Transforming Growth Factor β Inhibits Endomitosis in the Dami Human Megakaryocytic Cell Line

By Sheryl M. Greenberg, Chittaranjan Chandrasekhar, David E. Golan, and Robert I. Handin

Megakaryocyte development is a carefully controlled process that is at least partially regulated by cytokines. Previous investigations of megakaryocyte development have focused primarily on defining growth factors that induce or enhance differentiation. In this study we demonstrate that a specific cytokine, transforming growth factor β1 (TGFβ1), inhibits the phorbol myristate acetate (PMA)-induced differentiation of the Dami human megakaryocytic cell line. The addition of purified platelet TGFβ1 inhibits PMA-induced endomitosis in a dose-dependent manner. Inhibition of endomitosis occurs with as little as 0.4 pmol/L TGFβ1, is half-maximal at 6.4 pmol/L, and is maximal between 40 and 200 pmol/L TGFβ1. Inhibition does not require other growth factors or nonmegakaryocytic cells. Removal of TGFβ1 from the cultures decreases inhibition, suggesting that the continuous presence of TGFβ1 is required and that its effects are reversible. This effect occurs even though the Dami cells constitutively express TGFβ1 messenger RNA (mRNA) and the TGFβ1 mRNA levels are increased by PMA. TGFβ1 also has been shown to inhibit endomitosis during short-term culture of primary human megakaryocytes. These results suggest a model in which negative as well as positive regulatory factors modulate a critical stage of megakaryocyte development.

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MATERIALS AND METHODS

Cell culture. Dami cells were cultured as previously described. Briefly, cells were subcultured in Iscove's modified Dulbecco's medium (IMDM) containing 10% horse serum and 1% phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM). PMA was used at a final concentration of 500 nmol/L. TGFβ1 was purchased from R & D Systems (Minneapolis, MN). Normal human bone marrow was obtained through Dr Joseph Antin (Bone Marrow Transplant Program, Brigham and Women's Hospital, Boston, MA), fractionated on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and the nucleated cells were cultured overnight to remove adherent cells. The nonadherent cells were frozen for later use. The bone marrow cells were cultured in 10% fetal bovine serum in IMDM supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B. The cells were seeded into microtiter culture wells at 0.5 × 10⁴ cells/well and were grown for 7 days in a humidified incubator at 37°C in an atmosphere of 5% CO₂, 95% air.

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horse serum, 1% PHA-LCM, in IMDM as described for the Dami cells.

**Analysis of ploidy.** Dami cells were seeded at 200,000 cells/mL and fed again after 2 days. Cells were harvested with trypsin 3 days later, and the nuclei were isolated, stained with propidium iodide, and analyzed on a Becton Dickinson FACS Analyzer (Mountain View, CA) as previously described. Freshly prepared lymphocytes and propidium iodide standard beads (Flow Cytometry Standards Corp, Research Triangle Park, NC) were used to mark the position of the 2N cells. Cells were considered to be polyploid or undergoing endomitosis if the DNA content was greater than 4N.

**Northern blot analysis.** Total RNA was prepared by guanidine hydrochloride extraction and subjected to electrophoresis in 1% agarose formaldehyde denaturing gels as previously described. Equal amounts of total RNA (10μg) from each sample underwent electrophoresis and were transferred electrophoretically to GeneScreen (New England Nuclear, Boston, MA). A 28-base pair oligonucleotide which contains a sequence from the middle of exon 6 of the human TGFβ1 gene (R & D Systems), was labeled by kinase treatment of the 5' end, and used for hybridization. The filters were washed for 20 minutes twice with 6X SSC (1X SSC = 150 mmol/L sodium chloride, 1.0% sodium dodecyl sulfate, and 15 mmol/L sodium citrate) and twice in 4X SSC at room temperature, and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) for autoradiography.

**RESULTS**

The Dami cell line normally contains primarily 2N and 4N cells. In the presence of 5% human platelet-poor plasmaderived serum, PMA treatment for 5 days induces 30% to 40% of the Dami cells to increase their DNA content to greater than 4N. The induced cells fall into discrete ploidy groups containing 8N, 16N, and 32N complements of DNA. We found that the addition of between 1 and 5 ng/mL (40 and 200 pmol/L) TGFβ1 completely inhibited PMA-induced endomitosis (Fig 1). Concentrations of TGFβ1 ranging from 1 to 5 ng/mL (40 to 200 pmol/L) inhibited the PMA-induced endomitosis in a dose-dependent manner in the presence of the 5% plasma-derived serum. Half-maximal inhibition required 0.3 ng/mL (12 pmol/L) with the maximal inhibition at 1 to 5 ng/mL (40 to 200 pmol/L) (Fig 2).

In the presence of human serum, TGFβ1 was a more potent inhibitor of endomitosis with serum or plasma in the absence of TGFβ1. The denominator was corrected for the proportion of cells greater than 4N in the unstimulated control cultures. This is a representative experiment.
achieved at 0.16 ng/mL (6.4 pmol/L) TGFβ1 (Fig 2). As shown in Fig 3, concentrations of TGFβ1 that inhibited endomitosis had no effect on the proliferation of control or PMA-treated Dami cells. A slight (18%) decrease in Dami cell proliferation at the higher TGFβ1 concentrations was noted in the control cells. When cell viability was assessed by trypan blue exclusion, the percentage of dead cells did not change over the entire range of TGFβ1 concentrations tested (data not shown).

To determine the physiologic relevance of the TGFβ1-mediated inhibition of endomitosis, the effect of TGFβ1 on normal human megakaryocytes in crude preparations of bone marrow cells was also analyzed. Normal human bone marrow was prepared by fractionation on Ficoll-Paque and cultured overnight to remove adherent cells. Preliminary experiments demonstrated that the only bone marrow cells which were capable of achieving a greater than 4N ploidy state were megakaryocytic by their positive reaction with anti-platelet GPIb and GPIIb/IIIa monoclonal antibodies (MoAbs). The addition of 10 ng/mL TGFβ1 (400 pmol/L) decreased the proportion of nucleated cells that spontaneously achieved a ploidy state greater than 4N during 5 days of culture from 2.50% to 1.55% (Table 1), a 37% inhibition of baseline endomitosis in normal human megakaryocytes.

To determine whether the effects of TGFβ1 on endomitosis were reversible, Dami cells were cultured in the presence of PMA and TGFβ1 for 2 days followed by PMA alone for 3 days. The cells treated in this way became polyploid to the same extent as cells treated with PMA alone for 3 days (Table 2), suggesting that the mechanism by which TGFβ1 inhibits endomitosis is reversible. In cultures induced with PMA for 5 days, TGFβ1 treatment for the first 2 days resulted in a higher ploidy distribution than TGFβ1 treatment for the entire 5 days (Table 2). This suggests that the continuous presence of TGFβ1 is required for maximal inhibition. Decreasing the concentration of cells had no effect on the extent of PMA-induced endomitosis or on the TGFβ1 inhibition (data not shown).

To determine whether the Dami cells make TGFβ1 as has been demonstrated for normal megakaryocytes, total RNA was electrophoresed and transferred to filters for hybridization with a 28-nucleotide oligomer complementary to exon 6 of the human TGFβ1 gene. As shown in Fig 4, control and PMA-induced cells express the 2.2-kilobase TGFβ1 messenger RNA continuously with a slight increase in response to PMA. Incubation with an MoAb directed against TGFβ1 did not enhance the extent of endomitosis (data not shown).

### Table 1. Effect of TGFβ1 on Endomitosis in Normal Human Megakaryocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Cells &gt; 4N N = 4 (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.50 (± 0.47)</td>
</tr>
<tr>
<td>10 ng/mL TGFβ1</td>
<td>1.55 (± 0.45)</td>
</tr>
</tbody>
</table>

### Table 2. Reversibility of TGFβ1-Mediated Inhibition Endomitosis

<table>
<thead>
<tr>
<th>Treatment During Days 0–2</th>
<th>Treatment During Days 3–5</th>
<th>% Cells &gt; 4N N = 5 (± SD)</th>
<th>% Maximal Endomitosis N = 5 (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>8.1 (± 1.7)</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>PMA</td>
<td>20.0 (± 1.8)</td>
<td>43.8 (± 6.6)</td>
</tr>
<tr>
<td>PMA</td>
<td>PMA</td>
<td>35.4 (± 1.4)</td>
<td>100.0</td>
</tr>
<tr>
<td>PMA + TGFβ1</td>
<td>PMA</td>
<td>19.8 (± 1.2)</td>
<td>42.8 (± 8.0)</td>
</tr>
<tr>
<td>PMA + TGFβ1</td>
<td>PMA = TGFβ1</td>
<td>13.3 (± 2.1)</td>
<td>19.0 (± 7.6)</td>
</tr>
</tbody>
</table>

TGFβ1 was used at a final concentration of 5 ng/mL; PMA was used at a final concentration of 500 nmol/L.

*Percent maximal endomitosis was calculated as the percent of cells that achieved ploidy levels greater than 4N in the presence of TGFβ1 divided by the maximal percent of cells greater than 4N achievable in the absence of TGFβ1.

### DISCUSSION

Early megakaryocyte development is regulated by agents such as IL-3 that increase the proliferation of committed progenitor cells. As in other hematopoietic lineages, the proliferation of megakaryocyte progenitors in clonal assays is inhibited by TGFβ1. Late megakaryocyte differentiation, including expression of platelet cytoplasmic membranes, granules, and stored components in polyploid megakaryocytes, is enhanced or induced by less well-defined growth factors like MSF, TSF, or IL-6. TGFβ1 also inhibits this late stage of megakaryocyte maturation. Positive regulatory factors that act on megakaryocytes at an intermediate stage of differentiation have not been clearly defined. Based on data derived from primary mixed-cell rat bone marrow cultures, it has been suggested that there may be an inhibitor of endomitosis; although to date no direct and clear regulation of human megakaryocyte endomitosis has been demonstrated in a purified cell system. In this report, we have demonstrated that purified human TGFβ1 inhibits develop-
ment of the polyploid state of the human megakaryocytic Dami cell line and that this inhibitory effect does not require the presence of other hematopoietic cells. TGFβ1 similarly inhibited megakaryocyte endomitosis in primary cultures of normal human bone marrow that contained cells of other hematopoietic lineages.

Relatively high levels of TGFβ1 have been found in areas of active tissue differentiation that contain hematopoietic stem cells, such as bone marrow and fetal liver, and specifically in megakaryocytes. As has been demonstrated in other cell lines with megakaryocytic properties, TGFβ1 is expressed by the Dami cells. The level of expression is increased by PMA, an event that may be explained by the presence of three phorbol ester responsive elements (TREs) on the TGFβ1 gene. Incubation of Dami cells with a monoclonal TGFβ1 antibody at levels sufficient to neutralize 50 ng TGFβ1 did not enhance the extent of endomitosis, suggesting that it may not be released extracellularly, but this does not exclude the possibility of autocrine regulation.

It is well-known that TGFβ1 inhibits DNA synthesis and proliferation of B cells, epithelial cells, and early bone marrow progenitors. However, TGFβ1 did not affect Dami cell proliferation over the range of concentrations used to inhibit endomitosis. Thus, it appears that the mechanism of inhibition of endomitosis does not involve inhibition of DNA synthesis.

Of the four types of receptors for TGFβ that have been identified, two are well-defined. The high affinity TGFβ type I receptor is present on hematopoietic cells. It preferentially binds TGFβ1 and may be the principal cell surface molecule through which TGFβ1 induces its pleiotropic effects on signal transduction, altered gene expression, and changes in adhesion and growth control of hematopoietic cells. The type II receptor is also a high affinity TGFβ1 receptor, and may also play a role in hematopoiesis. It is possible that each receptor plays a specific role in mediating TGFβ1 action. Keller et al have suggested that if one of these were the principal receptor through which TGFβ1 mediates its effects on proliferation, loss of expression of this receptor might account for the loss of negative growth regulation in some leukemias. However, since we have shown that differentiation of the Dami cells, like that of normal megakaryocytes, is inhibited by TGFβ1, it is possible that a different TGFβ receptor may be involved in regulating megakaryocyte differentiation.

In summary, we have described how the human megakaryocytic Dami cell line can be used as a tool to examine the regulation of megakaryocyte differentiation. Specifically, we have demonstrated that purified, exogenous TGFβ1 directly inhibits endomitosis but not proliferation in a pure megakaryocyte cell system. Because platelets contain abundant amounts of TGFβ1, this data supports the concept that megakaryocyte endomitosis may be regulated by a negative autocrine feedback mechanism.

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