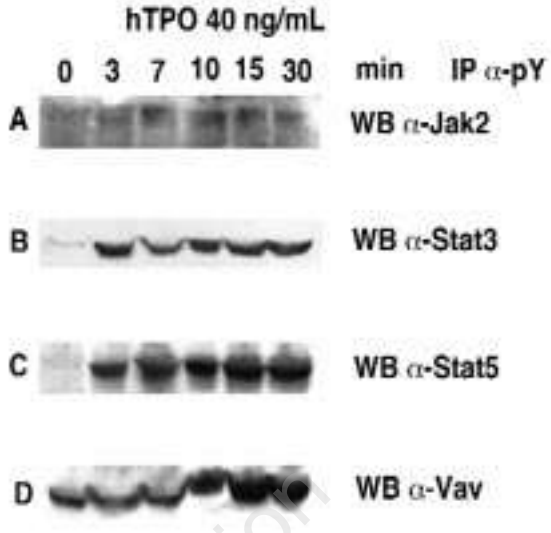

Figure 4. Activation of Vav and Stats in B1647 cells by TPO. B1647 cells were stimulated by TPO after starvation, cell lysate immunoprecipitated with anti-phosphotyrosine antibodies, and Western blot analysis was performed at different time points with anti-Stat3 and anti-Stat5 antibodies, as well as with anti-Vav antibodies.

Lyn kinase activity and its role in TPO, as well as in other hematopoietic growth factor signal transduction has not been fully considered. Lyn kinase is activated by the membrane proximal region of the intracytoplasmic domain of G-CSF-R and probably contributes to its dimerization,[11] at the same time starting a cascade of events, including Syk kinase phosphorylation.[12] TPO stimulation of the B1647 cells provoked a rapid and transient enhancement in tyrosine phosphorylation of Lyn kinase. Background Lyn activation could be detected also in unstimulated, spontaneously growing B1647 cells, confirming the importance of this protein in mediating proliferative signals, even autonomous and autocrine ones. These observations are in contrast to those described by other authors,[14] who did not detect any activation or enhancement of phosphorylation of Lyn after TPO stimulation of engineered lymphoid BAF cells. In our opinion this discrepancy might be generated by the substantial variances present in the signaling apparatus of different cell types. B1647 megakaryocytic cells constitute an almost physiologic model for TPO. Moreover, the pattern of TPO-induced tyrosine phosphorylation has been shown to vary even among normal megakaryocytes and leukemic cells transduced with c-Mpl,[15] indicating a high specificity. Quite differently from oth-

er hemopoietin receptors, for c-Mpl we could not demonstrate that Syk kinase was involved or associated with Lyn activation. This observation may indicate that the TPO/c-Mpl interaction elicits a specific alternative pathway of kinase activation. It has recently been shown that Tec kinase is phosphorylated upon TPO stimulation.[16] Tec is the prototype of a subfamily of non-receptor tyrosine kinases whose members (Tec, Btk, Itk, Txk, Bmx) have been demonstrated to play a role in the proliferation and differentiation of hematopoietic cells.[17] Tec is related to Lyn, and co-immunoprecipitates with it when activated.[18,19] It is possible that the cascade initiated by TPO-induced Lyn activation identifies an alternative kinase pathway complementary or parallel to the Jak-Stat pathway which is transducing TPO signals. In fact, our analysis of B1647 cell response to TPO confirmed what has been previously observed[20-23] Jak2, but not Jak1, is phosphorylated after TPO stimulation. Downstream subsequent activation of Stat protein was also present in B1647 cells. Although we observed slight discrepancies in Stat phosphorylation timing (less rapidly enhanced in B1647), the pattern of Stat3 and Stat5 activation demonstrated in B1647 cells is consistent with that described in previous reports.[24-27]

Neither Stat 1 nor Stat 4 was immunoprecipitated with phosphorylated proteins. Unstimulated, starved B1647 cells do express phosphorylated Stat 5, indicating that this molecule is essential in determining the proliferative activity of these cells. We may thus confirm the essential and ubiquitous role of Stat3 and Stat5 in TPO signaling. Although starved B1647 cells maintain the ability to proliferate spontaneously, TPO stimulation further promotes cell growth and produces a concomitant enhancement of Stat 5, as well as Lyn and Jak2 phosphorylation. This finding is somehow in contrast to the growth factor independence shown in megakaryocytic cell lines with Jak2/Stat5 constitutive activation.[28] We confirmed in this study that Shc is activated by TPO[21,29,30] and is linked to the docking protein Grb2, but also co-immunoprecipitates with phosphorylated Lyn kinase. Lyn could act by directly phosphorylating Shc, thus activating the raf-1 pathway. B1647 cells do phosphorylate Shc but are not able to mature following TPO stimulation.[13] These data are consistent with those observed in other cytokine receptor systems and other cellular models, in which Shc activation has been related to cell proliferation more than cell maturation.[31] We also observed Vav activation[32,33] during TPO proliferative signaling in B1647, but in our hands this protein was not directly bound to Lyn kinase.

The role of the Jak-Stat pathway in conveying TPO-growth signals has, in the past, been controversial.[14] Quite recently, however, sarcoma cell lines deficient in Jak2 or Tyk2 expression and engineered to express c-Mpl were employed to demonstrate that only Jak2 was essential to initiate TPO-mediated signaling.[23]

Our results suggest that TPO stimulation can induce the activation of redundant kinase pathways i.e., Jak2 and Lyn as well as Vav, leading to downstream phosphorylation of Shc, Stat3 and Stat5. We are at present investigating the role of PI3K in B1647 cells. In fact P13K has been shown to be stimulated by TPO in murine megakaryocytic cells.[34] Modulation of cross-talk and synergy among diverse protein-kinase families must be evaluated to elucidate the diversity of TPO signals in megakaryocytes and megakaryocyte progenitors, platelets, all cord blood progenitors, all cells expressing functional identical c-Mpl and nevertheless responding with specific and different biological events to TPO stimulation.

Contributions and Acknowledgments

VS designed and executed the experiments, and wrote the paper. BS designed and executed the experiments. All the other authors contributed with laboratory work and developed the paper. AG and LB are responsible for Figure 1. AG is responsible for Figure 2; VS and BS are equally responsible for all the other figures.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Funding

This work was supported by the Associazione Italiana Leucemie, 30 ore per la vita; MURST 1998. LB and GPB are supported by AIRC.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. José Luis Fernandez Luna, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Fernandez Luna and the Editors. Manuscript received August 13, 2002; accepted October 29, 2002.

What is already known on this topic

A number of molecular targets involved in proliferation, survival and differentiation have been shown to be phosphorylated via thrombopoietin stimulation.

What this study adds

It suggests novel insights into the proliferative signals triggered by TPO.

Potential implications for clinical practice

Understanding the signaling pathways that take place in megakaryocytic cells will help to unravel the mechanisms that may dysregulate the homeostasis of this cellular compartment.

José Luis Fernandez Luna, Associate Editor
(Santander, Spain)