Transforming Growth Factor β Inhibits Megakaryocyte Growth and Endomitosis

By David J. Kuter, Dianne M. Gminski, and Robert D. Rosenberg

Using a rat bone marrow culture system, the effect of transforming growth factor β1 (TGFβ1) on megakaryocyte growth and endoreduplication has been studied. Purified human platelet TGFβ1 inhibited the number of megakaryocytes that appeared in culture at a half-maximal concentration of 0.66 ± 0.21 ng/mL and inhibited megakaryocyte endoreduplication at a half-maximal concentration of 0.14 ± 0.08 ng/mL. Under identical conditions, growth of erythroid precursors was half-maximally inhibited at a concentration of 0.125 ng/mL while myeloid growth was not inhibited at concentrations of TGFβ1 up to 25 ng/mL. These profound inhibitory effects on megakaryocyte growth and endomitosis suggested that TGFβ might play a role in megakaryocytopenia. Therefore, we explored the effect of TGFβ in three different experimental situations by using a neutralizing antibody to TGFβ: (1) Serum but not plasma was found to inhibit the number and ploidy of megakaryocytes that grew in vitro. This inhibitory activity was completely neutralized by antibody to TGFβ or on treatment with dithiothreitol. (2) Plasma from thrombocytotic rats was observed to decrease megakaryocyte ploidy on culture but this effect was not prevented by the addition of antibody to TGFβ. (3) Plasma from thrombocytopenic but not normal rats increased megakaryocyte ploidy on culture. Addition of antibody to TGFβ did not alter these results. Therefore, TGFβ is a potent inhibitor of the number and ploidy of megakaryocytes and accounts for all the inhibition seen when megakaryocytes are cultured in serum. However, the differences in effect on megakaryocyte growth that we observe between normal, thrombocytopenic, and thrombocytotic plasmas are not due to variations in the amount of TGFβ. Furthermore, our results show that release of TGFβ from megakaryocytes during culture does not act as an autocrine regulator of megakaryocyte ploidy in vitro.

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THE MEGAKARYOCYTE is the precursor cell of the circulating platelet and is unique among bone marrow cells in possessing a polyploid nucleus. In response to an increased demand for platelets, the mean ploidy of megakaryocytes first increases3-5 and is then followed by a rise in the number of megakaryocytes.6 In contrast, the number of megakaryocytes and their mean ploidy decrease with a decreasing demand for platelets. The bone marrow megakaryocyte and the circulating platelet mass are linked by a feedback loop that is mediated by humoral mediator(s) whose activity can be measured by the extent of megakaryocyte polyploidization.7

The humoral mediator for platelet production has been assumed to be a stimulator of megakaryocyte growth.8 Such substances have been identified in human embryonic kidney conditioned medium7,11 as well as in thrombocytopenic plasma.8-15 While adequate amounts of none of these substances have been purified to allow further characterization, several well-defined growth factors have recently been shown to stimulate megakaryocyte growth. Recombinant human erythropoietin increases the number, size, and ploidy of megakaryocytes16-18 in vitro and pharmacologic doses in vivo may elevate the platelet count.19,20 Interleukin-6 (IL-6) in vitro increases megakaryocyte size21,22 and ploidy,22 and in the presence of IL-1α or IL-3 potentiates the number of CFU-Mk.23,24 In vivo, pharmacologic doses of IL-6 may increase the platelet count and megakaryocyte size.25,26

While most research has been directed toward finding substances that stimulate megakaryocyte growth and platelet production, far less attention has been paid to molecules that inhibit the growth of megakaryocytes. Indeed, the enhanced megakaryocyte growth seen with some of the partially purified substances noted previously may simply reflect the absence of inhibitory substances rather than the presence of stimulatory molecules. Several well-defined molecules have been shown to inhibit megakaryocyte growth. Platelet factor-4 (PF-4) and synthetic peptides of PF-426 as well as transforming growth factor β1 (TGFβ1)27-31 have been found to inhibit the growth of CFU-Mk. Recombinant α-interferon inhibits megakaryocyte growth in vitro32,33 and reduces platelet counts in patients with essential thrombocytoysis.34

We have recently devised18 a rat bone marrow culture system in which megakaryocyte endomitosis may be studied by using flow cytometry. We have reported that plasma from thrombocytopenic rats increased the ploidy of megakaryocytes that grew while plasma from thrombocytotic rats decreased the ploidy of megakaryocytes.5 During the course of these experiments we found that serum and platelet lysates but not plasma were potent inhibitors of the number and ploidy of the megakaryocytes which grew.18 Because TGFβ1 is present in large amounts in platelets,36,37 we sought to determine whether TGFβ1 might account for the differences between serum and plasma or between the plasmas obtained from normal, thrombocytotic, or thrombocytopenic rats.

MATERIALS AND METHODS

Reagents and animals. Recombinant human erythropoietin was purchased from Amgen (Thousand Oaks, CA). Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was from Genzyme (Boston, MA). Human platelet TGFβ1 and neutralizing rabbit antibodies to porcine TGFβ (lots J919 and J940), human platelet-derived growth factor (PDGF), and bovine

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acidic fibroblast growth factor (FGF) were obtained from R & D Systems (Minneapolis, MN). We have previously demonstrated that the antibody to porcine TGFβ also neutralizes TGFβ1 purified from rat platelets. Murine epidermal growth factor (EGF, citrated WB) were twice centrifuged at 3,000g for 15 minutes at 4°C. Rats were bled by cardiac puncture into citrate (final concentration, 0.38%). The citrated whole blood (WB) was centrifuged at 700g for 8 minutes at 4°C to prepare platelet-rich plasma (PRP). PRP or PPP-derived serum (PPPDS) were obtained from WB, PRP, and PPP, respectively, by recalcification in glass tubes and incubation at 37°C for 2 hours. Clots were removed and the specimens heated at 56°C for 1/2 hour. All samples were stored at −80°C and retained full growth-promoting or growth-inhibiting activity for over 2 years. Unless otherwise specified in the text, the terms “serum” and “plasma” refer to PRP-derived serum (PRPDS) and PPP-derived serum (PPPDS), respectively. Each experiment performed with PRPDS has also been done with S and identical results obtained.

Production of thrombocytopenic and thrombocytotic rats. As previously described, ether-anesthetized rats (250 to 300 g) were rendered acutely thrombocytopenic by injection of antiplatelet serum (APS). The amount of APS injected was titrated to produce a platelet count greater than 300% of normal that persisted for 24 hours, at which time the animals were bled by cardiac puncture. For platelet counting, blood samples were exposed to 0.066 M DTT for 1 hour at 18°C and subsequently dialyzed (3,000 molecular weight cutoff membranes) for 36 hours with three changes of a 500-fold excess of Hanks’ Balanced Salt Solution (without calcium or magnesium). Control rat plasma and serum were prepared by the Percoll density-gradient centrifugation method (Plasma Megakaryocyte Depletion Kit, Flow Cytometry, Inc., North Billerica, MA). Platelets were counted (two chambers per sample) by hemacytometer using a phase contrast microscope and the increase or decrease in count was expressed as a percentage of the normal, pretreatment count for each animal. Rats used in these experiments had a normal platelet count of 1.5 ± 0.16 × 10^11/L (average of 89 rats).

Bone marrow culture. Megakaryocyte-depleted bone marrow was prepared by the Percoll density-gradient centrifugation and filtration method. The megakaryocyte-depleted bone marrow cells were resuspended to a density of 7 × 10^8/mL (containing no more than 100 identifiable megakaryocytes/mL) in 3 mL of Iscove’s Modified Dulbecco’s Medium (GIBCO, Grand Island, NY) containing penicillin (200 U/mL), streptomycin (200 μg/mL), additional glutamine (0.592 mg/mL), and (unless otherwise indicated) 15% (vol/vol) rat PPPDS. Cultures were routinely grown for 3 days at 37°C in a 5% CO2 incubator. Subsequently, cells were harvested and stained for flow cytometry with APS and propidium iodide as described previously.

To stimulate megakaryocyte endomitosis, cultures were sometimes supplemented with GM-CSF and/or erythropoietin. Addition of GM-CSF (20 U/mL) and erythropoietin (2 U/mL) produced maximal megakaryocyte endomitosis as well as maximal myeloid and erythroid growth and are referred to as “GF-stimulated” cultures. Addition of erythropoietin alone (“epo-supplemented” cultures) at 0.33 U/mL resulted in half-maximal stimulation of megakaryocyte endomitosis.

The hemoglobin content of bone marrow cultures was determined using the 3,3’,5’,5’-tetramethylbenzidine method (Plasma Hemoglobin Assay [Sigma Chemical Co, St Louis, MO]).

Each experiment was performed from a single bone marrow preparation and allowed us to assay up to 20 individual specimens under identical culture conditions. Between identical plasma specimens assayed in the same preparation, there is routinely a coefficient of variation (CV) of less than 1%. Between identical plasma specimens assayed in different bone marrow preparations there is routinely a CV of 5% to 8%. (These differences reflect variations in the duration of culture and in the number of megakaryocyte precursors in the marrow [see reference 18].)

Antibody to TGFβ. Each lot of antibody to TGFβ was titrated against purified TGFβ1 to determine the optimal neutralizing concentration. Twenty to 160 μL of antibody was added to 0.45-mL aliquots of rat PPPDS containing 4 ng of TGFβ1, incubated at 18°C for 1 hour and then added to GF-stimulated cultures. The extent of neutralization of the TGFβ1-mediated megakaryocyte inhibition was determined.

To determine whether rat serum or plasma samples contained TGFβ, 0.45-mL aliquots were incubated at 18°C for 1 hour with antibody to TGFβ. The amount of antibody added was twice that calculated to be required if all the activity of the sample was due to inhibition by TGFβ. Specimens were then added to cultures with or without erythropoietin. Similarly, thrombocytopenic and thrombocytotic rat plasmas were incubated with antibody to TGFβ and added to cultures without erythropoietin.

Treatment of sera with dithiothreitol (DTT). Rat serum was exposed to 0.066 mol/L DTT for 1 hour at 18°C and subsequently dialyzed (3,000 molecular weight cutoff membranes) for 36 hours with three changes of a 500-fold excess of Hanks’ Balanced Salt Solution (without calcium or magnesium). Control rat plasma and serum were prepared in parallel experiments but without DTT exposure.

Flow cytometry. Flow cytometry was performed as previously described on a machine designed by H. Shapiro and built by Y.G. Caine. A coefficient of variation of the 2N peak was maintained at 2.2% to 3.0% by careful alignment of the optical system. Cells were routinely run at 800 to 1,200 cells/s and 1,000 megakaryocytes were analyzed.

Data analysis. Data from the flow cytometer were stored and analyzed on an Atari 130 XE computer (Sunnyvale, CA). Boundaries between each ploidy class were assigned from the DNA histogram and the number of megakaryocytes in each ploidy group was counted. Initially, a limitation in the computer program permitted counting of only 8N and higher megakaryocytes. During the course of these experiments, the program was altered to permit enumeration of the number of 4N megakaryocytes as well. The number of megakaryocytes in each ploidy class was then expressed as a percentage of the total number of megakaryocytes ≥4N or ≥8N. The geometric mean ploidy for each distribution 4N to 32N (Mean Ploidy4N) or 8N to 32N (Mean Ploidy8N) was determined as described by Arriaga et al.

Except for ploidy distributions, statistical analysis of differences was performed with Student’s t-test. Differences between ploidy distributions were tested for using the Mann-Whitney U test as previously described.

RESULTS

Analysis of megakaryocyte growth in liquid bone marrow cultures. After growth in liquid bone marrow culture, the total number of cells, the total number of megakaryocytes, and the ploidy distribution of the megakaryocytes were quantitated by flow cytometry (Table 1). Myeloid precursor cells were the primary cell type in these cultures and were
Megakaryocyte growth was quantitated by flow cytometry as the total number of cells. As indicated in Table 1, this number rose on addition of GM-CSF to the cultures. Megakaryocytes comprised the minority cell population in the culture but were precisely quantitated. After flow cytometry, the total number of megakaryocytes per culture and the ploidy distribution showed an 8N class containing 51.44% of the megakaryocytes. Addition of TGFβ1 resulted in a statistically significant (P < .001) decrease in the number of megakaryocytes to 63% of the plasma control and a small but statistically significant (P < .05) shift in the ploidy distribution toward lower ploidy classes. There was no effect on the total cell number. To assess further this suggested inhibitory effect of TGFβ1, cultures were stimulated with growth factors. The addition of GM-CSF to the cultures increased the total cell number, whereas the addition of erythropoietin produced a large shift in the ploidy distribution toward higher ploidy classes (Table 1).

When TGFβ1 was added to these GF-stimulated cultures there was no change in the total number of cells but the number of megakaryocytes decreased (Fig 1A) with a half-maximal inhibitory concentration of 0.66 ± 0.19 ng/mL (average of six experiments) and a maximal decrease of 15% (vol/vol) plasma and, where indicated, erythropoietin (2 U/mL), GM-CSF (20 U/mL), and/or TGFβ1 (5 ng/mL). Data are shown ± SD. n = The number of independent specimens analyzed for each condition in this experiment. The entire experiment has been performed over 10 times with the same results.

Statistical significance: *P < .01 compared with plasma alone; †P < .05 compared with plasma alone; ‡P < .01 compared with plasma alone; §P < .02 compared with plasma + EPO/GM; ||P < .05 compared with plasma + EPO/GM.

<p>| Table 1. Effect of Erythropoietin, GM-CSF, and TGFβ1 on the Number and Ploidy of Megakaryocytes In Vitro |
|------------------------------------------------------|-------------------|-----------------|-------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Total No. of Cells (× 10⁶)</th>
<th>Total No. of Megakaryocytes</th>
<th>% of Plasma Alone</th>
<th>8N (%)</th>
<th>16N (%)</th>
<th>32N (%)</th>
<th>Mean Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma alone (n = 3)</td>
<td>4.59 ± 0.48</td>
<td>8,826 ± 475</td>
<td>100 ± 5</td>
<td>51.44 ± 2.57</td>
<td>41.06 ± 3.62</td>
<td>7.50 ± 1.19</td>
<td>11.80 ± 1.34</td>
</tr>
<tr>
<td>Plasma + TGFβ1 (n = 3)</td>
<td>4.71 ± 0.48</td>
<td>5,530 ± 111</td>
<td>63 ± 1*</td>
<td>55.06 ± 2.22†</td>
<td>37.85 ± 1.67†</td>
<td>7.09 ± 1.62</td>
<td>11.48 ± 0.28†</td>
</tr>
<tr>
<td>Plasma + EPO/GM (n = 3)</td>
<td>5.72 ± 0.45</td>
<td>6,912 ± 360</td>
<td>78 ± 44</td>
<td>34.80 ± 1.35</td>
<td>49.15 ± 2.82</td>
<td>16.05 ± 2.24</td>
<td>14.05 ± 0.23†</td>
</tr>
<tr>
<td>Plasma + EPO/GM + TGFβ1 (n = 3)</td>
<td>6.21 ± 0.44</td>
<td>5,926 ± 178</td>
<td>67 ± 18</td>
<td>54.36 ± 3.06</td>
<td>37.98 ± 4.24</td>
<td>7.65 ± 1.16</td>
<td>11.58 ± 0.15†</td>
</tr>
</tbody>
</table>

Cultures were grown as described in Materials and Methods in 15% (vol/vol) plasma and, where indicated, erythropoietin (2 U/mL), GM-CSF (20 U/mL), and/or TGFβ1 (5 ng/mL). Data are shown ± SD. n = The number of independent specimens analyzed for each condition in this experiment. The entire experiment has been performed over 10 times with the same results.

Statistical significance: *P < .01 compared with plasma alone; †P < .05 compared with plasma alone; ‡P < .01 compared with plasma alone; §P < .02 compared with plasma + EPO/GM; ||P < .05 compared with plasma + EPO/GM.

Figure 1. The effect of recombinant TGFβ1 on the total number of megakaryocytes (A) and the ploidy distribution of megakaryocytes (B) in bone marrow cultures. GF-stimulated cultures were grown in the presence of increasing amounts of TGFβ1 as described in Materials and Methods and megakaryocyte growth quantitated by flow cytometry. The shaded areas represent the values of control samples cultured in the absence of TGFβ1 (± 2 SD). All data points outside the shaded areas differ significantly (P < .05) from controls. For (A), 100% = 7,076 megakaryocytes per culture. This experiment has been performed 10 times with the same results.
addition of TGFβ1 to concentrations up to 25 ng/mL produced no more inhibition. The effect on individual ploidy classes is shown in Fig 1B. As the concentration of TGFβ1 increased, the 8N compartment increased while the 16N and 32N compartments decreased in size. The Mean Ploidy_{8N} decreased from 12.12 ± 0.01 to 10.35 with a half-maximal inhibitory effect at 0.14 ± 0.08 ng/mL (average of four experiments). Addition of TGFβ1 up to 25 ng/mL failed to cause further inhibition.

These inhibitory effects of TGFβ1 were not potentiated by the addition of murine EGF. Concentrations of EGF ranging from 0.1 to 100 ng/mL in the presence of maximal (0.75 ng/mL) or submaximal (0.06 ng/mL) concentrations of TGFβ1 had no effect on the total cell number, the number of megakaryocytes, or the megakaryocyte ploidy distribution (data not shown).

**Purified TGFβ1 inhibits erythroid but not myeloid growth.** The effect of TGFβ1 on other lineages in the liquid culture system were also determined. While the GM-CSF–dependent increase in total cells (Table 1) was not inhibited by TGFβ1 up to 25 ng/mL, the erythropoietin-dependent hemoglobinization of the cultures was inhibited by TGFβ1 at a half-maximal concentration of 0.125 ng/mL (Fig 2). TGFβ1 produced a maximal decrease in hemoglobin production to 54% of control. These effects of TGFβ1 were not potentiated by the addition of murine EGF.

**Serum but not plasma inhibits the number and ploidy of megakaryocytes in culture.** When cultured at a concentration of 15% (vol/vol), rat serum produced 81% as many megakaryocytes (P < .001) with a lower Mean Ploidy_{8N} (P < .05) than did rat plasma (Table 2). When cultured at a concentration of 30% (vol/vol), the ploidy values were the same as at the lower protein concentration and still showed a statistically significant difference (P < .05). The relative difference in the number of megakaryocytes (P < .05) also persisted despite a protein concentration-dependent decrease in the total number of megakaryocytes seen in both plasma and serum. To demonstrate that the lesser growth in serum was due to the presence of an inhibitor, equivalent amounts of plasma and serum were mixed (final concentration = 30%). The ploidy distribution of megakaryocytes cultured in this 1:1 MIX was the same as in the serum. Taking into account the effect of the increase in the protein concentration, the total number of megakaryocytes in the 1:1 MIX was less than that in the plasma (30%) and equal to the serum (30%).

**TGFβ1 accounts for all the inhibitory activity found in serum.** Antibody to porcine TGFβ1 has previously been shown to neutralize TGFβ1 in serum extracts and in conditioned medium. To confirm that this antibody would also neutralize TGFβ1 in the presence of undiluted plasma, antibody was added to plasma samples containing a known amount of purified human platelet TGFβ1. As shown in Fig 3, complete neutralization of 4 ng of TGFβ1 was attained using 30 μg of antibody. Addition of equivalent amounts of rabbit antibody to human PDGF and bovine acidic FGF produced no neutralization of TGFβ1 activity.

On addition of antibody to TGFβ to serum (Table 3), the inhibitory effect of serum on the number and ploidy of megakaryocytes was fully neutralized. The Mean Ploidy_{8N} increased from 9.32 ± .33 to 11.98 ± .04 (plasma + antibody = 12.11 ± .15) while the number of megakaryocytes increased from 77% ± 10% to 104% ± 15% (plasma + antibody = 105% ± 9%). The addition of antibody to TGFβ to plasma produced a small, reproducible but statistically insignificant increase in the number of megakaryocytes and no effect on the ploidy distribution. Addition of control rabbit antibodies to human PDGF and bovine acidic FGF did not neutralize the inhibitory effect of serum.

Treatment of serum with DTT also completely eliminated the inhibitory effect on megakaryocyte number and ploidy (data not shown).

**TGFβ1 does not account for the differences found between thrombocytopenic and thrombocytotic rat plasma.** Plasma prepared from thrombocytopenic (Table 4) or thrombocytotic (Table 5) rats produced the same total number of cells and megakaryocytes in culture (data not presented) but had opposing effects on the ploidy of megakaryocytes grown. Plasma from thrombocytopenic rats (Table 4) promoted an increase in megakaryocyte ploidy compared with that found with plasma from normal rats (P < .05). Plasma from thrombocytotic rats (Table 5) failed to promote megakaryocyte ploidy growth equal to that seen with plasma from normal rats (P < .05). Addition of antibody to TGFβ to plasma from normal, thrombocytopenic, or thrombocytotic rats had no effect on the ploidy distributions. Addition of equivalent amounts of control rabbit antibodies to human PDGF and bovine acidic FGF was also without effect.

**DISCUSSION**

Although platelets contain by far the largest amount, TGFβ1 and its related compounds are produced in many tissues. The synthesized, biologically inactive, latent form becomes biologically active on exposure to proteases. Once activated, TGFβ displays an enormous range of biologic properties ranging from preventing the adipocyte differentiation of fibroblasts to reversing ischemic injury to

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**Fig 2.** The effect of recombinant TGFβ1 on erythroid growth in bone marrow cultures. Cultures were grown as described in Fig 1 and the amount of hemoglobin (±SD) quantitated. The shaded area represents the values of control samples (100% = 145 μg/culture) cultured in the absence of TGFβ1 (± 2 SD). All data points outside the shaded area differ significantly (P < .05) from controls. This experiment has been performed six times with the same results.
endothelial cells to mediating wound-related Rous sarcoma virus tumorigenesis.

In the hematopoietic system, TGFβ1 has so far been shown to have primarily inhibitory activity on early, but not late, growth factor-mediated responses. It inhibits the number of CFU-E, BFU-E, CFU-GEMM, and CFU-Mk. In addition, we have shown that at a half-maximal concentration of 0.14 ng/mL TGFβ1 also inhibits megakaryocyte endomitosis. Because megakaryocyte endomitosis is not an early event in megakaryocyte differentiation, our results show that this effect is an exception to the observation that TGFβ1 affects only early events in hematopoiesis. Moreover, the sensitivity of endomitosis to TGFβ1 may provide an explanation for the rapid, early decrease in the platelet count after the administration of purified TGFβ1 in vivo. In addition, we have shown that at a half-maximal concentration of 0.14 ng/mL TGFβ1 also inhibited megakaryocyte endomitosis. Because megakaryocyte endomitosis is not an early event in megakaryocyte differentiation, our results show that this effect is an exception to the observation that TGFβ1 affects only early events in hematopoiesis. Moreover, the sensitivity of endomitosis to TGFβ1 may provide an explanation for the rapid, early decrease in the platelet count after the administration of purified TGFβ1 in vivo.

The effects of TGFβ1 in our culture system are not lineage-specific. Erythropoietin-dependent erythropoiesis is inhibited half-maximally at a concentration (0.125 ng/mL) virtually identical with that for the inhibition of megakaryocyte endomitosis. But, as also reported by others, GM-CSF–dependent myeloid cell growth showed no inhibition by TGFβ1. Furthermore, we have demonstrated that unlike some other effects of TGFβ1, the inhibition of megakaryocyte and erythroid growth is not potentiated by EGF.

Given this potent effect of TGFβ1 on megakaryocyte growth, we subsequently sought to determine whether the presence of TGFβ1 might account for the inability of megakaryocytes to grow in several different experimental conditions. In the first of these situations, we have sought to explain the failure of megakaryocytes to grow in the presence of serum. Serum and platelet lysates but not plasma have been shown to inhibit CFU-Mk. Given the abundance of TGFβ1 and PF-4 in platelets, it has been suggested that TGFβ1 and/or PF-4 account for this inhibitory activity. We have used a neutralizing antibody to TGFβ in bone marrow cultures. Four nanograms of purified human TGFβ1 was added to 0.45-ml aliquots of rat plasma, incubated with various amounts of antibody to TGFβ, and then added to GF-stimulated cultures. Inhibition of megakaryocyte growth by TGFβ1 was quantitated by flow cytometry and the extent of neutralization of this inhibition by antibody was calculated. This experiment has been performed four times with the same results.

### Table 2. Rat Serum Contains an Inhibitor of Megakaryocyte Number and Ploidy

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total No. of Cells</th>
<th>% of Plasma (15%)</th>
<th>4N</th>
<th>8N</th>
<th>16N</th>
<th>32N</th>
<th>Mean Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (15%)</td>
<td>n = 3</td>
<td>5.33 ± 0.20</td>
<td>6.128 ± 74</td>
<td>100 ± 1</td>
<td>10.31 ± 0.4</td>
<td>34.27 ± 1.74</td>
<td>47.20 ± 0.33</td>
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<tr>
<td>Serum (15%)</td>
<td>n = 3</td>
<td>5.42 ± 0.37</td>
<td>4.953 ± 96</td>
<td>81 ± 1*</td>
<td>25.77 ± 1.54†</td>
<td>40.65 ± 3.19†</td>
<td>29.53 ± 1.38†</td>
</tr>
<tr>
<td>Plasma (30%)</td>
<td>n = 3</td>
<td>5.03 ± 0.01</td>
<td>3.975 ± 399</td>
<td>65 ± 7</td>
<td>23.05 ± 0.15</td>
<td>41.69 ± 0.56</td>
<td>31.92 ± 1.50</td>
</tr>
<tr>
<td>Serum (30%)</td>
<td>n = 3</td>
<td>5.13 ± 0.19</td>
<td>4.555 ± 421</td>
<td>74 ± 7</td>
<td>9.75 ± 0.58</td>
<td>34.52 ± 3.30</td>
<td>45.54 ± 1.76</td>
</tr>
<tr>
<td>1:1 MIX</td>
<td>n = 3</td>
<td>5.22 ± 0.3</td>
<td>3.852 ± 40</td>
<td>63 ± 1†</td>
<td>25.17 ± 2.85†</td>
<td>40.55 ± 1.50†</td>
<td>29.63 ± 1.76†</td>
</tr>
</tbody>
</table>

Cultures were grown as described in Materials and Methods in the presence of erythropoietin (2 U/mL). Data are shown SD. n = The number of independent specimens analyzed for each condition in this experiment. The entire experiment has been performed over 10 times with the same results.

Statistical significance: *P < .001 compared with plasma (15%); †P < .05 compared with plasma (15%); ‡P < .05 compared with plasma (30%).

Fig 3. Rabbit antibody to porcine TGFβ neutralizes the inhibitory effects of TGFβ1 in bone marrow cultures. Four nanograms of purified human TGFβ1 was added to 0.45-ml aliquots of rat plasma, incubated with various amounts of antibody to TGFβ, and then added to GF-stimulated cultures. Inhibition of megakaryocyte growth by TGFβ1 was quantitated by flow cytometry and the extent of neutralization of this inhibition by antibody was calculated. This experiment has been performed four times with the same results.
TGFβ to show that TGFβ1, not PF-4, accounts for all of the inhibitory effect of rat serum on megakaryocyte number and ploidy. Presumably latent TGFβ1 is released from platelet α granules during clotting and is acted on by platelet or serum proteases to produce the biologically active molecule.

We next examined the potential role of activated TGFβ1 as a circulating physiologic inhibitor of megakaryocyte growth. We have previously shown the existence of a humoral substance(s) in the circulation of the rat that inhibitory effect of any molecule is defined in the context of known or unknown countervailing stimulatory molecules, the optimal inhibitory effect of any molecule is demonstrated in vitro by using culture systems that are devoid of platelet products. If not possible, then treatment of samples with DTT or neutralizing antibody will eliminate activated TGFβ. Furthermore, when attempting to demonstrate circulating humoral activities directed against megakaryocytes, great care must be exercised in collecting blood from animals to prevent platelet secretion and subsequent TGFβ1 activation.

Because megakaryocytes synthesize abundant amounts of TGFβ1, the final situation we examined was whether autocrine release of activated TGFβ1 from megakaryocytes during culture might affect megakaryocytopeniosis in vitro. Under conditions of “GF-stimulated” or unstimulated megakaryocytopeniosis in our culture system, the addition of antibody to TGFβ did not alter the number or ploidy of megakaryocytes that grew. In addition, the medium conditioned by these cultures did not contain detectible amounts of TGFβ1 even after acidification (D.J.K., unpublished observation, July 1989). Therefore, these results do not support an autocrine role of activated TGFβ1 in megakaryocyte growth and endomitosis in vitro.

### Table 3. Antibody to TGFβ Neutralizes the Inhibitory Effect of Serum on Megakaryocyte Number and Ploidy

<table>
<thead>
<tr>
<th>Total No. Cells (×10^6)</th>
<th>Relative No. of Megakaryocytes (% of plasma)</th>
<th>4N (%</th>
<th>8N (%</th>
<th>16N (%</th>
<th>32N (%)</th>
<th>Mean Ploidy 4N-32N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (n = 3)</td>
<td>4.31 ± .25</td>
<td>100 ± 5</td>
<td>8.40 ± 1.17</td>
<td>35.56 ± .30</td>
<td>44.96 ± .86</td>
<td>11.09 ± 1.44</td>
</tr>
<tr>
<td>Serum (n = 3)</td>
<td>4.32 ± .11</td>
<td>77 ± 10*</td>
<td>21.20 ± 2.93†</td>
<td>41.06 ± 1.38†</td>
<td>32.27 ± 1.24†</td>
<td>5.47 ± .70†</td>
</tr>
<tr>
<td>Plasma + antibody (n = 3)</td>
<td>4.15 ± .10</td>
<td>105 ± 9</td>
<td>8.28 ± 1.20</td>
<td>34.30 ± .80</td>
<td>46.74 ± .67</td>
<td>10.68 ± .12</td>
</tr>
<tr>
<td>Serum + antibody (n = 3)</td>
<td>4.21 ± .26</td>
<td>104 ± 15</td>
<td>11.19 ± 1.16</td>
<td>28.86 ± 2.15</td>
<td>50.53 ± 1.16</td>
<td>9.43 ± .33</td>
</tr>
</tbody>
</table>

Cultures were grown as described in Materials and Methods in the presence of erythropoietin (2 U/mL). Data are shown ± SD. n = The number of independent specimens analyzed for each condition in this experiment. The entire experiment has been performed 10 times with the same results. For the relative number of megakaryocytes 100% = 6,184 ± 291.

Statistical significance: *P < .01 compared with plasma; †P < .05 compared with plasma.

### Table 4. Effect of Antibody to TGFβ on Plasma From Thrombocytopenic and Normal Rats

<table>
<thead>
<tr>
<th></th>
<th>4N (%)</th>
<th>8N (%)</th>
<th>16N (%)</th>
<th>32N (%)</th>
<th>Mean Ploidy 4N-32N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma (n = 3)</td>
<td>11.98 ± 1.88</td>
<td>39.62 ± 3.28</td>
<td>44.57 ± 2.94</td>
<td>4.34 ± 0.49</td>
<td>10.61 ± 0.24</td>
</tr>
<tr>
<td>Thrombocytopenic plasma (n = 3)</td>
<td>7.04 ± .95*</td>
<td>33.72 ± 2.01*</td>
<td>50.12 ± 1.87*</td>
<td>9.13 ± 1.28*</td>
<td>12.24 ± 0.34*</td>
</tr>
<tr>
<td>Normal plasma + antibody (n = 3)</td>
<td>13.06 ± .84</td>
<td>39.62 ± 1.25</td>
<td>43.03 ± 2.04</td>
<td>4.29 ± 0.69</td>
<td>10.45 ± 0.22</td>
</tr>
<tr>
<td>Thrombocytopenic plasma + antibody (n = 3)</td>
<td>8.23 ± 1.43†</td>
<td>33.08 ± 3.74†</td>
<td>50.25 ± 4.09†</td>
<td>8.44 ± 1.94†</td>
<td>12.05 ± 0.68†</td>
</tr>
</tbody>
</table>

Cultures were grown as described in Materials and Methods in 15% (vol/vol) plasma. Data are shown ± SD. n = The number of independent specimens analyzed for each condition in this experiment. This entire experiment has been performed four times with the same results. Statistical significance: *P < .05 compared with normal plasma; †P < .05 compared with normal plasma + antibody.
TGFβ INHIBITS MEGAKARYOCYTE GROWTH

We believe that they are relevant because the inhibitory effects of TGFβ1 are present (although of diminished magnitude) even in the absence of growth factors (Table 1) and because the effects shown in culture exactly parallel the effects in vivo.49

Although we can find no evidence using our culture system that activated TGFβ1 serves as a circulating regulator of megakaryocyte growth and endomitosis, we certainly cannot exclude a direct, local effect of TGFβ1 on megakaryocyte growth in vivo. Data are being accumulated which indicate that TGFβ production by stromal cells55,15 may regulate important early events in hematopoiesis, such as stem cell cycling.

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Transforming growth factor beta inhibits megakaryocyte growth and endomitosis

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