Recombinant thrombopoietin has been reported to stimulate megakaryocytopenesis and thrombopoiesis and it may be quite useful to treat patients with low platelet counts after chemotherapy. As little is known regarding the possible activation of platelets by thrombopoietin, we examined the effects of thrombopoietin on platelet aggregation induced by shear stress and various agonists in native platelets. Using hirudin as an anticoagulant, thrombopoietin (1 to 100 ng/mL) enhanced platelet aggregation induced by 2 μmol/L adenosine-diphosphate (ADP) in a dose dependent fashion. The enhancement was not affected by treatment of platelets with 1 μmol/L aspirin plus SQ-29548 (a thromboxane antagonist, 1 μmol/L) but was inhibited by a soluble form of the thrombopoietin receptor, suggesting that the enhancement was mediated by the specific receptors and does not require thromboxane production. Epinephrine (1 μmol/L), which does not induce platelet aggregation in hirudin platelet rich plasma (PRP), did so in the presence of thrombopoietin (10 ng/mL). Thrombopoietin (10 ng/mL) also enhanced or primed platelet aggregation induced by collagen (0.5 μg/mL), thrombin, serotonin, and vasopressin. Thrombopoietin does not induce any rise in cytosolic ionized calcium concentration nor activation of protein kinase C, as estimated by phosphorylation of prekstin, indicating that the priming effects of thrombopoietin does not require those processes. The ADP- or thrombin-induced rise in cytosolic ionized calcium concentration was not enhanced by thrombopoietin (100 ng/mL). Further, shear (ca. 90 dyn/cm²)-induced platelet aggregation was also potentiated by thrombopoietin. The priming effect on epinephrine-induced platelet aggregation in hirudin PRP was unique to thrombopoietin, with no effects seen using interleukin-6 (IL-6), IL-11, IL-3, erythropoietin, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, or c-kit ligand. These data indicate that monitoring of platelet functions may be necessary in the clinical trials of thrombopoietin.

**MATERIALS AND METHODS**

**Materials.** Aspirin, Arg-Gly-Asp-Ser (RGDS) peptide, bovine serum albumin (BSA), PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone), adenosine diphosphate (ADP), trisodium citrate, vasopressin, serotonin, and epinephrine were from Sigma (St Louis, MO). Recombinant hirudin was kindly provided by Japan Energy Co (Tokyo, Japan). Horse tendon collagen was from Hormon Chemie (Munich, Germany). SQ-29548 was from Cayman Chemical (Ann Arbor, MI). Heparin was from Kodama Co (Tokyo, Japan). Soluble murine c-Mpl was a gift from Amgen Inc (Thousand Oaks, CA). Interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF), erythropoietin, and c-kit ligand were provided from Kirin Brewery Co, Ltd (Tokyo, Japan). IL-11 was a gift from Sankyo Co, Ltd (Tokyo, Japan). Purified human thrombin was generously provided by Green Cross Co (Osaka, Japan). All cytokines and growth factors were highly purified and checked carefully for contaminants such as endotoxin by suppliers. An anti-glycoprotein Ib monoclonal antibody (GUR 83-35), which inhibits SIPA and ristocetin-induced platelet aggregation, was raised in our laboratory. 29-31 Fluo-3 acetoxymethylester (AM) was from Molecular Probes (Eugene, OR). Human recombinant thrombopoietin was expressed in Chinese hamster ovary cells as described previously.

**Sample collection.** Blood was drawn from healthy volunteers (age 23 to 43 years) who abstained from drugs known to affect platelet functions for 2 weeks before the studies. Blood was mixed with hirudin (final concentration, 100 unit/mL), heparin (final concentration, 20 unit/mL), or one tenth volume of 3.8% trisodium citrate. PRP was obtained by centrifugation at 150 x g for 15 minutes at room temperature. Platelets were adjusted to 3 x 10^5 to 5 x 10^5/L using autologous platelet poor plasma (PPP) prepared by centrifugation of blood at 3,000g for 20 minutes.

**Measurement of shear-induced platelet aggregation (SIPA).** The measurement of SIPA with a modified cone-plate viscometer was performed as previously described. Briefly, 400 μL of platelet rich plasma (PRP) was applied to the surface of a polymethylmethacrylate plate and exposed to shear stress at room temperature for 6 minutes. The rotation rate of the cone was 10 rpm for the first 15
seconds and was increased to 900 rpm within 1 second. Helium-neon laser light at 633 nm was passed through the streaming samples, and the transmitted light was recorded continuously. The percent aggregation was calculated according to the Lambert-Beer equation. To estimate shear stress, we measured the viscosity of plasma from healthy volunteers as described.16 The viscosity of citrated PRP was 1.72 ± 0.055 cp (mean ± SD, n = 10) at 22°C. Hirudin or heparin PRP also yielded similar values.16 Thus, at 900 rpm, the shear stress was approximately 90 dyn/cm² irrespective of the anticoagulant used.

Measurement of platelet aggregation. The measurement of platelet aggregation was performed with an aggregometer (Hema Tracer TM Model 601; Niko Bio Science, Tokyo, Japan) with continuous stirring (1,000 rpm) as described.17

Measurement of [Ca²⁺]i by flow cytometry. [Ca²⁺]i was measured by flow cytometry as described with following modifications.18,19 Hirudin PRP was incubated with fluo-3 AM (2 µmol/L) for 30 minutes at 37°C. Platelets were diluted 1:500 with the modified Heps-Tyrode buffer containing 1 mmol/L CaCl₂ at 37°C. Measurement of fluo-3 fluorescence was performed on an EPICS XL (Hialeah, FL). An argon laser beam was tuned to 488 nm/L to excite fluo-3 and its fluorescence emission was detected at 530 ± 20 nm/L. Platelets were identified by the typical light scattering profile.18,19 Agonists were added to the sample chamber, during a short interruption in flow, resulting in an approximately 10-second delay before collection of data after stimulation. Approximately 500 cells were examined per second.

RESULTS

The effects of thrombopoietin on ADP-induced platelet aggregation were examined in hirudin PRP. In most experiments, we employed hirudin PRP, because the concentrations of divalent cations remain physiological, unlike citrated PRP, and divalent cations are known to be critical for the regulation of integrins, including αIIbβ3.20 Thrombopoietin (1 to 100 ng/mL) alone did not induce platelet aggregation or shape change (Figs 1 and 3). However, it enhanced ADP (2 µmol/L)-induced platelet aggregation in a dose dependent fashion (Fig 1A). The enhancement of ADP (2 µmol/L)-induced platelet aggregation by thrombopoietin (10 ng/mL) was antagonized by a soluble form of the thrombopoietin receptor (c-mpl, 40 µg/mL) (Fig 1B), suggesting that the effect of thrombopoietin is through a specific interaction with its receptor. We next examined whether the enhancement of aggregation was dependent on the secondary production of thromboxane. When PRP was treated with aspirin (1 mmol/L) for 30 minutes and SQ-29548 (1 µmol/L) for 5 minutes, ADP (2 µmol/L)-induced platelet aggregation was still enhanced by thrombopoietin (10 ng/mL) (compare Fig 1A and Fig 1C). Thus, the effects of thrombopoietin were evident even in experimental conditions where the effects of thromboxane were eliminated.

We also examined the effects of thrombopoietin on platelet aggregation induced by agonists other than ADP. Epinephrine did not induce platelet aggregation in hirudin PRP, regardless of the presence of aspirin plus SQ-29548 (1 µmol/L), consistent with a previous report (Fig 2A).21 However, epinephrine induced platelet aggregation in the presence of thrombopoietin (10 ng/mL) (Fig 2A). Aspirin plus SQ-29548 (1 µmol/L) inhibited only the secondary phase of epinephrine-induced platelet aggregation. Thrombopoietin (10 ng/mL) also primed platelet aggregation induced by collagen (0.5 µg/mL) in hirudin PRP (Fig 2B). Collagen (0.5 µg/mL) induced only “shape change” (the decrease in the light transmission) in the presence of aspirin and SQ-29548 (Fig 2B, the lowest line), consistent with previous reports that, at low concentrations, collagen did not induce platelet aggregation in the presence of aspirin.2 However, in the presence of thrombopoietin (10 ng/mL), aggregation was induced by collagen (Fig 2B). In the absence of aspirin and SQ-29548 (Fig 2B, the upper two lines), thrombopoietin facilitated aggregation induced by collagen (compare the upper two lines). Similar enhancement was observed when serotonin or vasopressin was employed or when other anticoagulants such as heparin or P-PACK were used (data not shown). Thrombin (0.25 U/mL)-induced platelet aggregation was also enhanced by thrombopoietin (10 ng/mL) in citrated PRP (Fig 2C).

There have been reports that IL-6,24 c-kit ligand,25 or G-CSF26 enhanced platelet aggregation in citrated PRP. To our knowledge, the effects of these reagents on platelet aggregation have not been tested in the presence of physiological concentrations of divalent cations. Figure 3 shows that only thrombopoietin, but not IL-6, c-kit ligand, G-CSF, IL-11, or granulocyte macrophage-colony stimulating factor (GM-CSF) primed epinephrine-induced platelet aggregation in hirudin-PRP. IL-3 or erythropoietin also did not enhance platelet aggregation (data not shown).

There is increasing evidence suggesting that shear stress may be an important stimulus to platelet aggregation in partially occluded arteries or arterioles.10-12 Accordingly, we examined the effects of thrombopoietin on SIPA in a well characterized cone-plate aggregometer.13,14 When platelets in hirudin PRP were exposed to shear stress (at 900 rpm, ca 90 dyn/cm²), platelets started to aggregate slowly (Fig 4A). The rate and the extent of aggregation varied among different donors with a mean maximal extent of aggregation of 41.5 ± 7.3% (mean ± standard deviation [SD], N = 5 [Fig 3A]). Thrombopoietin increased the initial velocity as well as the maximum extent of SIPA in a dose dependent fashion (Fig 4A). The enhancement of SIPA by thrombopoietin (10 ng/mL) was abolished by soluble c-mpl (20 µg/mL) (Fig 4B). The SIPA in the presence of thrombopoietin was strongly inhibited by RGDS peptide (200 µg/mL) or GUR83-35 (an antiligicoprotein Iβ antibody, 20 µg/mL) (data not shown), suggesting that, under these conditions, the SIPA was mediated by binding of von Willebrand factor to αIIbβ3 and GP Ib/IX as previously described.11,12,14 When citrate was used as an anticoagulant instead of hirudin, the enhancing effects of thrombopoietin on SIPA were also noted (data not shown), suggesting that the effects of thrombopoietin on SIPA required neither physiological concentrations of extracellular calcium nor hirudin.

Agnostin-induced platelet aggregation is known to be mediated by activation of αIIIbβ3.21,22 Although the exact mechanism of activation is unclear, it has been accepted that calcium elevation and/or activation of protein kinase C may underlie the activation.5,21,22 Accordingly, we examined whether thrombopoietin elevates cytosolic ionized calcium concentration ([Ca²⁺]i). Because platelet responses to certain agonists like ADP is known to be affected during preparation of washed platelets,18,19 we performed flow cytometric measurement of [Ca²⁺]i with fluo-3, employing diluted PRP.18,19
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Thrombopoietin (100 ng/mL) did not significantly modify ADP- or thrombin-induced elevation of [Ca²⁺]ᵢ (Fig 5). Similar data were obtained in experiments employing washed platelets loaded with fura-2 (data not shown). Thrombopoietin also failed to induce phosphorylation of pleckstrin (a major substrate for protein kinase C) and myosin light chain in ³²Pi-loaded platelets (data not shown).

DISCUSSION

Our data shows that thrombopoietin enhances or primes platelet aggregation induced by various agonists and shear stress (Figs 1 through 4) even under conditions, where the extracellular calcium concentration is in the physiological range. The effects of thrombopoietin on agonist-induced platelet aggregation were not merely enhancing. Thus, epinephrine (1 μmol/L) or collagen (0.5 μg/mL) in the presence of aspirin and SQ-29548 did not induce platelet aggregation (Fig 2). However, in the presence of thrombopoietin (10 ng/mL), these agonists were capable of inducing platelet aggregation. Further, the augmentation and/or priming of platelet aggregation by thrombopoietin was only partially inhibited by aspirin plus SQ-29548 (1 μmol/L) in hirudin PRP (data not shown). When citrated PRP was used instead of hirudin, a similar enhancement of ADP-induced platelet aggregation was observed (Fig 2). However, this effect was almost completely abolished by SQ-29548 (data not shown), consistent with the report of Hammond et al. The reason for the difference in the results obtained in these anticoagulants is...
unknown. However, data similar to that obtained using hirudin was obtained with P-PACK or heparin PRP. We believe that the data obtained using hirudin PRP is more physiological as citrated PRP is known to exhibit aberrant behaviors in response to agonists. Specifically, ADP-induced secondary aggregation is known to occur only in citrated PRP or in a buffer with low calcium ion concentrations. Thus, the difference in the anticoagulants used could be responsible for our observation that enhancement of ADP-induced aggregation by thrombopoietin was abolished by SQ-29548 in citrated PRP but not hirudin PRP. Irrespective of the reason for the difference, it is clear from our studies that thrombopoietin enhanced agonist-induced platelet aggregation in the presence of physiological calcium ion concentrations in native plasma.

We also found that SIPA was enhanced by pretreatment of platelets with thrombopoietin (Fig 4). SIPA has previously been shown not to be affected by inhibition of thromboxane
production. These data further suggest that some of the effects of thrombopoietin on platelet aggregation are independent of thromboxane production.

Thus, thrombopoietin enhanced platelet aggregation induced by various stimuli. Moreover, the enhancement was at least partially independent of the effects of secondary effects of thromboxane. Because agonists (like ADP, or epinephrine)-induced thromboxane production is significantly dependent on aggregation, it is possible that thrombopoietin may initially augment the primary phase of platelet aggregation, which is required for production of thromboxane.

In hirudin PRP, the priming effect on epinephrine-induced platelet aggregation was a unique effect of thrombopoietin and it is not affected by IL-6, c-kit ligand, G-CSF, IL-11, GM-CSF, IL-3, or erythropoietin (Fig 3). Although the addition of epinephrine after thrombopoietin induced aggregation within 15 seconds (Fig 3A), in separate experiments, when the order of the agonists was reversed, there was a significant lag time of aggregation (approximately 30 to 45 seconds). The lag time probably reflects the time required for thrombopoietin binding to its receptor and subsequent signaling events within platelets.

The molecular mechanism of the priming of platelet aggregation by thrombopoietin is unknown. As an increase in fibrinogen binding to platelets is essential for platelet aggregation and is known to be mediated by activation of αIIbβ3 and calcium and/or activation of protein kinase C, we evaluated whether thrombopoietin induces elevation of $[Ca^{2+}]_i$ or phosphorylation of pleckstrin (a major endogenous substrate for protein kinase C in platelets). Unlike agonists such as thrombin or ADP, thrombopoietin does not induce elevation of $[Ca^{2+}]_i$ or phosphorylation of pleckstrin (Fig 5 and data not shown). Further, thrombopoietin (100 ng/mL) did not significantly enhance $[Ca^{2+}]_i$ induced by ADP or thrombin (Fig 5). On the other hand, we have found that thrombopoietin is capable of inducing tyrosine

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**Fig 3.** Thrombopoietin (10 ng/mL) but not IL-6 (100 ng/mL), c-kit ligand (100 ng/mL), G-CSF (100 ng/mL), IL-11 (100 ng/mL), or GM-CSF (100 ng/mL) primed epinephrine-induced platelet activation (hirudin-PRP). Each reagent was diluted in PPP from a stock solution and added at the times indicated by the arrow (*). After 5 minutes, epinephrine (1 μmol/L) was added and aggregation was measured as described in Fig 1A. A representative tracing of five or more experiments is shown.

**Fig 4.** (A) Effects of thrombopoietin on SIPA in hirudin PRP. Hirudin PRP (400 μL) was incubated with various concentrations of thrombopoietin (0.1 to 100 ng/mL) or with autologous PPP (= vehicle) for 10 minutes and were exposed to shear stress (900 rpm, ca 90 dyn/cm²) at 22°C, after recording the base line for 15 seconds. SIPA was measured with a modified viscometer and % aggregation was calculated as described previously. A representative tracing of five or more experiments is shown. (B) Soluble murine c-mpl (20 μg/mL) reversed the enhancement of SIPA by thrombopoietin (10 ng/mL). Hirudin PRP was incubated for 5 min with c-mpl or vehicle. Thrombopoietin (10 ng/mL) or autologous PPP (= vehicle 4 μL) was added to the PRP, and after 10 minutes, SIPA was measured at 900 rpm as in (A).
phosphorylation of Jak2, Tyk2, Shc, Stat3, and Stat5 in platelets.\textsuperscript{8,25} Protein tyrosine phosphorylation was observed at concentrations of thrombopoietin of 1 ng/mL and reached a maximum at 10 ng/mL.\textsuperscript{9} Further studies are necessary to determine whether tyrosine phosphorylation of proteins such as Jak2 is related to the priming effects on aggregation, possibly through modulation of activation of $a_{IIb/3}$.

The results of this in vitro study may not be reflective of the situation in vivo; however, a recent report showed that serum levels of thrombopoietin may reach 10 ng/mL in thrombocytopenic patients.\textsuperscript{29} To induce enhancement of platelet production in such patients, it is reasonable to assume that the therapeutic concentration of thrombopoietin must be similar to or exceed such a value. Thus, the concentration of thrombopoietin used in our experiments can be achieved in pathophysiological situations. If thrombopoietin primes platelets for aggregation in response to physiological platelet agonists in vivo, as suggested by our study, the results could be potentially detrimental. Our study indicates that platelet functions should be closely monitored in early clinical trials of thrombopoietin.

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Fig 5. Thrombopoietin (100 ng/mL) does not modify elevation of [Ca$^{2+}$]i induced by ADP or thrombin. Thrombopoietin was added to diluted PRP loaded with fluo-3, and fluorescence of fluo-3 as a measure of [Ca$^{2+}$]i was recorded. One minute after the beginning of the recording, ADP (0.1 or 0.3 $\mu$mol/L, [A]) or thrombin (0.01 or 0.03 U/mL, [B]) was added. Mean fluorescence of individual platelets is shown. Ordinate shows the intensity of mean fluo-3 fluorescence (arbitrary unit). Abscissa represents time. Representative of three separate experiments.


Thrombopoietin primes human platelet aggregation induced by shear stress and by multiple agonists

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