**Mechanism of Differential Inhibition of Factor-Dependent Cell Proliferation by Transforming Growth Factor-β1: Selective Uncoupling of FMS From MYC**

By Allen R. Chen and Larry R. Rohrschneider

Transforming growth factor-β1 (TGF-β1) selectively modulates hematopoietic cell proliferation. The proliferation of FDC-P1 clone MAC-11, a factor-dependent murine myeloid progenitor cell line, was inhibited differentially by TGF-β1: strongly in macrophage colony-stimulating factor (M-CSF), mildly in interleukin-3, and not at all in granulocyte-macrophage-CSF (GM-CSF). Flow cytometry and Western blots showed an unexpected increase in expression of FMS, the receptor for M-CSF, in response to TGF-β1. Metabolic labeling with 35S-methionine showed that synthesis of FMS protein accelerated in response to TGF-β1, whereas its degradation was unaffected. Northern analyses showed a rapid increase in c-fms RNA after the addition of TGF-β1. TGF-β1 did not affect kinase activity, cellular phosphotyrosine response, or internalization of FMS. However, TGF-β1 inhibited the induction by M-CSF of c-myc RNA analyzed on Northern blots and protein detected by radioimmunoprecipitation. TGF-β1 did not affect induction of c-myc expression by GM-CSF or induction of c-fos or c-jun by M-CSF. Therefore, FMS and the GM-CSF receptor induce c-myc via signal transduction pathways that differ in that only the former is inhibited by TGF-β1. This inhibition may account for the selective growth regulation by TGF-β1.

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**MATERIALS AND METHODS**

**Cells and cell culture.** FDC-P1 clone MAC-11 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), WEHI-3B conditioned medium (WCM) was used as a source of IL-3 at a concentration sufficient for maximal proliferation (2%). Recombinant murine M-CSF was provided by Oncogen (San Diego, CA). Recombinant murine GM-CSF was obtained from Immunex (Seattle, WA), and Recombinant human TGF-β1 was provided by Oncogen (Seattle, WA). Soft agar colony-forming unit (CFU) assays. Quadruplicate cultures were seeded with 200 cells in 1 mL 0.3% Bacto-agar containing 200 U/mL γM-CSF and various concentrations of TGF-β1. After 7 days at 37°C, 10% CO2, the cultures were fixed with 2.5% glutaraldehyde, dried onto glass slides, and stained with May-Grünwald Giemsa for colony counting.

**Flow cytometry.** For cell cycle analysis, cells were resuspended at 10⁶/mL in 1 mL ice-cold media, 0.25 mL staining solution (propidium iodide 125 μg/mL, 1% Triton X-100, RNAse 250,000 U/mL) was added, and the cells were incubated on ice for 5 to 45 minutes before analysis on the FACscan (Becton Dickinson, Mountain View, CA). Statistics on cell cycle phases were generated using the Multicycle computer program (Phoenix Flow Software, Phoenix, AZ).

To assess FMS expression, cells were washed in ice-cold phosphate-buffered saline (PBS) containing 5% FBS and 0.1% sodium azide, adjusted to 7.5 x 10⁶ cells/mL, blocked with normal goat Ig 1:250.

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Submitted November 12, 1992; accepted December 17, 1992.

Supported by National Institutes of Health Grants No. CA20551 and 5T32CA09351.

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for 30 minutes, incubated 60 minutes with a 1:250 dilution of tumor-bearing rat serum to the extracellular domain of FMS, and stained with phycoerythrin-conjugated goat antirat Ig 1:250 for 30 minutes. All incubations were performed on ice. Cells were analyzed on a FACScan and resulting data were analyzed using Reproman software (Truefacts Software, Seattle, WA).

**Immunoprecipitation and immunoblotting.** Cells were lysed with 1 mL radioimmunoprecipitation assay buffer with EDTA (150 mmol/L NaCl, 10 mmol/L Tris-Cl, pH 7.4, 0.1% SDS, 1% Triton X-100, 1% Droxycholate, 1% Trasylol, 5 mmol/L EDTA) buffer per 10^6 cells. FMS was immunoprecipitated with rabbit antiserum prepared against a peptide containing the tyrosine kinase domain of murine FMS expressed in Escherichia coli.22 For cell surface immunoprecipitation of FMS, tumor-bearing rat serum, after adsorption on FDC-P1 cells, was diluted 1:250 and incubated with intact cells that were then washed extensively and lysed. MYC was immunoprecipitated using affinity-purified rabbit antiserum raised against the species-specific C-terminal peptide, generously provided by Dr R. Eisenman (FHCRC). Control serum was blocked by preincubation with the immunizing peptide at 10 ng/mL.

Immunoprecipitated FMS and MYC were electrophoresed on 7.5% and 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, respectively. For Western transfer to nitrocellulose, a dry blot transfer system (Eillard, Seattle, WA) was used at 0.67 mA/cm^2 for 2 hours. Filters were blocked in TTBS (150 mmol/L NaCl, 25 mmol/L Tris-Cl, pH 8.0, and 0.5% Tween 20) with 3% gelatin, with 5% non-fat dry milk for chemiluminescence, and with 1% bovine serum albumin and 1% chicken ovalbumin for phosphotyrosine detection. FMS was detected using the rabbit antiserum; phosphotyrosine was detected using affinity-purified rabbit antiserum raised against the species-specific C-terminal peptide, generously provided by Dr R. Eisenman (FHCRC). Blots were washed at a final stringency of 0.1x SSC, 0.1% SDS, 65°C for 30 to 60 minutes.

**RESULTS**

Selective inhibition of proliferation by TGF-β1. TGF-β1 inhibits proliferation of various bone marrow progenitor populations differentially, depending on the CSF in which they are grown.11-14 This implies that TGF-β1 interacts selectively with certain CSF receptors or receptors selectively with certain cell types whose proliferation is selectively induced by the various CSFs. We investigated the possibility of selective interaction with CSF receptors by testing the effect of TGF-β1 on proliferation of the MAC-11 cell line. MAC-11 cells proliferate indefinitely as undifferentiated blast cells in either GM-CSF or IL-3. M-CSF induces both growth and some differentiation with limited expression of some macrophage markers.19 In the presence of TGF-β1, proliferation of MAC-11 cells was strongly inhibited in liquid cultures growing exponentially in M-CSF, whereas their proliferation was not inhibited when GM-CSF was the growth factor (Fig 1). Inhibition was intermediate in IL-3. A half-maximal effect occurred at about 1 ng/mL TGF-β1 (40 pmol/L). Differences in cell density were apparent within 24 hours and became more dramatic with time.

A change in cell morphology preceded detectable growth inhibition by TGF-β1. Cells in M-CSF became flatter and more adherent to tissue culture plastic or glass slides within 2 hours of addition of TGF-β1 (Fig 2). This raised the possibility that increased adherence may mediate slower growth. However, growth inhibition did not depend on induction of adherence, because TGF-β1 inhibited proliferation even when the cells were cultured in polypropylene tubes to which they could not adhere (Fig 3).

Growth inhibition observed in bulk liquid cultures may result from reduction of the proportion of cells proliferating or the rate of proliferation of each cell. To dissect the contributions of these mechanisms, we performed colony-forming assays. In 7-day soft agar cultures of MAC-11 cells in M-CSF (Table 1), low concentrations of TGF-β1 did not affect cloning efficiency, but reduced the size of colonies and altered colony morphology from compact to dispersed. At or above 10 ng/mL TGF-β1, no colonies formed in M-CSF.

Increased expression of FMS. Because proliferation in response to M-CSF was preferentially inhibited by TGF-β1, we expected that trans-modulation of FMS expression might be a mechanism of growth inhibition. However, both by flow cytometry (Fig 4A) and by Western blotting (Fig 4B) of MAC-11 cells cultured in M-CSF, we found an increase in FMS upon exposure to TGF-β1. An increase in the 140-Kd im-

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DIFFERENTIAL GROWTH INHIBITION BY TGF-β1

MAC-11 cells were cultured in Lab-Tek chamber slides in 2% WCM as a source of IL-3 or 200 U/mL γM-CSF without or with TGF-β1. After 24 hours, the media was aspirated gently, chambers removed, cover slips mounted in Elvanol, and phase-contrast photomicrographs obtained.

Fig. 2. Induction of adherence by TGF-β1. MAC-11 cells were cultured in Lab-Tek chamber slides in 2% WCM as a source of IL-3 or 200 U/mL γM-CSF without or with TGF-β1. After 24 hours, the media was aspirated gently, chambers removed, cover slips mounted in Elvanol, and phase-contrast photomicrographs obtained.

Fig. 3. Selective growth inhibition by TGF-β1 without adherence. Exponentially dividing MAC-11 cells were cultured in 12 × 75 mm polypropylene tubes containing various concentrations of TGF-β1 and either GM-CSF, M-CSF, or IL-3. Relative cell counts were determined by dividing the hemacytometer count at 72 hours by the inoculating cell count.

Metabolic labeling experiments in which cells were pulsed for 30 minutes with 35S-methionine and chased for 0 to 4 hours showed that, after TGF-β1 exposure, the signal increased in each FMS band, signifying more rapid synthesis (Fig 5). TGF-β1 exposure did not affect the rate of degradation. When M-CSF was present, most FMS disappeared from the cell surface within 60 minutes of synthesis, and without M-CSF the half-life was about 4 hours. The diminished signal at the 40-minute time point (Fig 5) without M-CSF and without TGF-β1 was not typical.

Table 1. MAC-11 7-Day Soft Agar Cultures

<table>
<thead>
<tr>
<th>TGF-β1 Concentration, ng/ml</th>
<th>Compact</th>
<th>Intermediate</th>
<th>Dispersed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>64 (53%)</td>
<td>46 (38%)</td>
<td>10 (8%)</td>
<td>120</td>
</tr>
<tr>
<td>0.1</td>
<td>6 (6%)</td>
<td>57 (60%)</td>
<td>32 (34%)</td>
<td>95</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>49 (39%)</td>
<td>77 (61%)</td>
<td>126</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1 (100%)</td>
<td>1</td>
</tr>
</tbody>
</table>

Each 35 mm plate was seeded with 200 cells in 1 mL 0.3% agar containing 200 U/mL γM-CSF.

* Concentration of TGF-β1 in nanograms per milliliter.
Northern blot analysis (Fig 6) showed an increase in c-fms RNA beginning within 30 minutes after the addition of TGF-β1, preceding the increase in protein synthesis. The response to TGF-β1 did not require simultaneous presence of M-CSF (data not shown). We have previously shown that both GM-CSF and IL-3 can trans-downmodulate c-fms expression at the RNA level. The effect of GM-CSF is dominant to M-CSF and is mediated by induction of RNase activity. The results in Fig 6 also indicate that GM-CSF-dependent trans-downmodulation was dominant to the TGF-β1–induced increase in c-fms expression.

Preservation of FMS kinase activity. We considered the possibility that, although FMS was expressed at higher levels in cells exposed to TGF-β1, it may be functionally inhibited by posttranslational modification or other factors induced by TGF-β1. However, assays of endogenous kinase activity performed on immunoprecipitated receptor showed that incorporation was in all cases proportionate to the amount of FMS detectable in parallel Western blots (Fig 7). To exclude the possibility that FMS may be regulated by interactions that take place only in intact cells, we also examined the cellular phosphoprotein response to M-CSF. TGF-β1 did not affect the amount of phosphotyrosine detectable by Western blots of immunoprecipitated FMS after cells were stimulated by M-CSF. In cells lysed within 10 minutes after M-CSF stimulation, the strongest phosphotyrosine signal is from transphosphorylated FMS, but one can detect tyrosine phosphorylation of several other proteins, some of which presumably are substrates of FMS. When whole lysates of cells stimulated with M-CSF were immunoblotted for phosphotyrosine, the pattern of bands in the presence of TGF-β1 was indistinguishable from controls without TGF-β1 (data not shown).

Preservation of FMS internalization. After stimulation by M-CSF, v-FMS is rapidly internalized in clathrin-coated pits and transported via endosomes to lysosomes where it is degraded. In addition to terminating the signal, it is possible that association with coated pits and internalization play an active role in receptor function by providing access to...
enhance c-fms RNA expression upon stimulation with TGF-β1. MAC-11 cells were growing exponentially in M-CSF or GM-CSF. At time 0, 10 ng/mL TGF-β1 was added to each 15 cm plate of cells. At the indicated times, cells were collected by gentle pipetting and their RNA extracted. Ten micrograms of RNA was loaded in each lane. The blot was hybridized with a nick-translated probe for c-fms and was subsequently stripped and reprobed for β-actin.

Because we observed that cells deprived of M-CSF upmodulated expression of FMS to a level comparable to that of cells exposed to both TGF-β1 and M-CSF (Fig 7), we considered the possibility that TGF-β1 may prevent M-CSF-induced downmodulation of FMS. We measured internalization of FMS as the loss of receptor immunoprecipitable from the cell surface upon stimulation with M-CSF. Incubation with TGF-β1 for up to 48 hours before the internalization assay had no effect, consistent with the results of the metabolic labeling experiments that showed a primary effect on synthesis, not degradation (data not shown).

Specific inhibition by TGF-β1 of FMS-induced c-myc expression. Because TGF-β1 selectively inhibited M-CSF-driven proliferation without reducing FMS expression or inhibiting early functions of the receptor, we surmised that downstream signal transduction events must be inhibited. A mutant of c-fms that fails to induce c-myc is defective in proliferation and can be rescued by coexpression of c-myc, implying that MYC performs functions that are essential for FMS-stimulated proliferation. Because c-myc is also a target of TGF-β1 inhibition in keratinocytes, we tested its role in our hematopoietic system. In untreated MAC-11 cells made quiescent by CSF starvation overnight, stimulation with either M-CSF or GM-CSF induced expression of early response genes c-fos, c-jun, and c-myc within 30 minutes. TGF-β1 pretreatment for 24 hours prevented M-CSF-induced expression of c-myc at both the RNA and protein levels (Fig 8); interestingly, c-myc expression induced by GM-CSF was slightly increased after TGF-β1 treatment. TGF-β1 did not inhibit induction of c-fos or c-jun expression by M-CSF.

Cell cycle analysis. Because c-myc function is required for transition from S phase to G2/M, as well as for entry to S from G1, we asked in what phase of the cell cycle MAC-11 cells cultured in M-CSF would accumulate after incubation with TGF-β1. The doubling time of these cells is about 24 hours in M-CSF and about 12 hours in GM-CSF or IL-3. After 24 hours of exposure to TGF-β1, MAC-11 cells grown in M-CSF accumulated in G0/G1 (Table 2 and Fig 9). TGF-β1 did not affect cell cycle progression of MAC-11 cells stimulated by GM-CSF.

DISCUSSION

TGF-β1 is a potent inhibitor of proliferation of most hematopoietic cells; however, its mechanism of action remains to be fully elucidated. Because responses differ among cell types and depend on the level of maturation and on which CSFs are present, it was predicted that modulation of CSF receptors might be a mechanism whereby TGF-β1 inhibits proliferation. Indeed, several examples illustrate this
phenomenon. The present study shows the antithesis: although TGF-β1 maximally inhibits proliferation of MAC-11 cells when they are growing in response to M-CSF, expression of FMS, the receptor for M-CSF, was increased by TGF-β1. Instead, TGF-β1 appeared to inhibit FMS-dependent expression of c-myc, a proto-oncogene involved in growth regulation.

We have used the MAC-11 cloned continuous cell line, which remains dependent on any of three CSFs for survival and proliferation. Its proliferative behavior was consistent with that of freshly isolated bone marrow progenitors and tissue macrophages with inhibition in M-CSF and responses in GM-CSF ranging from little effect to synergistic stimulation. These observations suggest the validity of the MAC-11 cell line as a model for bipotential progenitor cells. Our use of this cloned cell line excludes the possibility of indirect effects mediated by contaminating stromal elements and the possibility that the cells that continue to proliferate in GM-CSF represent a subpopulation distinct from those whose proliferation in M-CSF is inhibited.

In an effort to resolve the apparent paradox between increased FMS expression and decreased FMS-mediated proliferation, we studied the known early events in receptor tyrosine kinase-mediated signal transduction. TGF-β1 affected neither in vitro kinase activity of FMS nor tyrosine phosphorylation of FMS and other cellular targets in cells stimulated with M-CSF. Furthermore, M-CSF-induced internalization of FMS proceeded as usual, despite TGF-β1 treatment. However, TGF-β1 pretreatment rendered MAC-11 cells incapable of responding to M-CSF with increased c-myc expression, although c-fos and c-jun were induced to normal levels with normal kinetics. Roussel et al. observed an identical pattern of early response gene induction in murine fibroblasts that express a point mutant of human c-fms in which the major autophosphorylation site, tyrosine 809, was eliminated by mutation to phenylalanine. In that system, constitutive overexpression of c-myc complements FMS Y809F for proliferation. Although TGF-β1 may inhibit more than one function of FMS, c-myc induction was the only function we observed to be inhibited. Moreover, based on the observation that FMS requires cooperation from MYC and on the lack of examples of cell proliferation without MYC function, we believe that the inhibition by TGF-β1 of FMS-dependent c-myc induction can account for its growth inhibition. Formal proof will require overexpression of c-myc to rescue cells from growth inhibition by TGF-β1, and these experiments are in progress.

MYC function is required for entry into S phase, and recent evidence suggests it also is required for exit from S phase to G2/M. We considered the possibility that inhibition of c-myc expression might arrest cells in late S phase, but found that the addition of TGF-β1 to exponentially growing cultures of MAC-11 in M-CSF led to arrest in G0/G1, as was shown for mink lung epithelial cells.

Inhibition of c-myc expression by antisense oligonucleotides inhibits proliferation and promotes granulocytic differentiaton.

### Table 2. Cell Cycle Analysis of MAC-11 Cells

<table>
<thead>
<tr>
<th></th>
<th>CSF</th>
<th>TGF-β1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>0</td>
<td>64.0</td>
<td>30.4</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>10 ng/mL, 24 h</td>
<td>86.3</td>
<td>9.9</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>10 ng/mL, 48 h</td>
<td>91.4</td>
<td>6.5</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>1 ng/mL, 48 h</td>
<td>91.8</td>
<td>5.6</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>0</td>
<td>29.9</td>
<td>70.1</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>10 ng/mL, 24 h</td>
<td>36.7</td>
<td>63.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>10 ng/mL, 48 h</td>
<td>33.7</td>
<td>54.4</td>
<td>11.9</td>
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<tr>
<td>GM</td>
<td>1 ng/mL, 48 h</td>
<td>30.6</td>
<td>67.5</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>
We observed some features of increased differentiation in MAC-11 cells treated with TGF-β1: larger size, more granularity, increased adherence, increased FMS expression, and decreased proliferation. However, the cells did not acquire phagocytic activity, nor did they clearly alter their expression of differentiation antigens after treatment with TGF-β1 (data not shown). It is possible that this pattern represents the defective differentiation of a continuous cell line, or other factors may be required for complete differentiation.

Our results are similar to those of Pietenpol et al.32 who have shown that in keratinocytes stimulated by epidermal growth factor (EGF) TGF-β1 inhibits proliferation by inhibiting transcription of c-myc. They have identified a TGF-β1 response element between −86 and −63 relative to the c-myc promoter P1. We have yet to determine the mechanism of regulation of c-myc RNA levels in our model system. The work of Sherr et al.31 suggests that a signal transducer that interacts with FMS phosphotyrosine 809 goes on to induce c-myc. Interruption of any element in this pathway by TGF-β1 could account for our observations. FMS Y809 is homologous with the residue of the plasma-derived growth factor receptor (PDGF-R) required for binding of pp6Osrc (Y857);0 raising the possibility that pp6Osr participates in this pathway. Because there is no apparent homology between FMS Y809 and the mapped tyrosine phosphorylation sites of the EGF receptor, the initial signals for c-myc induction in EGF-stimulated keratinocytes and in M-CSF–stimulated hematopoietic cells appear to differ. If subsequent elements of the signalling pathway from FMS and the EGF-R to c-myc also differ, then the TGF-β1 control element identified by Pietenpol et al.32 may not be active in our system.

In lymphoid cells, a mutant IL-2Rβ that fails to interact with the src-family tyrosine kinase p56ck and does not affect cellular phosphotyrosine nevertheless induces c-myc in response to IL-2.33 Therefore, lymphoid cells possess a tyrosine kinase-independent signal transduction pathway from a member of the hematopoietin receptor family to c-myc. Fibroblasts possess a signal transduction pathway from FMS to c-myc that involves the FMS autoprophosphorylation site Y809.31 The present study shows for the first time that a single cell possesses distinct pathways of c-myc induction by a member of the hematopoietin receptor family (the GM-CSF receptor) and a receptor tyrosine kinase (FMS), and that these pathways constitute a mechanism of selective growth inhibition by TGF-β1. Because early hematopoietic cells express receptors that are highly homologous to FMS, this may be a general mechanism whereby TGF-β1 selectively inhibits proliferation of early hematopoietic cells.

ACKNOWLEDGMENT

We thank Drs Brian Gliniak, Irwin Bernstein, and Elizabeth Blackwood for their helpful suggestions; Kristen Carlberg, Jayne Goldstein, and Gary Myles for critically reviewing the manuscript; and Christine Loucks for secretarial assistance.

REFERENCES


23. Lyman SD, Rohrschneider LR: The kinase activity of the v-fms encoded protein have a low pH optimum. Oncogene Res 4:149, 1989


39. Wickstrom EL, Bacon TA, Gonzalez A, Freeman DL, Lyman GH, Wickstrom E: Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecanoxyxynucleotide targeted against c-myc mRNA. Proc Natl Acad Sci USA 85:1028, 1977

Mechanism of differential inhibition of factor-dependent cell proliferation by transforming growth factor-beta 1: selective uncoupling of FMS from MYC

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