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Stimulation of granulopoiesis by transforming growth factor β: Synergy with granulocyte/macrophage-colony-stimulating factor

(receptor regulation/granulocyte burst-forming unit)

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ABSTRACT **Transforming growth factor β1 (TGF-β1) is known to inhibit the growth of immature hematopoietic progenitor cells, whereas more mature, lineage-restricted progenitors are not inhibited. In contrast, in the presence of saturating concentrations of granulocyte/macrophage-colony-stimulating factor (GM-CSF), TGF-β promoted a 3- to 5-fold increase in the number and size (>0.5 mm) of bone marrow colonies in a dose-dependent manner with an ED_{50} of 10–20 pM; TGF-β1 alone had no effect. Morphological examination showed an increase in granulocyte colonies. In suspension cultures, TGF-β1 and GM-CSF stimulated an increase in total viable cells with markedly enhanced neutrophilic differentiation and a concomitant decrease in the number of monocytes/macrophages by day 6 in culture. Limiting dilution analysis demonstrated a 2- to 5-fold increase in the frequency of progenitor cells that responded to GM-CSF plus TGF-β1 vs. GM-CSF alone. Bone marrow progenitors obtained from mice 3 days after treatment with 5-fluorouracil responded to a combination of GM-CSF and TGF-β1, whereas either factor alone had no effect. A single-cell assay identified a progenitor cell that directly responded to TGF-β and GM-CSF. TGF-β increased the number of GM-CSF receptors on bone marrow cells. Thus, TGF-β1 can act as a bifunctional mediator of hematopoietic cell growth, and TGF-β1 and GM-CSF act together to stimulate granulopoiesis as measured by large granulocyte colony formation; the progenitor cell is tentatively designated granulocyte burst-forming unit.**

Transforming growth factor β (TGF-β) is a member of a family of polypeptides that regulate cell growth and differentiation (1–3). TGF-β is a multifunctional growth regulator whose action is dependent on the cell type, the growth conditions, the state of differentiation, and presence of other factors.

We and others have demonstrated inhibitory effects of TGF-β on hematopoiesis (4–9). In particular, we have shown that TGF-β is a selective inhibitor of colony-stimulating factor (CSF)-driven growth of both murine (4) and human (5) hematopoietic cells. TGF-β was found to directly inhibit the growth of multipotential hematopoietic cells, such as the interleukin 3 (IL-3)-induced growth of purified, lineage-negative (Lin^-), Thy-1-positive progenitor cells and high-proliferative-potential colony-forming cells, whereas the more differentiated cells, committed to a single lineage, were insensitive to TGF-β inhibition (6). Similar inhibitory effects on hematopoietic progenitor cells were seen by the *in vivo* administration of recombinant TGF-β (10).

In addition to the inhibitory action of TGF-β, we now report that TGF-β also exerts stimulatory effects on hematopoietic progenitors. In particular, TGF-β can enhance the colony growth of murine bone marrow cells in response to granulocyte/macrophage (GM)-CSF. In addition, TGF-β preferentially stimulates GM-CSF-induced granulocyte differentiation over monocytic differentiation. We also provide evidence that TGF-β can act in a two-signal model with GM-CSF to stimulate a progenitor cell that promotes increased granulopoiesis.

MATERIALS AND METHODS

Mice. All mice were obtained from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center.

Growth Factors. Purified recombinant murine GM-CSF was supplied by Steven Gillis (Immunex, Seattle). Purified recombinant granulocyte (G-CSF) was supplied by Lawrence Souza (Amgen Biologicals). Purified recombinant IL-4 (BSF-1) was purchased from Genzyme, and macrophage-CSF (CSF-1) from Cellular Products. Recombinant murine IL-3 was produced by COS-7 cells transfected with a plasmid containing the IL-3 cDNA, and the culture supernatant was harvested (11). TGF-β1 was purified to homogeneity by a published procedure (12).

Colony Formation in Soft Agar. A modification of the method of Stanley *et al.* (13) was used to measure colony formation of murine bone marrow cells. In brief, bone marrow cells were flushed from the femurs of normal BALB/c mice or from mice given a single i.v. injection of 5-fluorouracil (5-FU, 150 mg/kg of body weight). The cells were washed twice in Iscove's modified Dulbecco's medium (IMDM); resuspended in 1 ml of IMDM with 10% fetal bovine serum, glutamine, and antibiotics (complete IMDM) containing 0.3% SeaPlaque agarose (FMC); and incubated in 35-mm Lux Petri dishes (Miles Scientific) at 37°C in 5% CO_2/95% air until scored for colony growth.

Morphological Characterizations. Cells were either obtained from liquid culture or picked from agar cultures and then centrifuged onto glass slides. Alternatively, agar plugs from 35-mm Lux dishes were dried directly onto glass slides. The cells were stained with Jenner–Giemsa reagents and at least 200 cells were identified morphologically.

Purification of Lin^- Bone Marrow Cells. Lin^- cells were selected using published techniques (14). In brief, bone marrow cells were obtained from BALB/c mouse femurs and separated into low-density cells on lymphocyte separation medium (Organon Teknika). Cells (10^6) were resuspended in Hanks' balanced salts solution and incubated for 30 min at

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Abbreviations: TGF-β, transforming growth factor β; CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage-CSF; G-CSF, granulocyte-CSF; CSF-1, macrophage-CSF; IL, interleukin; 5-FU, 5-fluorouracil; Lin^-, lineage-negative.

Table 1. Effect of TGF-β1 on the morphology of GM-CSF-induced hematopoietic colonies

TGF-β1	Colony-forming units, no. (% total)			
	No.	Granulocyte	Macrophage	Granulocyte/ macrophage
	Colonies of diameter < 0.5 mM			
−	24 ± 2	4 ± 2 (18)	4 ± 1 (18)	14 ± 3 (64)
+	54 ± 4	20 ± 2 (42)	7 ± 1 (15)	21 ± 2 (43)
	Colonies of diameter > 0.5 mM			
−	2 ± 1	0	0	2 ± 1
+	10 ± 2	8 ± 1	0	2 ± 1

Normal bone marrow cells were plated at a density of 5×10^4 per ml in IMDM containing 10% fetal bovine serum and 0.3% SeaPlaque agarose (soft agar) supplemented with GM-CSF (20 ng/ml) in the presence or absence of TGF-β1 (20 ng/ml). After a 6- to 8-day incubation (37°C, 5% CO_2), cultures were scored for colony growth greater than 50 cells. The agar plug was removed from the 35-mm Petri dish and dried onto a 75 × 50-mm microscope slide, and colony morphology was determined by Jenner–Giemsa stain on 10 slides for each treatment. In addition, individual colonies were retrieved, cytocentrifuged, and stained for naphthyl-ASD-chloroacetate esterase and nonspecific esterase to confirm their morphology.

4°C with a mixture of antibodies: RA3-6B2 (B220 antigen) and RA3-8C5 (GR-1 antigen) (gifts of R. Coffman, DNAX); MAC-1 (purchased from Boehringer Mannheim); Lyt-2 (CD8) and L3T4 (CD4) (purchased from Becton Dickinson). Cells were washed twice in Hanks' balanced salts solution and then resuspended in the same buffer (10^8 cells per ml). Magnetic beads (Dynal, Great Neck, NY) were added at a ratio of 40:1 (beads/cell) and the suspension was incubated for 30 min at 4°C on a rotator. The cells were then magnetically separated with a magnetic particle concentrator (Dynal) and Lin^- cells were obtained. The Lin^- cells were seeded in Terasaki plates (Nunc) at a concentration of 1 cell per well in 20 μl of complete IMDM. Wells containing 2 cells or more were eliminated, while those with single cells were scored for cell growth (>10 cells) after 6–8 days at 37°C, 5% CO_2.

^{125}I-GM-CSF Binding Assays. GM-CSF was radioiodinated by a modification of the chloramine-T method (15). The specific activity of the biologically active ^{125}I-GM-CSF was $4.0–7.1 \times 10^6$ cpm/pmol. Binding was assayed by a phthalate oil separation method (16, 17). Equilibrium binding data were analyzed by the Scatchard method (18) and by computerized linear regression analysis. Duplicate determinations were done for each concentration of GM-CSF.

RESULTS

Effect of TGF-β on GM-CSF-Induced Bone Marrow Colony Formation. In contrast to its ability to inhibit IL-3-stimulated colony formation (4–9), TGF-β promoted a 3- to 5-fold increase in the number of bone marrow colonies in soft agar in the presence of saturating concentrations of GM-CSF (Table 1). The effect of TGF-β was dose-dependent with an ED_{50} of 10–20 pM (data not shown). In addition to the enhanced colony formation in the presence of TGF-β, there was a concomitant increase in the size of colonies >0.5 mm in diameter (Table 1; Fig. 1 *A* and *B*).

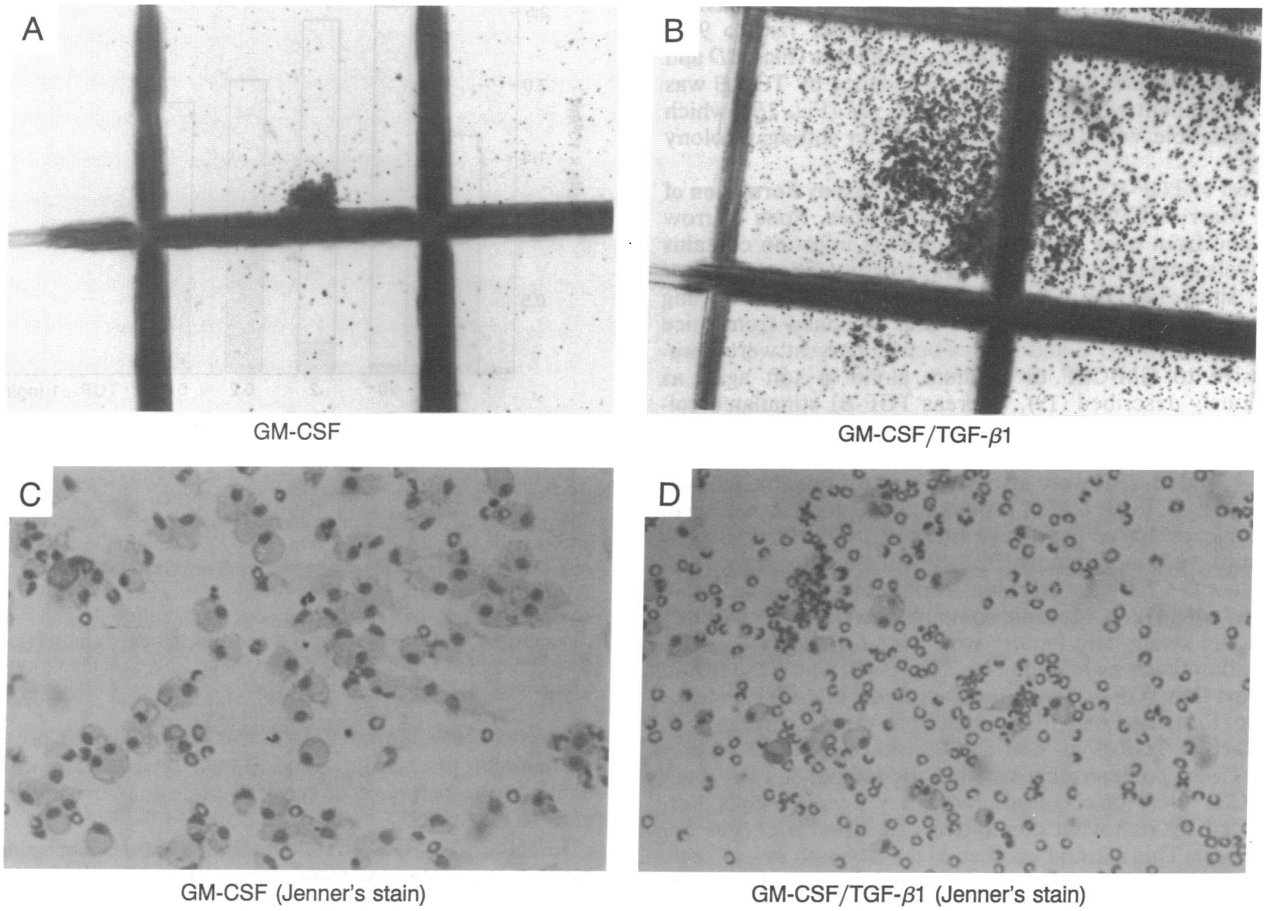

FIG. 1. Murine bone marrow cells in soft agar and liquid culture. Normal bone marrow cells were plated (5×10^4 cells per 35-mm dish) in soft agar medium containing GM-CSF (20 ng/ml) and cultured for 7–10 days in the absence (*A*) or presence (*B*) of TGF-β (20 ng/ml). Bone marrow cells were also plated (1.5×10^6 cells per ml) in liquid culture containing GM-CSF (20 ng/ml) for 6 days in the absence (*C*) or presence (*D*) of TGF-β (20 ng/ml). (*A* and *B*, ×75; *C* and *D*, ×300.)

To characterize the GM-CSF-induced bone marrow colonies grown in the presence or absence of TGF-β, the soft-agar colonies were dried onto glass slides and colony morphology was examined by Jenner–Giemsa and other histochemical stains (Table 1). There was a marked increase in both the number and the percentage of granulocyte-colony-forming unit cells. However, while we observed a decrease in the percentage of macrophage-colony-forming units and granulocyte/macrophage-colony-forming units in cultures supplemented with GM-CSF plus TGF-β vs. GM-CSF alone (Table 1), there was a modest increase (<2-fold) in the number of these colonies when compared with cultures with GM-CSF alone (Table 1). Cytocentrifuge preparations of large colonies (>0.5 mm) picked from soft-agar cultures supplemented with GM-CSF and TGF-β contained either pure or >90% granulocytes with the remaining cells being macrophages. Thus, TGF-β promotes an increase in the number and size of GM-CSF-induced bone marrow colonies in soft agar, with the largest colonies consisting predominantly of granulocytes.

Effect of TGF-β on the Growth of Bone Marrow Cells in Liquid Culture Supplemented with GM-CSF. Bone marrow cells were seeded at high density in liquid culture supplemented with GM-CSF in the presence or absence of TGF-β. Cultures supplemented with GM-CSF plus TGF-β showed an increase in total viable cells compared with cultures supplemented with GM-CSF alone, while cultures containing medium alone rapidly lost viability (Fig. 2*A*). Morphological and histochemical examination of cytocentrifuge preparations of bone marrow cells from 6-day cultures demonstrated that cultures supplemented with GM-CSF alone consisted of 70–75% monocyte/macrophages and 25–30% granulocytes (Figs. 1*C* and 2*B*). In marked contrast, cultures supplemented with GM-CSF plus TGF-β (20 ng/ml) gave rise to 90% granulocytes and 10% monocyte/macrophages (Figs. 1*D* and 2*B*). The increase in granulopoiesis induced by TGF-β was dose-dependent with an ED_{50} of 20–30 pM (Fig. 2*B*), which is comparable with the ED_{50} observed in soft-agar colony assays.

Effect of TGF-β on GM-CSF-Induced Colony Formation of Bone Marrow Cells from 5-FU-Treated Mice. Bone marrow obtained from mice 2–3 days after 5-FU injection contains relatively immature populations of hematopoietic progenitor cells, since 5-FU is cytotoxic for more committed cycling progenitors (19). Bone marrow cells obtained from mice injected with 5-FU 2 and 3 days before harvest were unresponsive to GM-CSF or medium alone in soft agar, as previously described (19), whereas TGF-β1 stimulated colony formation in a dose-dependent manner, in saturating concentrations of GM-CSF (Table 2). IL-3, IL-4, IL-6, CSF-1, and G-CSF were also examined for possible interactions with TGF-β1. CSF-1 promoted the colony formation of bone marrow harvested 2 days after 5-FU injection and these colonies were inhibited by TGF-β, while neither IL-3, IL-4, IL-6, nor G-CSF induced colony formation in the presence or absence of TGF-β. Multiple combinations of these cytokines including synergistic factors were able to stimulate colony formation that was inhibited by TGF-β (6). Thus, the results indicate that TGF-β is required to promote GM-CSF-induced colony formation of 5-FU-treated bone marrow progenitors.

Effect of TGF-β on GM-CSF-Driven Growth of Isolated Lin^- Cells. To determine whether the effects of TGF-β and GM-CSF were direct, bone marrow progenitor cells were enriched by removing committed cells (see *Materials and Methods*). This method resulted in the isolation of Lin^- cells representing 1–2% of the unfractionated marrow. These cells were sufficiently purified such that 1 in 23 isolated Lin^- cells proliferated in response to GM-CSF when seeded into Terasaki plates at a density of 1 cell in 20 μl per well (Table 3). When these cells were grown in the presence of TGF-β plus GM-CSF, this response increased to 1 in 13 cells, an increase of 76%. No cell growth was detected in the presence of medium or TGF-β alone. Thus, TGF-β can directly enhance the response of purified bone marrow progenitor cells to GM-CSF.

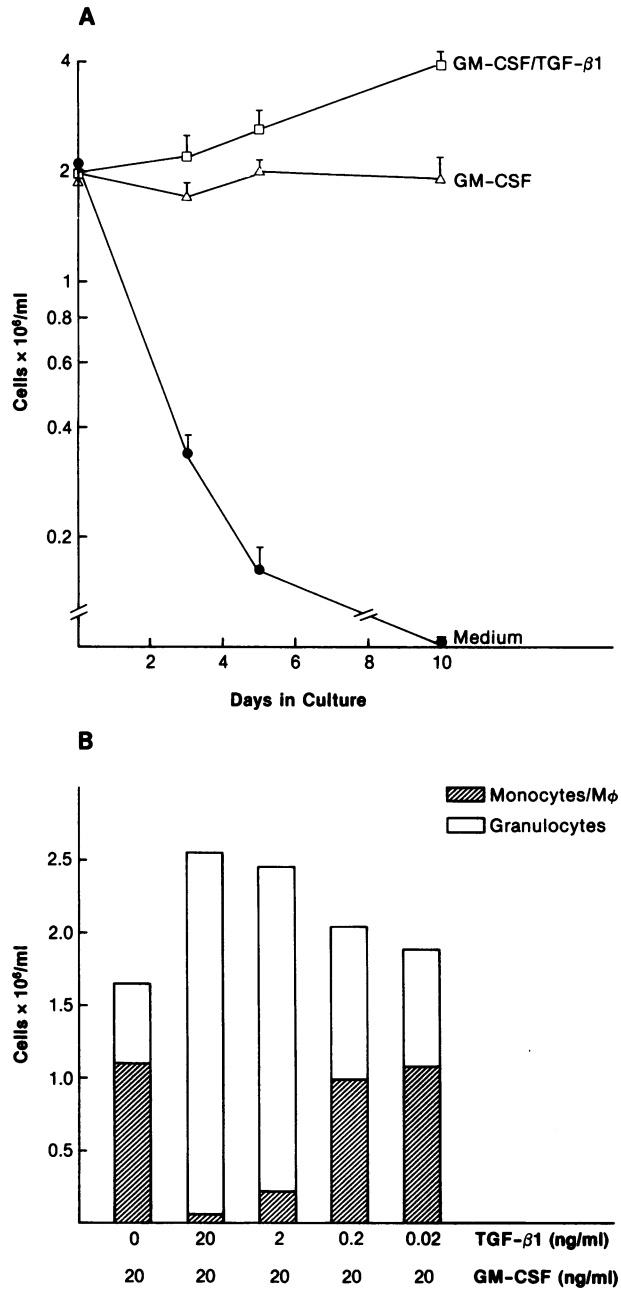

FIG. 2. (*A*) Effect of TGF-β1 on the viability of normal bone marrow cells cultured with GM-CSF. Normal bone marrow cells were plated at 2 × 10^6 per ml in 24-well Costar plates in medium alone (●), medium plus GM-CSF (20 ng/ml) (△), or medium plus GM-CSF and TGF-β1 (□). (*B*) Effect of TGF-β1 on GM-CSF-induced bone marrow differentiation. Bone marrow cells were plated with GM-CSF (20 ng/ml) as described above and cultured with various concentrations of TGF-β1 for 6 days. Granulocyte and monocyte/macrophage (Mφ) content was determined by examining cytocentrifuge preparations.

Effect of TGF-β1 on GM-CSF Receptor Expression on Normal Bone Marrow Cells. Since TGF-β1 enhanced GM-CSF-induced colony formation and increased the frequency of GM-CSF-responsive bone marrow progenitors, it was possible that TGF-β1 might increase the level of GM-CSF receptor expression. GM-CSF specific binding was not altered following up to 48 hr of incubation in medium alone but

Table 2. Effect of TGF-β1, GM-CSF, and other cytokines on colony formation of bone marrow cells from 5-FU-treated mice

Growth factor*	TGF-β1 ng/ml	Colony forming units, no. 2 days after 5-FU	3 days after 5-FU
Medium	0	0	0
	20	0	0
GM-CSF	0	0	0.5 ± 0.5
	20	9 ± 2	82 ± 2
	2	10 ± 2	78 ± 3
	0.2	5 ± 1	43 ± 5
	0.02	0	2 ± 1
CSF-1	0	7 ± 1	ND
	20	0.5 ± 0.5	ND
IL-3	0	0	0.5 ± 0.5
	20	0	0.5 ± 0.5
G-CSF	0	0	0
	20	0	0
IL-4	0	0	0
	20	0	0
IL-6	0	0	0
	20	0	0

Bone marrow cells were obtained from mice 2 and 3 days after injection with 5-FU (150 mg/kg, i.v.) and plated at a density of 5×10^4 per ml as described in Table 1. Cultures were scored for colony growth after a 7- to 10-day incubation (37°C, 5% CO_2).

*Cultures were supplemented with homogeneous preparations of recombinant GM-CSF (20 ng/ml), recombinant G-CSF (20 ng/ml), CSF-1 (100 units/ml), IL-3 (100 units/ml), IL-4 (100 units/ml; Genzyme), and recombinant IL-6 (100 units/ml; gift of Rick Norton, National Cancer Institute).

increased 20–30% after 24 hr and 50–60% after 48 hr of treatment with TGF-β (data not shown). Scatchard analysis of equilibrium binding data after 48 hr showed that bone marrow cells in medium alone had 78 high-affinity binding sites per cell, whereas cultures containing TGF-β1 had 123 sites per cell without a change in receptor affinity (Fig. 3). Similarly, the number of low-affinity GM-CSF receptor sites was also increased, from 186 to 267, in the presence of TGF-β1. Thus, TGF-β1 stimulated greater cell surface expression of GM-CSF receptors in cultures of bone marrow cells.

Table 3. Direct effect of TGF-β1 on purified bone marrow progenitors (Lin^- cells)

GM-CSF	TGF-β1	1/f*
−	−	0
−	+	0
+	−	23 ± 2
+	+	13 ± 1

Bone marrow cells were separated as outlined in *Materials and Methods*, and Lin^- cells were seeded into Terasaki plates (one cell per well). Culture medium was supplemented with GM-CSF (20 ng/ml) and/or TGF-β (20 ng/ml) as indicated.

*Frequency of growth to >10 cells (mean ± SE of at least three experiments; minimum of 1000 wells were scored).

DISCUSSION

Initial reports indicated that TGF-β was a potent negative regulator of murine and human CSF-driven hematopoietic cell growth (4–9). We now provide evidence that TGF-β is a direct multifunctional regulator of hematopoiesis whose action is dependent on the type of CSF present and the nature of the interacting cell. In particular, while TGF-β had no effect alone, it significantly enhanced GM-CSF-driven colony formation *in vitro*. As a consequence of stimulation by both TGF-β and GM-CSF, there is a marked increase in the production of differentiated granulocytes both in suspension and in soft-agar cultures. Counting the number and percentage of granulocytes per colony results in the same preferential increase in granulopoiesis as seen in suspension cultures. In the light of these observations, we propose that TGF-β and GM-CSF can act directly in a two-signal model to promote granulopoiesis through a granulocyte burst-forming unit (BFU-G) analogous to the erythroid burst-forming unit (BFU-E) that requires IL-3 and erythropoietin (20). This model is reinforced by two observations. (*i*) Both GM-CSF and TGF-β are required to promote the formation of BFU-G colonies by bone marrow cells obtained from mice 3 days after the 5-FU treatment that are unresponsive to either factor alone. (*ii*) Single-cell cloning experiments indicate that

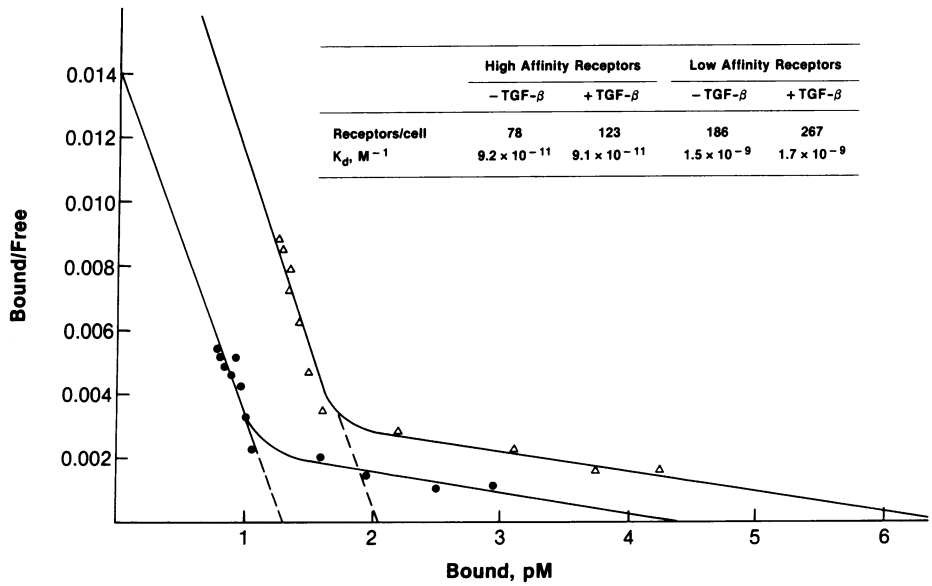

FIG. 3. Effect of TGF-β on GM-CSF receptor expression on bone marrow cells. Bone marrow cells were incubated in the presence or absence of TGF-β (20 ng/ml). Equilibrium binding and Scatchard analysis were performed as described in *Materials and Methods*. The Scatchard analysis is plotted as the mean of duplicate determinations and is representative of three separate experiments. (*Inset*) Number and affinities of GM-CSF receptors on TGF-β-treated and untreated cells.

this is a direct effect of TGF-β and GM-CSF on this progenitor cell. Both types of experiments show that there are hematopoietic progenitor cells that are unresponsive to GM-CSF alone and require two signals (TGF-β and GM-CSF) to promote their growth and differentiation. In addition, TGF-β does not appear to be the same as other previously described synergistic factors (21), since TGF-β inhibits their colony formation (6).

One possible mechanism accounting for TGF-β effect on GM-CSF-stimulated granulopoiesis is the ability of TGF-β to modulate cell surface expression of GM-CSF receptors. This would be analogous to the upregulation of CSF-1 receptors by hemopoietin 1 seen on high-proliferative-potential colony-forming cells (22). In the present study, TGF-β increased the number of GM-CSF receptors on bone marrow cells by 24 and 48 hr. Increased differentiation probably does not account for the observed increase in GM-CSF receptor expression, since no change in morphology was observed over the incubation period. In addition, GM-CSF receptor expression has been reported to decrease on myeloid cells undergoing differentiation (23).

It was unexpected to find that TGF-β could greatly augment GM-CSF induction of granulopoiesis, in light of previous observations that it was a potent inhibitor of primitive hematopoietic cells. However, studies examining the effects of TGF-β *in vivo* support the multifunctional nature of TGF-β and its stimulatory effect on granulopoiesis. Chronic administration of 5 μg of TGF-β for 14 consecutive days into mice resulted in a 40–50% reduction in the red-cell mass and platelet number, while the bone marrow and spleen showed enhanced neutrophil proliferation and differentiation (24). Thus, TGF-β must have a stimulatory effect on granulocyte production *in vivo*.

Finally, the requirement of TGF-β for optimal GM-CSF-induced granulopoiesis could play an important physiological role in maintaining and accelerating granulopoietic activity in the host. Further, the ability of TGF-β to inhibit stem-cell proliferation while stimulating and accelerating granulopoiesis suggests that it may be useful as a protective agent against myelotoxicity of chemotherapeutic drugs and as a potential restorative agent to increase granulocyte recovery after chemotherapeutic treatment.

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1. Roberts, A. B., Flanders, K. C., Kondaiah, P., Thompson, N. L., Van Obberghen-Schilling, E., Wakefield, L., Rossi, P., De Crombrugghe, B., Heine, U. & Sporn, M. B. (1988) *Recent Prog. Horm. Res.* **44**, 157–197.
2. Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F. & Sporn, M. B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 119–123.
3. Hill, D. J., Strain, A. J., Elstow, S. F., Swenne, I. & Milner, R. D. G. (1986) *J. Cell. Physiol.* **128**, 322–328.
4. Keller, J. R., Mantel, C., Sing, G. K., Ellingsworth, L. R., Ruscetti, S. K. & Ruscetti, F. W. (1988) *J. Exp. Med.* **168**, 737–750.
5. Sing, G. K., Keller, J. R., Ellingsworth, L. R. & Ruscetti, F. W. (1988) *Blood* **72**, 1504–1511.
6. Keller, J. R., McNiece, I. K., Sill, K. T., Ellingsworth, L. R., Quesenberry, P. J., Sing, G. K. & Ruscetti, F. W. (1990) *Blood* **75**, 596–602.
7. Ishibashi, T., Miller, S. L. & Burstein, S. A. (1987) *Blood* **69**, 1737–1741.
8. Ohta, M., Greenberger, J. S., Anklesaria, P., Bassols, A. & Massague, J. (1987) *Nature (London)* **329**, 539–541.
9. Ottmann, O. G. & Pelus, L. M. (1988) *J. Immunol.* **140**, 2661–2665.
10. Goey, H., Keller, J. R., Back, T., Longo, D. L., Ruscetti, F. W. & Wiltrout, R. H. (1989) *J. Immunol.* **143**, 877–880.
11. Okayama, H., Kawaichi, M., Brownstein, M., Lee, F., Yokota, T. & Arai, K. (1987) *Methods Enzymol.* **154**, 3–29.
12. Ellingsworth, L. R., Brennan, J. E., Fok, D., Rosen, D. M., Bentz, H., Piez, K. A. & Seyedin, S. M. (1986) *J. Biol. Chem.* **261**, 12362–12367.
13. Stanley, E. R., Metcalf, D., Marite, J. S. & Yeo, G. F. (1972) *J. Lab. Clin. Med.* **79**, 657–660.
14. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. (1988) *Science* **241**, 58–63.
15. Ihle, J. N., Keller, J., Henderson, L., Klein, F. & Palaszynski, E. (1982) *J. Immunol.* **129**, 2431–2436.
16. Dower, S. K., Ozato, K. & Segal, D. M. (1984) *J. Immunol.* **132**, 751–754.
17. Jacobsen, S. E. W., Ruscetti, F. W., Dubois, C. M., Lee, J., Boone, T. M. & Keller, J. R. (1991) *Blood* **77**, 1706–1716.
18. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–665.
19. Bradley, T. R., Hodgson, G. S., Kriegler, A. B. & McNiece, I. K. (1985) *Prog. Clin. Biol. Res.* **184**, 39–54.
20. Iscove, N. N., Siebere, F. & Winterhalter, K. H. (1979) *Am. J. Cell. Physiol.* **83**, 309–314.
21. McNiece, I. K., Bertoncello, I., Kriegler, A. G. & Quesenberry, P. J. (1990) *Int. J. Cell Cloning* **8**, 146–160.
22. Bartelmez, S. H. & Stanley, E. R. (1985) *J. Cell. Physiol.* **122**, 370–378.
23. Nicola, N. A. (1989) *Annu. Rev. Biochem.* **58**, 45–77.
24. Carlino, J. A., Higley, H. R., Avis, P. D. & Ellingsworth, L. E. (1990) *Ann. N.Y. Acad. Sci.* **593**, 330–333.