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ORIGINAL ARTICLE

Effect of Thrombopoietin and Granulocyte Colony-Stimulating Factor on Platelets and Polymorphonuclear Leukocytes

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Abstract

Thrombopoietin (TPO) and granulocyte colony-stimulating factor (G-CSF) may be administered together in aplastic patients. We evaluated the effect of both cytokines alone or combined on platelets and polymorphonuclear leukocytes (PMN) functional responses. TPO, G-CSF, or the combination of both cytokines, induced neither platelet nor PMN activation. TPO but not G-CSF synergized with threshold ADP concentrations to induce maximal aggregation and ATP release. The synergistic effect of TPO with ADP was not modified by the presence of G-CSF. Flow cytometry studies have shown that thrombin-induced loss of GPIb from platelet surface was significantly increased by pretreatment of platelets with TPO, G-CSF, or both cytokines. P-selectin expression induced by thrombin was augmented by TPO, but not by G-CSF. Coincubation of the cells with TPO and G-CSF did not modify the values obtained with TPO alone. Expression of CD11b on PMN surface was augmented by G-CSF or fMLP. G-CSF-treated PMN increased the effect of fMLP on CD11b expression. TPO did not modify either basal levels of CD11b or the increased expression induced by G-CSF or fMLP. Incubation of PMN with both cytokines showed no differences compared to G-CSF alone. Platelet-PMN aggregates induced by thrombin in whole blood were augmented by TPO. G-CSF alone neither synergized with thrombin nor changed the results observed with TPO. These data show that in vitro functional responses of platelets, or PMN induced by TPO or G-CSF alone, were neither further increased nor inhibited by treatment of the cells with both cytokines.

Key Words: Thrombopoietin; Granulocyte colony-stimulating factor; Platelets; Polymorphonuclear leukocytes

Thrombopoietin (TPO) is the primary physiologic regulator of megakaryocytopoiesis. TPO stimulates megakaryocyte colony growth, promotes megakaryocyte maturation, and supports the formation of functional platelets in vitro and in vivo [1–5]. Its potential as a therapeutic agent in ameliorating thrombocytopenia associated with primary disorders of bone marrow or post chemotherapy is enormous. In most cases,

Abbreviations: TPO, thrombopoietin; G-CSF, granulocyte colony-stimulating factor; PMN, polymorphonuclear leukocytes; GP, glycoprotein; MoAbs, monoclonal antibodies; fMLP, n-formyl-met-leu-phe; PE, phycoerythrin; FITC, fluorescein isothiocyanate; rHu, recombinant human; PRP, platelet-rich plasma; dPBS, dulbeco phosphate-buffered saline; BSA, bovine serum albumin; HBSS, 150 mM NaCl, 5 mM KCl, 1 mM SO_4Mg, 10 mM Hepes pH=7.4; CD, cluster differentiation; RT, room temperature.

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TPO would be administered in combination with G-CSF.

It is known that growth factors have a wide range of biological activities in addition to their main role as regulators of hematopoiesis. Thrombopoietin, although not active by itself, is able to prime platelet aggregation, 5-hidroxitriptamine release, and thromboxane B2 formation induced by other platelet agonists [6,7]. It induces tyrosine phosphorylation of several cellular proteins including the TPO receptor, c-Mpl [8]. It has recently been shown that besides its effects on platelets, TPO also regulates PMN activation by a specific interaction with its receptor expressed on PMN surface [9]. G-CSF is not only the humoral factor responsible for granulocyte formation but also increases the host defense activity of PMN by priming these cells to enhance responses to several inflammatory substances [10–12]. Moreover, treatment of platelets with G-CSF results in an increased platelet reactivity toward a second platelet stimulus [13].

All these data suggest that simultaneous administration of both cytokines could promote platelet and/or leukocyte activation, which in turn would increase the risk of occurrences of thrombotic episodes. In this study, we investigated the effects of cytokines, either alone or in combination, on platelet and PMN biological responses.

1. Materials and Methods

1.1. Reagents and Antibodies

Monoclonal antibodies (MoAbs) were obtained from Immunotech (Marseille, France): phycoerythrin (PE)-labeled anti-CD42b (GPIb) and fluorescein isothiocyanate (FITC)-labeleled-anti-CD62p (P-selectin) were used as platelet markers. FITC-anti-CD11b and FITC-anti-CD45 were used as leukocyte markers. All experiments included irrelevant isotype-specific mouse MoAbs as negative controls.

Recombinant human (rHu) TPO was obtained from R&D Systems (Abington, UK); rHu G-CSF, ADP, human thrombin, and fMLP were obtained from Sigma (St Louis, MO, USA).

1.2. Blood Samples

Blood samples were obtained from healthy donors who had taken no medication for at least 10 days before the day of sampling. Blood was obtained by venepuncture of the forearm vein, and it was drawn directly into plastic tubes.

1.3. Platelet Aggregation

Platelet-rich plasma (PRP) was prepared by centrifugation of the citrated blood samples at $180\times g$ for 10 minutes. Platelet aggregation was recorded in a Chrono-Log lumiaggregometer. Aggregation was measured at 37°C under continuous stirring in 0.4-mL aliquots of PRP, and it was monitored for 5–30 minutes. Aggregation was expressed as a percentage of maximal light transmission.

1.4. Platelet Adenosine Triphosphate Release

Adenosine triphosphate (ATP) release was measured simultaneously with platelet aggregation using luciferin-luciferase as the luminescent reagent.

1.5. Platelet Glycoprotein Expression

EDTA anticoagulated blood samples were allowed to sediment and, when ready, 45 uL of a 1:10 diluted suspension of platelets in dulbeco phosphate-buffered saline (dPBS) were treated with TPO, G-CSF, both cytokines or dPBS/bovine serum albumine (dPBS/BSA) (0.1%) (cytokine vehicle) for 15 minutes at room temperature (RT). Then, 5 uL of samples were transferred to 50 uL of dPBS containing saturating concentrations of PE-CD42b or FITC-CD62p antibodies, arginyl-glycyl-aspartyl-serine (1 mg/mL), and incubated for 30 minutes at RT. After fixation with paraformaldheide 1%, platelets were analyzed by flow cytometry. In some experiments, after platelet incubation with cytokines, platelets were stimulated by the addition of thrombin for 3 minutes.

1.6. PMN CD11b Expression

Human PMN were isolated by Ficoll-Isopaque ($d=1.077$ g/cm^3) gradient. Red cells were lysed by hypotonic shock. The purity of PMN suspension was approximately 90–95%. Expression of CD11b with or without cytokines was evaluated in a similar manner as described for platelet glycoproteins.

1.7. PMN-Platelet Aggregates

Mixed aggregates were measured as described by Goodall and Hjemdahl [14]. Briefly, aliquots (5 uL) of citrated blood samples were transferred to 50 uL of buffer (150 mM NaCl, 5 mM KCl, 1 mM SO_4Mg, 10 mM Hepes pH=7.4, HBSS) containing saturating concentrations of PE-CD42b, FITC-CD45, arginyl-glycyl-aspartyl-serine (1 mg/mL), and TPO, G-CSF, both cytokines or dPBS/BSA (0.1%) (cytokine vehicle). After 15 minutes of incubation at RT, mixed aggregates formation was stimulated by the addition of thrombin for 3 minutes. Then, 5 uL of the sample were added to 400 uL of HBSS and immediately analyzed by flow cytometry.

1.8. Flow Cytometry

Flow cytometric analysis was performed using a Becton Dickinson flow cytometer. Fluorescence attributable to FITC and PE-labeled antibodies was excited by an argon laser operating at 488 nm. Emission from fluorescein and PE was measured using bandpass filters of 525 nm and 585 nm, respectively. Platelets or PMN were first analyzed by forward and side scatter, and the gates were set to include the majority of the cells and to exclude larger particles. A fluorescence histogram was obtained for 10000 cells. Mixed aggregates were analyzed as described by Goodall and Hjemdahl [14]. Briefly, live gating on leukocyte-sized events was performed to exclude single platelets using a combination of forward and side scatter, and positive anti/CD45 fluorescence. Leukocyte subsets, i.e. monocytes, PMN, and lymphocytes, were distinguished from one another on the basis of these characteristics. The $CD45^+$ population was then analyzed in an FL1 vs. FL2 dot-plot for the presence of the CD42b (FL2) signal, indicative of leukocyte binding to one or more activated platelets.

1.9. Statistical Analysis

Student's paired *t*-test was used to determine the significance of differences between means, and $p<0.05$ was considered statistically significant.

2. Results

2.1. Effect of TPO and G-CSF on Platelet Aggregation and ATP Release

The addition of TPO up to 500 ng/mL to a PRP was not able to induce either platelet aggregation or ATP release ($n=7$). However, preincubation of PRP for 30 minutes with TPO (100 ng/mL) significantly potentiated platelet aggregation (70±6% vs. 23±3%, $n=7$, $p<0.001$) and ATP release (2.3±0.7 uM vs. 0.2±0.1 uM, $n=4$, $p<0.05$) induced by threshold concentrations of ADP (0.5±1 uM). In fact, in platelets pretreated with TPO, a first wave of aggregation, with no ATP release induced by ADP, was transformed into a second wave of aggregation accompanied by ATP release. Treatment of platelets with 10 ng/mL of TPO did not modify either platelet aggregation or ATP release induced by ADP ($n=5$). TPO at 50 ng/mL augmented aggregation and ATP release induced by ADP only in two out of five experiments. Since the synergism between TPO at 100 ng/mL and ADP was observed in all cases, this TPO concentration was used in the rest of the experiments.

G-CSF alone up to 500 ng/mL was not able to induce any platelet response ($n=7$) and, in contrast to TPO, it was completely unable to enhance platelet aggregation (32±3% vs. 23±3%, $n=5$) or ATP release (0.2±0.1 uM vs. 0.2±0.1 uM, $n=5$) induced by ADP. The TPO enhancement of aggregation and ATP release induced by ADP was not modified (72±6% and 2.9±0.9 uM of ATP, $n=5$) by the simultaneous treatment of platelets with G-CSF (100 ng/mL) and TPO (100 ng/mL) before platelet stimulation by ADP. No effect of G-CSF was observed even when this cytokine was used at 500 ng/mL.

2.2. Effect of TPO and G-CSF on Platelet GPIb and P-Selectin

We analyzed the pattern of glycoprotein expression in response to TPO and G-CSF. Incubation of platelets with TPO (10–500 ng/mL), G-CSF (10–500 ng/mL), or both cytokines, did not induce significant changes in the level of GPIb or P-selectin on the platelet surface (data not shown, $n=7$). However, Figure 1 shows that loss of GPIb induced by thrombin (0.5 U/mL) was significantly aug-

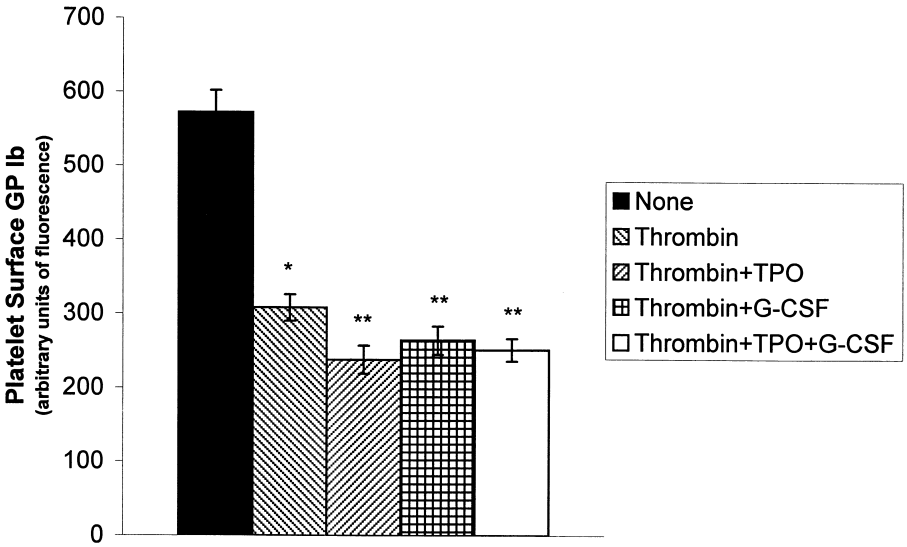

Fig. 1. Effect of TPO and G-CSF on platelet GPIb expression. Each value represents the mean±SEM of four separate experiments. *$p<0.05$ when comparing with nonstimulated platelets; **$p<0.05$ when comparing with thrombin stimulated platelets.

mented when platelets were treated with TPO, G-CSF, or both cytokines (100 ng/mL).

P-selectin expression induced by thrombin (0.5 U/mL) was increased by TPO (100 ng/mL), but not by G-CSF (100–500 ng/mL) (Figure 2). This increase was not modified by simultaneous incubation of the platelets with TPO and G-CSF.

2.3. Effect of TPO and G-CSF on PMN CD11b Expression

Expression of CD11b on PMN surface was significantly augmented by G-CSF (100 ng/mL) or fMLP (5×10^{-7} M) (Figure 3). TPO (100–500 ng/mL) was not able to modify either the basal levels of CD11b or the augmented expression induced by G-CSF. It can be seen from Figure 3 that preincubation of PMN with G-CSF increased the effect of fMLP on CD11b expression, but TPO had no effect. Coincubation of leukocytes with TPO and G-CSF gave similar results as those obtained with G-CSF alone.

2.4. Effect of TPO and G-CSF on PMN-Platelet Mixed Aggregates Formation

Because we found that TPO increased P-selectin expression induced by thrombin, we finally studied

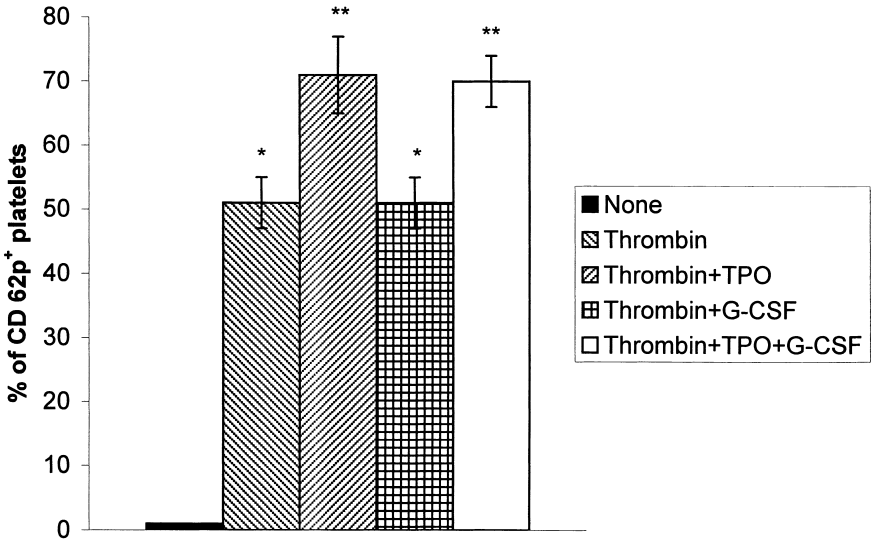

Fig. 2. Effect of TPO and G-CSF on P-selectin expression. Each value represents the mean±SEM of four separate experiments. *$p<0.05$ when comparing with nonstimulated platelets; **$p<0.05$ when comparing with thrombin stimulated platelets.

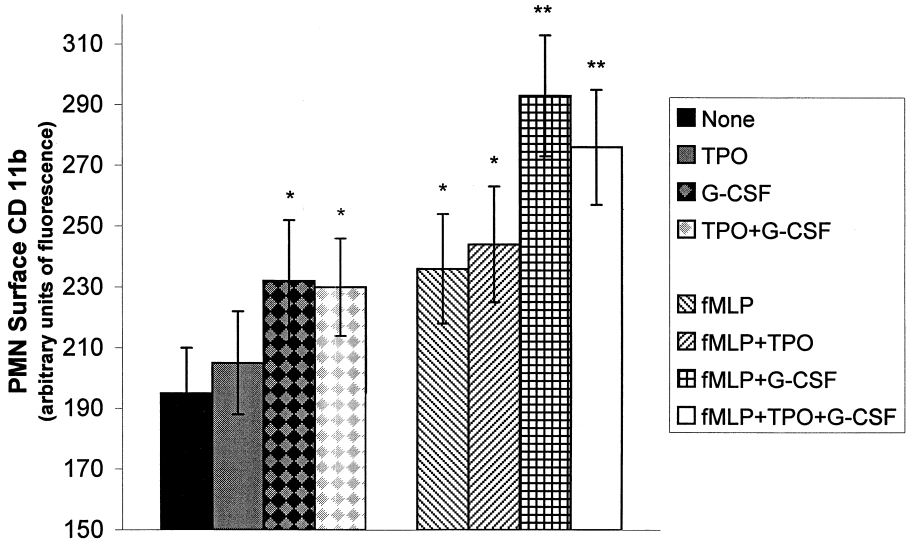

Fig. 3. Effect of TPO and G-CSF on CD11b PMN expression. Each value represents the mean±SEM of five separate experiments. *$p<0.05$ when comparing with nonstimulated PMN; **$p<0.05$ when comparing with fMLP-stimulated PMN.

the ability of TPO and G-CSF to induce PMN-platelet aggregates. To quantify the interaction of platelets and leukocytes, we used a double immunofluorescence staining that permits the simultaneous detection of two different types of single cells and of their presence in mixed/type cell aggregates. While thrombin (0.5 U/mL) induced 40% of mixed aggregates, neither TPO, G-CSF, nor both cytokines together were able to stimulate adhesion of platelets to PMN. Mixed aggregates induced by thrombin were significantly enhanced by the presence of TPO, but not by G-CSF (Figure 4).

3. Discussion

The results of the present study confirm previous findings, showing that TPO by itself was not able to activate platelets, but it increased aggregation and ATP release induced by ADP [6,7]. The role of G-CSF on platelet activation is not yet fully clarified. Shimoda et al. demonstrated that G-CSF did not exert a direct effect on platelets but that it primed platelets to be fully activated by other platelet agonists through a receptor-specific mechanism [13]. On the contrary, Oda et al. [6], using

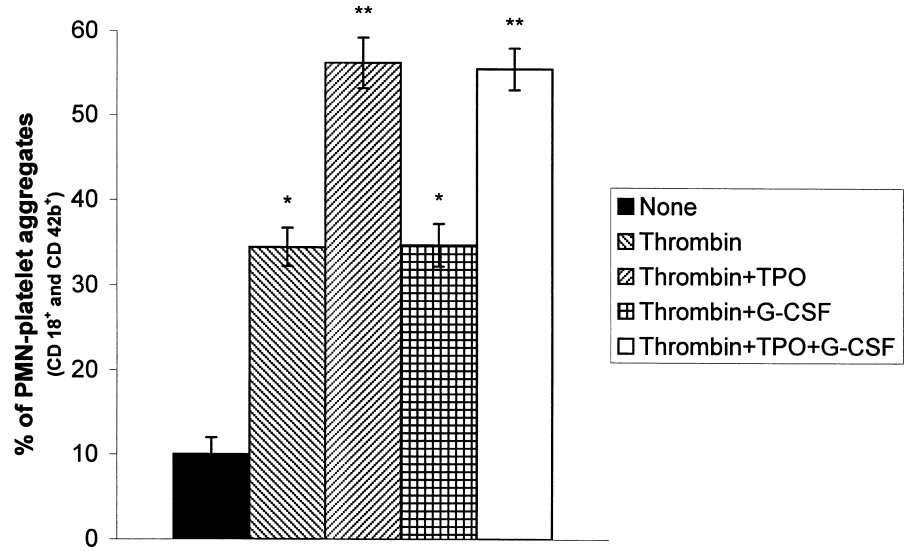

Fig. 4. Effect of TPO and G-CSF on PMN-platelet aggregates. Each value represents the mean±SEM of four separate experiments. *$p<0.05$ when comparing with nonstimulated blood; **$p<0.05$ when comparing with thrombin stimulated whole blood.

physiologic Ca^{2+} concentrations, were not able to confirm this synergistic effect of G-CSF. A moderate direct effect of G-CSF on platelet P-selectin expression was shown only at supraphysiological G-CSF concentrations by Wun et al. [15]. Under our experimental conditions, we were unable to observe a G-CSF effect either on platelet aggregation or on ATP-release, whether it was used alone or in combination with ADP. Moreover, the presence of G-CSF did not modify the TPO-enhanced aggregation and ATP release induced by ADP.

It has been demonstrated that platelet activation by thrombin modulates glycoprotein expression on the platelet membrane: While GPIb is internalized from the membrane surface, P-selectin normally stored in alpha granules is expressed on the platelet surface [16,17]. A previous report demonstrated that TPO and, in a lesser degree, G-CSF, stimulated CD62p expression on normal human platelets [15]. We now further demonstrate that TPO and G-CSF differentially modulated thrombin-induced changes in GPIb and P-selectin expression. While TPO augmented the internalization of GPIb and the externalization of P-selectin induced by thrombin, G-CSF only affect the loss of GPIb. The observation that G-CSF only regulates GPIb expression could be related to the fact that downregulation of GPIb-IX complex and P-selectin expression are uncoupled phenomena. In fact, it was shown that ADP induces a marked loss of GPIb from the platelet membrane, but it is a weak inducer of P-selectin expression [17]. In agreement with Wun et al. [15], we did not find a synergy between TPO and G-CSF in the expression of either GPIb or P-selectin.

The TPO receptor c-Mpl is mainly expressed in platelets, megakaryocytes, $CD34^+$ cells, and endothelial cells [18]. Recently, Brizzi et al. [9] demonstrated that PMN also express c-Mpl and that TPO can regulate PMN activation. These authors have shown that TPO synergized with fMLP in the production of an early oxidative burst, and that TPO directly induced the release and synthesis of IL-8 from PMN. We were not able to observe any effect of TPO in the regulation of PMN surface expression of CD11b whether it was used alone or in combination with fLMP. Furthermore, TPO did not modify the G-CSF-induced increased expression of CD11b, suggesting that TPO might not be involved in the regulation of PMN integrin expression.

It has been suggested that platelet–neutrophil interactions may play a role in several diseases, such as the acute respiratory distress syndrome, cardiopulmonary bypass, and atherosclerosis [19, 20]. Thus platelet–neutrophil adhesion is likely of physiologic importance, not only for the targeting of both cell types to appropriate inflammatory and/or hemostatic sites but also for functional alterations in these cells. We found that TPO increased the formation of mixed aggregates induced by thrombin. Since we also found that TPO synergizes with thrombin in the upregulation of P-selectin, it seems likely that that expression of this adhesive protein might be the mechanism involved in TPO increased formation of mixed aggregates.

In conclusion, these data demonstrate that despite the fact that TPO and G-CSF were able to trigger platelet and PMN activation responses, there was no synergism between both cytokines. It is important to point out that the concentrations of TPO and G-CSF used in this study were in the range of those obtained after the administration of these drugs to patients [21,22]. The results of this in vitro study may not be reflective of the situation in vivo. However, since the combination of both cytokines is likely to be used therapeutically, clinical trials are needed to ascertain the interactions between both of them.

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