Physiologic production of platelets is thought to require at least two humoral growth factors that regulate proliferation and maturation of megakaryocytes. One is a megakaryocyte colony-stimulating factor (meg-CSF), which induces the proliferation and differentiation of megakaryocyte progenitors. The second is thrombopoietin, a novel megakaryocyte growth and development factor, which promotes the maturation of megakaryocytes. It has been suggested that meg-CSF and thrombopoietin regulate megakaryocyte maturation by binding to the c-Mpl, a member of the hematopoietic receptor family encoded by the proto-oncogene c-mpl.

After cloning of human and murine complementary DNA for thrombopoietin, several groups have investigated in vitro and in vivo the biologic properties of the recombinant megakaryocyte growth and development factor (MGDF), a protein related to thrombopoietin. The MGDF has been shown to bind the c-Mpl receptor and to stimulate platelet production when injected in mice and in nonhuman primates. Recently, it has been reported that thrombopoietin receptors were expressed on human platelets. Therefore, in light of the potential clinical use of the MGDF, the question of whether it may affect platelet functions arises.

In the present study we evaluated the effect of MGDF on platelet aggregation in platelet-rich plasma (PRP) and in whole blood. The results obtained indicate that MGDF by itself did not affect platelet aggregation. However, when added before other agonists such as adenosine diphosphates (ADP), epinephrine (EPI), and thrombin (THR), it rendered platelets more sensitive. This "priming" effect of MGDF was dependent on the dose and on the time of platelet preincubation, and it occurred both in PRP and in whole-blood platelet aggregation. MGDF also "primed" the release of adenosine triphosphates and the production of thromboxane B2 by platelets stimulated with ADP, EPI, and THR. When added 15 seconds after the preincubation of platelets with subthreshold concentrations of ADP, EPI, and THR, MGDF exhibited a synergism with these agonists. Moreover, we observed a "priming" effect of MGDF on the phosphorylation of p-42 mitogen-activated protein kinase promoted by ADP, EPI, and THR. These observations suggest that thrombopoietin may play a physiologic role in modulating the response of platelets to several stimuli and thereby their hemostatic potential.

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THROMBOPOIETIN AND PLATELET ACTIVATION

TXB₂ levels were determined by ELISA. Obtained using either methods.

During aggregation was compared with the luminescence induced 6 minutes after addition of aggregating agents. Similar results were obtained using either methods. Moreover, the synergic effect of MGDF on ADP, EPI, and THR was evaluated by using subthreshold concentrations of these aggregating agents, followed 15 seconds later, by the addition of MGDF.

When platelet aggregation in PRP and whole blood were measured repeatedly the variation versus the previous measurement was, respectively, within 5% and 8%.

In selected experiments luciferin-luciferase reagent was added to PRP 60 seconds before the addition of aggregating agents. Luminescence caused by the release of adenosine triphosphates (ATP) during aggregation was compared with the luminescence induced by 2 mmol of ATP standard as described by Feinman et al.

Measurement of thromboxane B₂ (TXB₂) production. The generation of TXB₂ by stimulated platelets in PRP was evaluated as previously described. Briefly, at the indicated time after the addition of agonists the reaction was stopped by addition of 5 μmol/L indomethacin, platelets were centrifuged at 12,000 g for 2 minutes, and plasma was removed and stored at -60°C. After appropriate dilution TXB₂ levels were determined by ELISA.

Western blot analysis and immunoprecipitation studies. Platelets (4 × 10⁹/mL) were incubated at 37°C for 5 minutes with or without ADP (0.5 μmol/L), EPI (2 μmol/L), or THR (0.6 U/mL) in the presence or absence of MGDF (40 ng/mL). At the end of incubation, platelets were extracted with cold DM buffer (50 mmol/L PIPES pH 6.8, 100 mmol/L NaCl, 5 mmol/L MgCl₂, 300 mmol/L sucrose, 5 mmol/L EGTA, 2 mmol/L orthovanadate) containing 1% Triton X-100 (Sigma Chemical Co) and a mixture of proteinase inhibitors (1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 0.15 U/mL aprotinin, 1 μg/mL pepstatin A) for 20 minutes at 4°C, and centrifuged at 15,000 g for 20 minutes. The protein concentration of platelet lysates was determined according to Bradford’s technique and the protein content of the samples was normalized by appropriate dilution with cold DM buffer. The supernatant was preincubated with 0.4 pmol/L ADP, 1.6 pmol/L EPI, or 0.6 pmol/L THR after pretreatment (5 minutes) with various doses of MGDF. The results are expressed as mean ± SE of four individual experiments.
TXB2 production (Fig 4). A "synergic" effect of MGDF on ADP, EPI, and THR was evident when subthreshold concentrations of these agents were added 15 seconds before MGDF (Fig 5). The minimal effective dose of MGDF required for the "synergic" effect was 20 to 40 ng/mL. All these effects of MGDF were abrogated by 5 minutes of boiling. No effects were observed when platelets were incubated with the vehicle alone instead of MGDF.

Effect of MGDF on platelet aggregation in whole blood. MGDF (1 to 80 ng/mL) by itself did not affect platelet aggregation in whole blood but, as for PRP, "primed" the action of ADP and EPI. As shown in Fig 6, the "priming" effect of MGDF on aggregation induced by ADP and EPI was dependent on the dose and on the time of preincubation. The minimal effective dose of MGDF added to blood 5 minutes before stimulation with the agonists was 0.5 ng/mL.

Effect of MGDF on p-42 mitogen-activated protein kinase (MAPK) tyrosine phosphorylation. Platelets contain high levels of tyrosine kinase activity and show many changes in the pattern of protein tyrosine phosphorylation upon agonist-induced activation.26,28 Recently, tyrosine phosphorylation of p42 MAPK isoform of MAPK has been involved in the signal transduction pathway of aggregation induced by THR.29 To evaluate the phosphorylation of MAPK, platelets were stimulated in the lumi-aggregometer with ADP, EPI, or THR in the presence or the absence of MGDF. Platelets recovered from the aggregation assay were lysed and immunoprecipitated with antiphosphotyrosine antibody. The filters were then probed either with a monoclonal or a polyclonal antibody anti-p42 MAPK isoform of MAPK. In parallel experiments platelet lysates were immunoprecipitated with monoclonal antibody anti-p42 MAPK and the filters were primed with the same antibody to detect the band corresponding to the p42 MAPK isoform of MAPK that comigrates with the tyrosine

Fig 2. Effect of time of preincubation with MGDF on the aggregation of platelets induced by ADP in PRP. (A) Platelets preincubated for various times with MGDF (0.25 and 1 ng/mL) or with the vehicle alone (RPM1 containing 0.5% BSA) were stimulated with 0.4 pmol/L ADP and aggregation was recorded. These results are a representative example of four individual experiments. (B) "Priming" index calculated on aggregation of PRP stimulated with 0.8 μmol/L ADP after pretreatment for various period of time with 0.25 (●) or 1 ng/mL (○) MGDF. The results are expressed as mean ± SE of four individual experiments.

Fig 3. Effect of time of preincubation with MGDF on platelet aggregation induced by EPI in PRP. (A) Platelets preincubated for various times with MGDF (0.25 and 1 ng/mL) or with the vehicle alone (RPM1 containing 0.5% BSA) were stimulated with 1 μmol/L EPI and aggregation was recorded. These results are a representative example of four individual experiments. (B) "Priming" index calculated on aggregation of PRP stimulated with 1 μmol/L EPI after pretreatment for various period of time with 0.25 (●) or 1 ng/mL (○) MGDF. The results are expressed as mean ± SE of four individual experiments.
Table 1. Effects of MGDF on the Release of ATP From Platelets Stimulated With ADP, EPI, and THR

<table>
<thead>
<tr>
<th>Pretreatment (5 min)</th>
<th>Stimuli</th>
<th>ATP Release (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>ADP</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Vehicle</td>
<td>EPI</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Vehicle</td>
<td>THR</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>MGDF</td>
<td>ADP</td>
<td>1.98 ± 0.3*</td>
</tr>
<tr>
<td>MGDF</td>
<td>EPI</td>
<td>2.22 ± 0.2*</td>
</tr>
<tr>
<td>MGDF</td>
<td>THR</td>
<td>4.65 ± 0.3*</td>
</tr>
</tbody>
</table>

Platelets preincubated 5 minutes with 40 ng/mL MGDF or with the vehicle alone (RPMI containing 0.5% BSA) were stimulated with 0.4 μmol/L ADP, 1 μmol/L EPI, or 0.2 U/mL THR and the release of ATP was measured in lumi-aggregometer as described in Materials and Methods. These results are a representative example of four individual experiments. Student’s t-test was performed between vehicle alone plus stimulus and MGDF plus stimulus: * P < .05.

phosphorylated band observed after immunoprecipitation with anti-phosphotyrosine antibody. As shown in Fig 7, the level of phosphorylated p42* was low in platelets stimulated with MGDF, ADP, EPI, or THR alone as well as in unstimulated platelets. In contrast, when platelets were costimulated with MGDF and ADP, EPI, or THR a higher level of tyrosine phosphorylated p42* was detected.

DISCUSSION

Thrombopoietin is considered a lineage-dominant cytokine that regulates the proliferation and maturation of committed cells leading to the production of megakaryocytes and platelets. Recently, complementary DNAs for both human and murine thrombopoietin have been cloned and shown to encode a glycoprotein that has selective actions on megakaryocyte proliferation. This glycoprotein has a novel two-domain structure with an amino-terminal domain homologous with erythropoietin and a carboxy-terminal domain rich in serine, threonine, and proline residues and containing seven potential N-linked glycosylation sites. Several lines of evidence strongly suggest that the c-Mpl protein encoded by the c-mlp proto-oncogene acts as a receptor for thrombopoietin. The c-Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to mature platelets. In c-mpl−deficient mice a decreased number of platelets but not of other hematopoietic cell types was observed.

The impressive feature of MGDF, the recombinant thrombopoietin, is its ability to increase the number of platelets so that it may find several potential clinical applications in thrombocytopenic states.

We report herein that MGDF was able to affect the function of mature platelets. We observed that MGDF by itself did not induce platelet aggregation. However, when added before other agonists such as ADP, EPI, and THR, it rendered platelets more sensitive. This “priming” effect of MGDF was dose dependent and it occurred not only in PRP but also in whole-blood platelet aggregation, which better reflects normal physiology. The minimal effective dose was similar to that reported for proliferation of c-mlp−expressing, responsive cell lines. A “synergetic” effect of MGDF on ADP, EPI, and THR was also evident when subthreshold concentrations of these agents were added 15 seconds before MGDF. However, the minimal effective dose of MGDF required for the “synergetic” effect was 80 to 160 times higher then the minimal “priming” dose. Similar effects on platelets were previously observed with human stem cell factor which is a hematopoietic growth factor produced by mesenchymal cells acting on c-Kit receptor.

The concept that proliferative cytokines may affect the function of the mature end cells has been demonstrated by studies on the role of granulocyte-macrophage colony-stimulating factor and of granulocyte colony-stimulating factor in “priming” the leukocyte response to activating agents. The results of present study suggest a modulatory role of thrombopoietin on the function of circulating platelets. This may imply that production of thrombopoietin could be stimulated not only by the reduction of circulating platelets but also by platelet dysfunction.

Recently, it has been suggested that thrombopoietin is released in response to changes in the platelet mass and to poorly functioning platelets. In these conditions the humoral factor stimulates megakaryocytes to increase platelet production.
In our study the priming effect of MGDF was dependent on the time of platelet preincubation as it was already detectable after 30 seconds, reached its maximal after 5 to 30 minutes, and lasted up to 3 hours. This may have clinical implication as treatment with MGDF will involve exposure to this hormone for prolonged period of time. It remains to be determined whether such an increase in platelet responsiveness may be beneficial or detrimental in various clinical thrombocytopenic states.

The intracellular events that occur after thrombopoietin binding to its receptor are poorly understood. Tyrosine phosphorylation of specific intracellular proteins are essential for the action of many hematopoietic growth factors that are known to share common signal transduction cascade. Recent studies have shown an increase in tyrosine kinase activity on THR-, ADP-, tromboxane A2- or collagen-induced platelet activation with phosphorylation of several members of Src and Syk families and of MAPK. It has been recently shown that platelets express two forms of MAPK, p42 MAPK and p44 MAPK. During THR-induced platelet activation, p42 MAPK but not p44 MAPK becomes phosphorylated on serine, threonine, and tyrosine, and functionally activated.

In the present study we have evaluated tyrosine phosphorylation of p42 MAPK as a marker of increased tyrosine kinase activity. The results obtained indicate that MGDF enhanced phosphorylation of p42 MAPK promoted by ADP, EPI, and THR.

It has been suggested that in platelet p42 MAPK could modulate cytoskeletal protein functions in secretion and adhesion. Another potential substrate for p42 MAPK in platelets is cytosolic phospholipase A2. It has been shown that MAPK belongs to the platelet signal transduction pathway involved in the activation of phospholipase A2 and in the mobilization of arachidonic acid. Miyakawa et al reported that MGDF induces rapid protein tyrosine phosphorylation of Janus kinase 2 and Shc in human blood platelets. Our results indicating that MGDF ‘‘primes’’ the phosphorylation of p42 MAPK further support the contention of Miyakawa et al that the ligand binding to c-mpl activates signal transduction in human platelets. It is possible that Jak2, Shc, and p42 MAPK may be involved in the increase in platelet sensitivity to physiologic agonists induced by MGDF.

In conclusion, these results suggest that thrombopoietin has pleiotropic effects, because, besides acting as an hematopoietic growth factor, it potentiates platelet activation. There-
fore, thrombopoietin may play a physiologic role in modulating the response of platelets to several stimuli and thereby their hemostatic potential.

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Effects of recombinant human megakaryocyte growth and development factor on platelet activation

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