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 megakaryocyteopoiesis. 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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acidic fibroblast growth factor (FGF) were obtained from R & D Systems (Minneapolis, MN). We have previously demonstrated that the antibody to porcine TGFB also neutralizes TGFB1 purified from rat platelets. Murine epidermal growth factor (EGF, receptor grade) was from Collaborative Research, Inc (Bedford, MA). Sprague-Dawley-derived (CD) rats were obtained from Charles River Breeding Laboratories, Inc (Wilmington, MA) and were housed in single cages with free access to food and water for at least 1 week before use. New Zealand White rabbits (3 kg) were from Hazleton Research Products, Inc (Denver, PA).

Preparation of plasma and serum. Under ether anesthesia, male 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 acidic fibroblast growth factor (FGF) were obtained from R & Systems (Minneapolis, MN). We have previously demonstrated that the antibody to porcine TGFB also neutralizes TGFB1.

Preparation of platelet-poor plasma (PPP), serum (APS), and 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 15 minutes at 4°C to prepare platelet-poor plasma (PPP). Serum (S), PRP-derived serum (PRPDS), and 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.

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 TGFB. Each lot of antibody to TGFB was titrated against purified TGFB1 to determine the optimal neutralizing concentration. Twenty to 160 µL of antibody was added to 0.45-mL aliquots of rat PPPDS containing 4ng of TGFB1, incubated at 18°C for 1 hour and then added to GF-stimulated cultures. The extent of neutralization of the TGFB1-mediated megakaryocyte inhibition was determined.

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

Treatment of sera with diethyldithritol (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.

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
measured 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 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 ± 0.21 ng/mL (average of four experiments) and a maximal 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 (P < .02) and the megakaryocyte ploidy distribution dropped from a Mean Ploidy (± SD) of 14.05 to 11.58 (P < .05).

Using these GF-stimulated cultures, the concentration dependence of this effect of TGFβ1 was explored (Fig 1). There was no effect on the total number of cells (data not shown) but the number of megakaryocytes decreased (Fig 1A) with a half-maximal inhibitory concentration of 0.66 ± 0.21 ng/mL (average of four experiments) and a maximal decrease in the number of megakaryocytes to 55.8% ± 9.2% (average of six experiments) of normal. Further

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**Table 1. Effect of Erythropoietin, GM-CSF, and TGFβ1 on the Number and Ploidy of Megakaryocytes In Vitro**

<table>
<thead>
<tr>
<th>Plasma alone (n = 3)</th>
<th>Plasma + TGFβ1 (n = 3)</th>
<th>Plasma + EPO/GM (n = 3)</th>
<th>Plasma + EPO/GM + TGFβ1 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of Cells (× 10⁶)</td>
<td>4.59 ± 0.48</td>
<td>4.71 ± 0.08</td>
<td>5.72 ± 0.45</td>
</tr>
<tr>
<td>Total No. of Megakaryocytes</td>
<td>8,826 ± 475</td>
<td>5,530 ± 111</td>
<td>6,912 ± 360</td>
</tr>
<tr>
<td>% of Plasma Alone</td>
<td>100 ± 5</td>
<td>63 ± 1*</td>
<td>78 ± 4*</td>
</tr>
<tr>
<td>8N (%)</td>
<td>51.44 ± 2.57</td>
<td>55.06 ± 2.22*</td>
<td>34.80 ± 1.35*</td>
</tr>
<tr>
<td>16N (%)</td>
<td>41.06 ± 3.62</td>
<td>37.85 ± 1.67*</td>
<td>49.15 ± 2.82*</td>
</tr>
<tr>
<td>32N (%)</td>
<td>7.50 ± 1.19</td>
<td>7.09 ± 1.62</td>
<td>16.05 ± 2.24*</td>
</tr>
<tr>
<td>Mean Ploidy 8N-32N</td>
<td>11.80 ± 0.44</td>
<td>11.48 ± 0.28*</td>
<td>14.05 ± 0.23*</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 < .001 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.

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**Fig 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 TGFβ 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...
TGFB INHIBITS MEGAKARYOCYTE GROWTH

In the hematopoietic system, TGFB1 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, and CFU-GEMM in a liquid culture assay and reduced the size of isolated single megakaryocytes. Mitjavila et al also found that purified TGFB1 inhibited the number of megakaryocytes that grew at a half-maximal concentration of 0.14 ng/mL. In addition, we have shown that at a half-maximal concentration of 0.125 ng/mL, TGFB1 decreased the amount of acetylcholinesterase (a marker of murine megakaryocytes) in a liquid culture assay and reduced the size of isolated single megakaryocytes. Mitjavila et al also found that TGFB1 reduced megakaryocyte cell size. However, Solberg et al did not find any suppression of megakaryocyte DNA synthesis with TGFB1. When administered to mice in vivo, TGFB1 produced a 95% decrease in the platelet count within 7 days as well as a 50% drop in hematocrit with a 100% increase in the white blood cell count over 14 days.

We have recently developed a rat bone marrow culture system in which the number and ploidy of megakaryocytes that grow can be precisely quantitated using flow cytometry. With this assay we have examined the effect of purified TGFB1 on megakaryocyte growth. We found that purified TGFB1 inhibited the number of megakaryocytes which grew at a half-maximal concentration of 0.66 ng/mL, a concentration at which others have observed inhibition of CFU-Mk. In addition, we have shown that at a half-maximal concentration of 0.14 ng/mL TGFB1 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 TGFB1 affects only early events in hematopoiesis. Moreover, the sensitivity of endomitosis to TGFB1 may provide an explanation for the rapid, early decrease in the platelet count after the administration of TGFB1 in vivo.

The effects of TGFB1 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 TGFB1. Furthermore, we have demonstrated that unlike some other effects of TGFB1, the inhibition of megakaryocyte and erythroid growth is not potentiated by EGF.

Given this potent effect of TGFB1 on megakaryocyte growth, we subsequently sought to determine whether the presence of TGFB1 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 growth. Given the abundance of TGFB1 and PF-4 in platelets, it has been suggested that TGFB1 and/or PF-4 account for this inhibitory activity. We have used a neutralizing antibody to

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total No. of Cells (&gt;10⁶)</th>
<th>Total No. of Megakaryocytes</th>
<th>% of Plasma (15%)</th>
<th>4N (µg/mL)</th>
<th>8N (µg/mL)</th>
<th>16N (µg/mL)</th>
<th>32N (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (15%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<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 ± 3.3</td>
<td>8.23 ± 1.44</td>
</tr>
<tr>
<td>Serum (15%)</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>n = 3</td>
<td>5.42 ± 0.37</td>
<td>4.953 ± 28</td>
<td>81 ± 1</td>
<td>25.77 ± 1.54</td>
<td>40.65 ± 3.19</td>
<td>29.53 ± 1.38</td>
<td>4.06 ± 0.27</td>
</tr>
<tr>
<td>1:1 MIX 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 ± 0.55</td>
<td>31.92 ± 1.50</td>
<td>3.36 ± 1.10</td>
</tr>
<tr>
<td>Plasma (30%)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<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>45.64 ± 1.76</td>
<td>10.20 ± 0.96</td>
</tr>
<tr>
<td>Serum (30%)</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 3</td>
<td>5.22 ± 0.03</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>
<td>4.68 ± 0.42</td>
</tr>
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</table>

Cultures were grown as described in Materials and Methods in the presence of erythropoietin (2 U/mL). Data are shown as mean ± 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%); tP < .05 compared with plasma (15%); #P < .05 compared with plasma (30%).

Fig 3. Rabbit antibody to porcine TGFB neutralizes the inhibitory effects of TGFB1 in bone marrow cultures. Four nanograms of purified human TGFB1 was added to 0.45-ml aliquots of rat plasma, incubated with various amounts of antibody to TGFB1, and then added to G-stimulated cultures. Inhibition of megakaryocyte growth by TGFB1 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 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. 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.

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 megakaryocytopoiesis in vitro. Under conditions of "GF-stimulated" or unstimulated megakaryocytopoiesis 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 |
|---|---|---|---|---|---|---|
| Total No. of Cells (×10^6) | Relative No. of Megakaryocytes (% of plasma) | 4N (%) | 8N (%) | 16N (%) | 32N (%) | Mean Ploidy 4N-32N |
| Plasma (n = 3) | 4.31 ± .25 | 100 ± 5 | 8.40 ± 1.17 | 35.56 ± .30 | 44.96 ± .86 | 11.99 ± 1.44 | 12.02 ± .33 |
| Serum (n = 3) | 4.32 ± .11 | 77 ± 10 | 21.20 ± 2.93† | 41.06 ± 1.38† | 32.27 ± 1.24† | 5.47 ± .70† | 9.32 ± .33† |
| Plasma + antibody (n = 3) | 4.15 ± .10 | 105 ± 9 | 8.28 ± 1.20 | 34.30 ± .80 | 46.74 ± .67 | 10.68 ± .12 | 12.11 ± .15 |
| Serum + antibody (n = 3) | 4.21 ± .26 | 104 ± 15 | 11.19 ± 1.16 | 28.86 ± 2.15 | 50.53 ± 1.16 | 9.43 ± .33 | 11.98 ± .04 |

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.

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 megakaryocytopoiesis in vitro. Under conditions of "GF-stimulated" or unstimulated megakaryocytopoiesis 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.

The potent inhibitory effect of TGFβ1 poses a number of problems for those studying megakaryocytes and the regulation of their growth. At a practical level, effort must be made to use plasma (or plasma-derived serum) that is 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 the 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 maximally stimulated. To maximize megakaryocyte growth we have used erythropoietin while others have used IL-3 or medium conditioned by pokeweed mitogen-stimulated spleen cells or by various cell lines. However, because the physiologic stimulator of megakaryocyte growth has not yet been identified, it is important to ask whether the described effects of TGFβ1 have physiologic relevance.

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

| Mean Ploidy 4N-32N |
|---|---|---|---|---|---|---|
| Normal plasma (n = 3) | 11.98 ± 1.88 | 39.62 ± 3.28 | 44.57 ± 2.94 | 4.34 ± .49 | 10.61 ± .24 |
| Thrombocytopenic plasma (n = 3) | 7.04 ± .95* | 33.72 ± 2.01* | 50.12 ± 1.87* | 9.13 ± 1.28* | 12.24 ± .34* |
| Normal plasma + antibody (n = 3) | 13.06 ± .84 | 39.62 ± 1.25 | 43.03 ± 2.04 | 4.29 ± .69 | 10.45 ± .22 |
| Thrombocytopenic plasma + antibody (n = 3) | 8.23 ± 1.43† | 33.08 ± 3.74† | 50.25 ± 4.09† | 8.44 ± 1.94† | 12.05 ± .68† |

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.
We believe that they are relevant because the inhibitory effects of TGFβ1 are present (although 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 cells56-57 may regulate important early events in hematopoiesis, such as stem cell cycling.

ACKNOWLEDGMENT

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Transforming growth factor beta inhibits megakaryocyte growth and endomitosis

DJ Kuter, DM Gminski and RD Rosenberg