Type β Transforming Growth Factor Is a Potent Inhibitor of Murine Megakaryocytopoiesis In Vitro

By Toshiyuki Ishibashi, Susan L. Miller, and Samuel A. Burstein

To investigate the potential role of platelets in the inhibition of megakaryocytopoiesis, freeze-thawed extracts of human platelets were added to serumless liquid cultures of murine marrow. When acetylcholinesterase (AchE), a marker of megakaryocytic differentiation in mice, was assayed, a significant inhibition of enzymatic activity was noted in cultures containing the equivalent of greater than 5 × 10^6 solubilized platelets per milliliter. Freeze-thawed extracts of granulocytes had significantly less inhibitory effect than did platelets. Transforming growth factor β (TGF-β), a growth factor known to be inhibitory to some cell lineages and to be found at relatively high concentrations in platelets, was then added to liquid marrow cultures. A similar inhibition of AchE activity was detected when cultures were stimulated with mitogen-stimulated conditioned medium. The effect was potent with 50% inhibition of AchE activity observed at 4 pmol TGF-β/L. To determine if TGF-β inhibited specifically one aspect of megakaryocytic differentiation, the factor was added to isolated single megakaryocytes in serumless culture induced by interleukin 3 (IL3) to increase in size. The number of megakaryocytes increasing in size in response to IL3 exposure was reduced from 68% to 20% when both factors were simultaneously added to cultures. Colony assays showed that megakaryocytic and granulocyte-macrophage colony detection was inhibited at picomolar concentrations of the factor. These data suggest that TGF-β is a potent in vitro inhibitor of the murine megakaryocytic lineage, although its effects are not limited to this lineage.

© 1987 by Grune & Stratton, Inc.
supplemented with 1% Nutricyte (J. Brooks Laboratories, San Diego), a serum-free medium supplement containing 2% pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) produced under serum-free conditions and various concentrations of platelet extract or transforming growth factor-beta (TGF-β, 96% pure; R & D Systems Inc, Minneapolis). One microgram of TGF-β was reconstituted in 1 mL of 4 mmol/L HCl and diluted to the desired concentrations with 4 mmol/L HCl containing 50 μg/mL of crystalline bovine serum albumin (BSA; Sigma Chemical Co, St. Louis). Control wells contained either the cellular extract diluent (PBS) or TGF-β diluent (4 mmol/L HCl + 50 μg/mL BSA).

Acetylcholinesterase (AchE) assay. AchE activity of liquid marrow cultures was measured fluorometrically to quantitate megakaryocytopoiesis. After four days of culture, the cells were centrifuged at 700 g for ten minutes, and the supernatant was supplemented with 1% Nutricyte (J. Brooks Laboratories, San Diego) a serum-free medium supplement containing 2% pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) produced under serum-free conditions and various concentrations of platelet extract or transforming growth factor-beta (TGF-β, 96% pure; R & D Systems Inc, Minneapolis). One microgram of TGF-β was reconstituted in 1 mL of 4 mmol/L HCl and diluted to the desired concentrations with 4 mmol/L HCl containing 50 μg/mL of crystalline bovine serum albumin (BSA; Sigma Chemical Co, St. Louis). Control wells contained either the cellular extract diluent (PBS) or TGF-β diluent (4 mmol/L HCl + 50 μg/mL BSA).

Acetylcholinesterase (AchE) assay. AchE activity of liquid marrow cultures was measured fluorometrically to quantitate megakaryocytopoiesis. After four days of culture, the cells were centrifuged at 700 g for ten minutes, and the supernatant was discarded. Two-tenths milliliter of a solution of 0.2% Triton X-100 in 1 mmol/L EDTA, 0.12 mol/L NaCl, and 50 mmol/L HEPEs pH 7.5 was added to each well followed by the addition of 20 μL of 5.9 mmol/L acetylthiocholine iodide. After four hours of incubation, 10 μL of 0.4 mmol/L 3-mercaptoethanol (3-ME). Fewer than five colonies were counted for each experiment. TGF-β was added to each well at conc. 5 x 10 platelets/mL (P < 0.05; Student’s t test). One of six similar experiments. The error bars represent ± 1 SD of five replicate wells. AU, arbitrary fluorescence units.

Whole marrow cells treated with PWM-SCM and various concentrations of platelet extracts. As shown in Fig 1, platelet extracts inhibit AchE activity in murine liquid marrow cultures. A significant reduction was observed employing ≥ 5 x 10⁶/mL of solubilized platelets (P < 0.05). This decrease in AchE activity was accompanied by a decrease in the size and apparent number of megakaryocytes when observed microscopically. Figure 2 shows the inhibition of AchE production of platelet ν granulocyte extracts. A log-log transformation was performed to linearize the data, as other means of representing the data yielded curvilinear plots. Platelet extract induced a significantly greater degree

Fig 1. The effect of freeze-thawed platelet extracts on AchE activity in liquid marrow cultures stimulated with PWM-SCM. A significant inhibition of AchE activity is observed at a concentration ≥ 5 x 10⁶ platelets/mL (P < 0.05; Student’s t test). One of six similar experiments. The error bars represent ± 1 SD of five replicate wells. AU, arbitrary fluorescence units.

Statistical analysis. Statistical analyses were performed by Student’s t-test and chi-square analysis. Comparison between platelet and granulocyte extracts was performed by log-log transformation of the raw data to linear plots and subsequent linear regression analysis of the differences between slopes.

RESULTS

Effect of cellular extracts on AchE activity in liquid culture. AchE activity was assayed fluorometrically on

Fig 2. Comparison of inhibitory activity of freeze-thawed platelet ϒ granulocyte extracts. Extracts prepared as described in the Materials and Methods section. The abscissa represents the log of the protein concentration, while the ordinate shows the log (log[(% of control OD)/100 − % of control OD]), representing the AchE activity. A logit of zero thus indicates 50% inhibition. The slopes of these computer-generated lines are significantly different (P < 0.001; linear regression analysis). The solid line (platelet extract) is a composite of six experiments (each experiment was done with five different protein concentrations; at each concentration five replicate wells were assayed), while the dashed line (granulocyte extract) is a composite of four experiments done identically. Fifty percent inhibition of AchE activity is observed with 90 μg/mL of platelet extract. None of the tested concentrations of granulocyte extract induced 50% inhibition. Twenty-eight percent inhibition was observed at 90 μg/mL of granulocyte extract.
of inhibition than an equivalent amount of granulocyte extract (P < 0.001).

**Influence of purified TGF-β on AchE activity in liquid culture.** Figure 3 shows a significant decrease in AchE activity at a concentration of 2 pmol/L TGF-β, with further reductions at higher concentrations (P < 0.05). Four pmol/L TGF-β was required for 50% inhibition of AchE activity. Visual inspection of these cultures showed a decrease both in the size and apparent number of megakaryocytes when compared to control cultures. When IL 3 was substituted for PWM-SCM, the identical inhibitory effects of TGF-β were observed (data not shown). To determine whether the inhibition of AchE activity was due to cytotoxicity of TGF-β, cell viability after culture was measured by trypan blue exclusion. No significant difference was observed between cells cultured with or without 400 pmol/L TGF-β (P < 0.01).

**Cell diameter of single megakaryocytes isolated from CFU-MK exposed to TGF-β.** Single megakaryocytes were cultured with 5 U/mL IL 3, 20 pmol/L TGF-β, or both factors at the indicated concentrations. Cell diameter was measured on 123 megakaryocytes before and after 24 hours of culture. As shown in Table 1, 68% of the cells increased in diameter in response to IL 3, whereas only 20% of the cells increased in response to the combination of IL 3 and TGF-β. The response to IL 3 was significantly inhibited by TGF-β (X² = 14.6, P < 0.0001).

**Colony assays.** CFU-MK and CFU-GM were enumerated in situ after six days of culture. The effect of TGF-β on progenitor cells is shown in Table 2. No change in the numbers of CFU-MK- and CFU-GM-derived colonies were detected at concentrations ≤4 pmol/L. Inhibition of colony formation by both progenitor cell types was observed at concentrations ≥20 pmol/L (P < 0.01). However, at maximum inhibitory concentrations, CFU-MK numbers were decreased by 77%, whereas CFU-GM were decreased by 50%. Moreover, the numbers of cells per megakaryocyte colony and the size of the individual megakaryocytes within colonies were decreased in cultures containing TGF-β (data not shown).

**DISCUSSION**

Although inhibitors of hematopoiesis have been postulated for many years, it has only been recently demonstrated that specific purified factors such as lactoferrin and acid isoferri- tin inhibit growth factor-driven hematopoietic cellular proliferation.20-23 Suggestions that megakaryocytic proliferation might be subject to a significant inhibitory mechanism were provided by the studies of Messner et al.1 Vainchenker et al.,2 Kimura et al.,3 and Solberg et al.4 These investigators observed that megakaryocytic colony growth was markedly improved when the culture medium was supplemented with human plasma rather than serum and that the inhibitory activity appeared to be derived from platelets.

To determine if a platelet-derived factor might inhibit murine megakaryocytopenesis, we added freeze-thawed extracts of human platelets to serumless liquid cultures of murine marrow. We measured AchE, a relatively specific marker of the murine megakaryocytic lineage by a fluorometric method that is sensitive to low concentrations of the enzyme.17,24,25 Measurement of enzyme activity presumably reflects both the number and the state of differentiation of megakaryocytes.19,20 At concentrations of extract equivalent to 5 × 10⁴ platelets per milliliter, a significant inhibition of AchE activity was noted. These data together with the human colony data are somewhat at odds with the findings of Williams et al.26 These workers could detect no effect of the addition of freeze-thawed platelet extracts on the numbers of murine megakaryocytic colonies detectable in agar cultures. It is possible that the use of fetal calf serum or the conditioned medium used by Williams et al might account for the differences observed.

**Table 2. Effect of TGF-β on Colony Growth**

<table>
<thead>
<tr>
<th>TGF-β (pmol/L)</th>
<th>CFU-MK/10⁵ Cells</th>
<th>CFU-GM/10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22 ± 2</td>
<td>123 ± 13</td>
</tr>
<tr>
<td>0.4</td>
<td>19 ± 2</td>
<td>107 ± 9</td>
</tr>
<tr>
<td>4</td>
<td>19 ± 2</td>
<td>101 ± 16</td>
</tr>
<tr>
<td>20</td>
<td>15 ± 3</td>
<td>95 ± 8</td>
</tr>
<tr>
<td>40</td>
<td>12 ± 3</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>100</td>
<td>6 ± 1</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>200</td>
<td>5 ± 2</td>
<td>70 ± 13</td>
</tr>
<tr>
<td>400</td>
<td>6 ± 2</td>
<td>61 ± 10</td>
</tr>
<tr>
<td>800</td>
<td>(7)</td>
<td>(76)</td>
</tr>
</tbody>
</table>

The data represent the mean ± 1 SD of six experiments except for the numbers in parentheses (one experiment). Three to five replicate plates were enumerated for each experiment. A significant difference was observed at 20 pM TGF-β for both CFU-MK and CFU-GM (P < 0.01; Student’s t test).

An increase in diameter was defined as an increment of greater than 0.5 μm. The range of increments was 0.5 to 7.2 μm. The data represent the summation of all cells analyzed in five experiments. The difference between cells treated with IL 3 + TGF-β and IL 3 alone is highly significant (P < 0.0001; chi-square analysis).
However, studies of inhibition employing whole cell extracts are complex. Inhibition may be specific and have significance with respect to regulation. Conversely, inhibition may be nonspecific or trivial and related to proteolysis, pH changes, or other potential effects of whole cell extracts. Since extracts of cells other than platelets probably contain numerous constituents not present in platelet extracts, appropriate controls for this type of experiment are difficult to devise. On a per weight basis, however, platelet extracts contain more inhibitory activity than granulocyte extracts. Nevertheless, a more convincing experiment might be the documentation of inhibition by a purified factor at concentrations physiologically achievable.

Recently a factor, designated transforming growth-factor beta, has been purified to homogeneity and molecularly identified 

and related to proteolysis, pH changes, or other potential effects of whole cell extracts. 

Above marrow can be separated by physical techniques into megakaryocyte-rich or CFU-MK-rich fractions, addition of TGF-β2 to such separated marrow would not rule out the possibility that the factor was acting indirectly via accessory marrow cells. Therefore to determine specifically the influence of TGF-β on one aspect of megakaryocytic differentiation, we studied the effect of the factor on isolated single megakaryocytes in serumless culture. As shown previously, IL 3 is one factor that promotes megakaryocytic differentiation as assessed by increments in cell size, ploidy, and AchE content. 

At the tested concentration of 20 pmol/L, the percentage of cells expected to increase in size in response to IL 3 was markedly inhibited by TGF-β.

The inability to isolate single CFU-MK precludes a similar analysis of the influence of TGF-β on megakaryocytic progenitor cell proliferation. Nevertheless, picomolar concentrations of TGF-β inhibited both megakaryocytic and granulocyte-macrophage colony formation in a standard agar culture system. Preliminary data from Solberg et al suggest that TGF-β inhibits early erythroid and multilineage colony formation in addition to megakaryocytic colony formation in man. TGF-β may thus be a general inhibitor of hematopoiesis, although its effects may not be directly on the progenitor cells themselves.

The data show that TGF-β is a potent in vitro inhibitor of murine megakaryocytopoiesis. Moreover, TGF-β is an inhibitor of the granulocytic lineage, at least at the level of the colony-forming cell. These data do not show, however, that TGF-β is the only platelet-derived inhibitor. Other factors that inhibit megakaryocytopoiesis may be present in platelet extracts, perhaps with greater lineage specificity, and further investigation of this possibility will be required. Thus the overall importance of TGF-β in the suppression of hematopoiesis in general and megakaryocytopoiesis in particular remains unclear.

Studies of the influence of TGF-β on in vivo megakaryocytopoiesis will be of interest. Hypertransfusion of platelets into experimental animals has been shown by a number of investigators to result in a decrease in the size, ploidy, and number of megakaryocytes, an effect attributed to suppression of a thrombopoietic hormone. 

Krizza et al have reported that administration of freeze-thawed platelet extracts also inhibits megakaryocytopoiesis. 

It is conceivable that these in vivo effects of platelets or platelet products may not be related to suppression of a hormone but rather mediated via platelet-derived inhibitor(s), perhaps including TGF-β.

REFERENCES

10. Tucker RF, Volkenant ME, Branum EL, Moses HL: Comparison of intra- and extracellular transforming growth factors from
TGF-β INHIBITS MEGAKARYOCYTOPOIESIS

1741


Type beta transforming growth factor is a potent inhibitor of murine megakaryocytopoiesis in vitro

T Ishibashi, SL Miller and SA Burstein