Transforming Growth Factor-β1 Bifunctionally Regulates Murine Macrophage Proliferation

By Kai Fan, Qingyun Ruan, Lyle Sensenbrenner, and Ben Chen

Transforming growth factor-β (TGF-β) is a family of polypeptide growth factors with multiple functional activities. Recent studies suggest that TGF-β is a selective inhibitor of hematopoietic cells. In this report, we study the effect of TGF-β1 on the proliferation of murine peritoneal exudate macrophages (PEM) in response to purified recombinant granulocyte-macrophage colony-stimulating factor (rMuGM-CSF) and human recombinant M-CSF (rHuM-CSF). In mice, PEM and other types of tissue macrophages display multiple types of receptors for CSFs and respond to them, either alone or in combination, to undergo extensive proliferation in vitro. Recombinant human TGF-β1 (rHuTGF-β1) (0.1 to 1.0 ng/mL) markedly enhanced the growth of PEM in response to rMuGM-CSF but inhibited their responsiveness to rHuM-CSF. Similar effects of rHuTGF-β1 were also detected using murine pulmonary alveolar macrophages (PAM) and bone marrow-derived macrophages (BMDM). Receptor binding assays using iodinated rMuGM-CSF and rHuM-CSF showed that rHuTGF-β1 treatment greatly enhanced the expression of GM-CSF receptors in PEM, in a time- and dose-dependent manner, suggesting a possible mechanism for the synergistic effect of TGF-β1. On the other hand, the expression of M-CSF receptors was not affected by TGF-β1 treatment. Analysis by mRNA PCR showed that the synergistic effect of TGF-β1 is not due to autocrine CSFS produced by treated cells. Our results suggest that TGF-β1 is an important regulator of macrophage proliferation. Depending on the types of CSFs present, TGF-β1 may act either as a growth promoter or inhibitor.

© 1992 by The American Society of Hematology.

MATERIALS AND METHODS

Mice. Female C3H/HeJ mice, 8 to 12 weeks old, were obtained from the Jackson Laboratories (Bar Harbor, ME). Cells obtained from this strain of mice are refractory to endotoxin stimulation.

Reagents. Purified murine recombinant GM-CSF (rMuGM-CSF) (specific activity >4 × 10⁶ U/mg protein) was kindly provided by Immunex Corp (Seattle, WA). In some experiments, rMuGM-CSF purchased from Biosource International (Westlake Village, CA) was used with exactly the same result. Human recombinant M-CSF (rHuM-CSF, specific activity: 5 × 10⁷ U/mg protein) was kindly provided by Cetus Co (Emeryville, CA). Human recombinant TGF-β1 (biological activity: 6,000 U/μg protein) was a gift of the National Cancer Institute (Bethesda, MA). GeneAmp RNA PCR kit was obtained from Perkin-Elmer Cetus Co (Norwalk, CT) and mouse Cytokine MAPPing Amplimers (GM-CSF, β-actin) were products of Clontech Laboratories Inc (Palo Alto, CA). Fetal calf serum (FCS) was obtained from Sterile System, Inc (Logan, UT).

Cells and proliferation assay. Murine PEM were harvested by peritoneal lavage with 5 mL α-minimal essential medium (α-MEM) 3 days after one intraperitoneal (IP) injection of 1 mL thioglycolate medium (Difco Laboratories, Detroit, MI). Murine pulmonary alveolar macrophages (PAM) were collected by pulmonary lavage using a modified method of Brain and Frank. Briefly, mice were anesthetized with sodium pentobarbital and killed by exsanguination. A sterile polyethylene tube was inserted into the trachea and the lungs were washed with 5 mL of cold saline solution. The yield of cells varied from 1 × 10⁶ to 2.5 × 10⁷ per lavage, and over 90% to 95% of them were identified as macrophages. To obtain

From the Division of Hematology/Oncology, Department of Internal Medicine, Wayne State University School of Medicine, Detroit, MI. Submitted August 12, 1991; accepted November 18, 1991.

Supported by Public Health Service Grant CA 47424 awarded by the National Cancer Institute and AI 23499 awarded by the National Institute of Allergy and Infectious Diseases, Department of Health and Human Services.

Address reprint requests to Ben Chen, PhD, PO Box 02188, Wayne State University School of Medicine, Detroit, MI 48202.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/7907-0010$0.10
bone marrow macrophage precursor cells, we cultured bone marrow cells in T-75 Falcon flasks in 10 mL of warm aMEM containing 10% FCS (α-10). Two hours later, nonadherent (NA) cells were removed, washed, and resuspended in α-10. Thereafter, 10^5 NA cells per well were plated in 24-well Falcon culture plates (Becton Dickinson, Oxnard, CA). After incubating for an additional hour, cells were further washed with warm α-10 to remove NA cells. Over 90% of the cells attached to the wells (approximately 2 x 10^5/well) displayed monocyte-like morphology and were considered as bone marrow-derived macrophage (BMDM) precursors.

Proliferation assays were performed by incubating 2 x 10^3 cells/well in 1 mL of α-10 containing either 0.5 ng/mL rMuGM-CSF or 100 ng/mL rHuM-CSF and various concentrations of rHuTGF-β1 in triplicate. After incubation for 7 to 9 days at 37°C in a humidified incubator with 7.5% CO₂ in air, the number of macrophage progeny in cultures containing rHuTGF-β1 in addition to rMuGM-CSF. The enhancing effect of rHuTGF-β1 is dose-dependent; as little as 0.01 ng/mL of rHuTGF-β1 was sufficient to induce a synergistic response in PEM to rMuGM-CSF (Fig 1B). Contrarily, the proliferative response of PEM to rHuM-CSF (100 ng/mL) was inhibited by its presence, also in a dose-dependent manner (Fig 2).

Such dichotomous effects of TGF-β1 on cell proliferation was inhibited by its presence, also in a dose-dependent manner (Fig 2).

RESULTS

TGF-β1 synergizes with GM-CSF to stimulate macrophage proliferation. The effect of rHuTGF-β1 on the proliferation by murine PEM in response to either rMuGM-CSF or rHuM-CSF was examined. As shown in Fig 1A, TGF-β1 alone did not have growth stimulating activity on PEM. However, when combined with rMuGM-CSF it greatly enhanced their proliferative response to rMuGM-CSF. On day 9 of incubation, there was a twofold increase in total number of macrophage progeny in cultures containing rHuTGF-β1 in addition to rMuGM-CSF. The enhancing effect of rHuTGF-β1 is dose-dependent; as little as 0.01 ng/mL of rHuTGF-β1 was sufficient to induce a synergistic response in PEM to rMuGM-CSF (Fig 1B). Contrarily, the proliferative response of PEM to rHuM-CSF (100 ng/mL) was inhibited by its presence, also in a dose-dependent manner (Fig 2).
TGF-β1 AND MACROPHAGE PROLIFERATION

125

- n

100

50

25

0

25

0

0.1 1.0

0.0 0.01 0.05 0.1

0.0 0.001 0.01 0.5 1.0

A

rHuTGF−β1 (ng/ml)

Cell Number (x10^3/ml)

Fig 2. Antiproliferative effect of TGF-β1 on PEM growth in response to M-CSF. C3H/HeJ PEM (2,000/well) were incubated in the presence of 0.5 ng/mL rMuGM-CSF or 100 ng/mL rHuM-CSF plus rHuTGF-β1 as indicated for 9 days at 37°C, cell numbers were determined using a Coulter Counter (Hialeah, FL). (A) Bifunctional effects of TGF-β1 on PEM growth stimulated by GM-CSF and M-CSF, respectively. (B) Dose dependence of TGF-β1 inhibition on PEM growth induced by M-CSF.

are not limited to PEM. In separate experiments, we examined the effect of TGF-β1 on proliferation of murine alveolar macrophages and BMDM, which also respond to both GM-CSF and M-CSF. As shown in Fig 3, proliferation of both PAM and BMDM were also regulated by rHuTGF-β1 in a manner similar to that of PEM. However, the sensitivities of their responsiveness to TGF-β1 appeared to be less than that of PEM.

TGF-β1 upregulates GM-CSF receptors. The preceding experiments demonstrated that TGF-β1 is a bifunctional regulator of macrophage proliferation. Depending on the types of growth factors, it can act either as a stimulator or as an inhibitor. To better understand the regulatory mechanisms of TGF-β1 in this process, we asked whether the action of TGF-β1 may be mediated at the receptor level. We treated PEM with rHuTGF-β1 for various time periods, after which the levels of both GM-CSF and M-CSF receptors were determined. As shown in Fig 4A, treatment of PEM with rHuTGF-β1 for 24 hours resulted in an enhanced specific binding of ^[125]I-rMuGM-CSF to PEM by more than 80%. However, under the same experimental conditions specific binding of ^[125]I-rHuM-CSF to PEM was not affected even at the highest dose of rHuTGF-β1 used in this study (Fig 4B). Prolonged treatment for up to 72 hours also failed to suppress the levels of M-CSF receptors (data not shown). The effect of TGF-β1 on the upregulation of GM-CSF receptors was detected within 4 hours after treatment and reached a maximal level 24 hours later (Fig 5). Scatchard plot analysis was performed to determine whether TGF-β1 affects GM-CSF receptor affinity or the number of receptors in PEM. As shown in Fig 6, TGF-β1 treatment led to an increased number of GM-CSF receptors in PEM without changing their affinity (kd = 34 pmol/L in both treated and control groups). The number of GM-CSF receptors in PEM after rHuTGF-β1 (1.0 ng/mL) treatment for 24 hours increased from 7,200 to 14,200/cell. In addition, we also detected some low-affinity GM-CSF receptors in PEM as the concentrations of the iodinated ligand was raised to over 140 pmol/L. However, the number of low-affinity GM-CSF receptors did not change appreciably after TGF-β1 treatment.

TGF-β1 does not induce GM-CSF synthesis in PEM. We have shown previously that rMuGM-CSF can upregulate its own receptors in PEM (unpublished data, May 1991). To rule out the possibility that the synergistic effect of TGF-β1 seen in the preceding experiments is caused indirectly by autocrine GM-CSF produced by treated PEM, we used the PCR technique to determine whether or not TGF-β1

Fig 3. Comparison of TGF-β1 effects on PEM, PAM, and BMDM growth. C3H/HeJ PEM, PAM, and BMDM (approximately 2,000/well) were incubated in the presence of 0.5 ng/mL rMuGM-CSF or 100 ng/mL rHuM-CSF plus rHuTGF-β1 at a concentration of 1.0 ng/mL for 9 days at 37°C. Data represent means from two identical experiments.
hematopoietic cells are regulated by multiple CSFs and cytokines, we investigated whether the action of TGF-β1 in this process is dependent on the types of exogenous CSFs. This study was performed using murine PEM and other types of tissue macrophages, which we have shown previously that display multiple types of CSF receptors and can be stimulated by each one of them to undergo clonal growth in vitro. Our results demonstrate that TGF-β1 is an important regulator of macrophage proliferation. However, the action of TGF-β1 is strictly dependent on the type of CSF present. It enhances the proliferative response of PEM to rMuGM-CSF yet inhibits their responsiveness to lineage-specific rHuM-CSF. These findings provide a model of bifunctional regulation of TGF-β in controlling macrophage proliferation and differentiation. It’s noteworthy that the bifunctional effects of TGF-β have been noticed in other cell types as well.5,7

In addition to PEM, TGF-β1 also exerts similar but less effects on the growth of PAM and BMDM. The biologic basis for the differential sensitivity to TGF-β1 among different types of tissue macrophage are not known at present. Because inflammatory macrophages such as PEM represent a more mature cell population than either resident alveolar macrophages or BMDM, our findings imply that the action of TGF-β1 may also depend on the degree of macrophage differentiation. One possibility is that TGF-β1 induces a “de-differentiation” on the more mature PEM and render them more susceptible to the stimulation
treatment induces expression of GM-CSF transcripts in PEM. As shown in Fig 7, no GM-CSF transcripts were detected in PEM cultures with or without rhuTGF-β1 treatment. On the other hand, expression of GM-CSF mRNA was readily detected in PEM that had been treated with rMuIL-1β (10 ng/mL) for 24 hours.

DISCUSSION

Previous studies have shown that TGF-β is a potent growth inhibitor when tested in normal hematopoietic progenitor cells and several established leukemic cell lines.18-23 Because the proliferation and differentiation of

---

**Fig 4.** Effect of TGF-β1 on specific binding of radiolabeled GM-CSF and M-CSF to PEM. C3H/HeJ PEM (2 x 10^6/mL) were treated with various amounts of rhuTGF-β1 as indicated or medium alone for 24 hours. Cells were washed with cold PBS and replenished with cold binding buffer containing 20,000 cpm/well 125I-rMuGM-CSF (A) or 100,000 cpm/well 125I-rHuM-CSF (B), respectively. Equilibrium binding for both receptors was performed as described in Materials and Methods. Specific bindings in control cultures were 800 ± 67 cpm for GM-CSF receptors and 3,750 ± 208 cpm for M-CSF receptors, respectively. Data are means ± SD of triplicate determinations.

**Fig 5.** Time course of TGF-β1 upregulating specific GM-CSF receptor binding. C3H/HeJ PEM (2 x 10^6/mL) were treated with rhuTGF-β1 (1.0 ng/mL) or medium alone at 37°C for various time periods. The specific 125I-rMuGM-CSF binding was determined as described under Materials and Methods. Data are means of triplicate cultures from one representative experiment.
of GM-CSF, which is a less lineage-restricted growth factor. In supporting this view, recent studies have shown that TGF-β is a potent macrophage deactivation factor. It is also of relevance to mention that TGF-β was originally identified by its ability to induce anchorage independent growth of several non-neoplastic cell lines in vitro.

The effects of TGF-β1 on proliferation of bone marrow CFU-GM have been reported by several investigators. Strassmann et al. found that TGF-β inhibited CSF-1 (M-CSF)-dependent macrophage precursor proliferation in a manner similar to that of tissue-derived macrophage. Hampson et al. also reported that TGF-β inhibits the colony formation by progenitors recruited by the lineage restricted factor, M-CSF, but to a lesser degree by the bipotential progenitors recruited by GM-CSF. In a similar study, Aglietta et al. reported that TGF-β1 stimulates the growth of day 7 CFU-GM in normal bone marrow cells but not that of day 14 CFU-GM. Ottmann and Pelus also reported that rHuTGF-β1 enhanced the growth of human CFU-GM stimulated by rHuG-CSF, rHuGM-CSF, and rHuIL-3. Additionally, Keller et al. reported that TGF-β promotes a threefold to fivefold increase in the size of bone marrow CFU-GM colonies in the presence of murine GM-CSF, due to an increase in the number of mature granulocytes and a concomitant decrease in the number of monocyte-macrophages. Our study extends the action of TGF-β1, as a hematopoietic regulator, to include the more mature tissue-derived macrophage precursors. Compared with their bone marrow counterparts, tissue macrophages offer several advantages: (1) they are unipotential and give rise to macrophage progeny only, regardless of the types of growth factors used, and (2) they represent a relatively pure and homogeneous cell population, thus, minimizing possible accessory cell effects.

Receptor binding assays using iodinated rMuGM-CSF and rHuM-CSF showed that TGF-β1 treatment enhances the expression of GM-CSF receptors but not that of M-CSF receptors (Fig 4). Scatchard analysis shows that TGF-β1 does not change GM-CSF receptor affinity (both $k_d = 34$ pmol/L) but increases the number of receptor sites. Although some low affinity GM-CSF receptors were detected in PEM as the concentration of the binding ligand was raised to over 140 pmol/L, the number of these receptor sites did not appear to change after TGF-β1 treatment. The upregulation of GM-CSF receptors in PEM by TGF-β1 is both time- and dose-dependent and correlated well with their growth phenotype, thus suggesting a possible mechanism for the synergistic activity of TGF-β1 in promoting the macrophage proliferative responses to rMuGM-CSF. On the other hand, recent studies by others have demonstrated that TGF-β1 inhibits not only the expression of IL-3, but also...
GM-CSF, and G-CSF receptors, but cell proliferation as well in some murine factor-dependent myeloid progenitor cell lines.20,21 Thus, the regulatory effect of TGF-β on the expression of GM-CSF receptors in normal cells may differ from that in established cell lines. Current thoughts favor the concept that there is a hierarchical interaction between distinct types of growth factor receptors. Conceivably, such interaction may be defective or nonexistent in leukemic and transformed cell lines so that they are unable to respond properly to the action of TGF-β. On the other hand, the levels of M-CSF receptors were not affected by TGF-β treatment, indicating that the inhibitory effect of TGF-β on the mitogenic effect of M-CSF may involve the postreceptor metabolic events downstream the signaling transduction pathway.

We have shown recently that rMuGM-CSF upregulates its own receptors in PEM (unpublished observation, May 1991). The fact that no GM-CSF transcripts are detected in either normal or TGF-β-treated PEM rules out the possibility that autocrine production of GM-CSF is responsible for the synergistic effect seen in this study. Interestingly, although IL-1β treatment induces GM-CSF mRNA expression in PEM, it does not have any growth modulating effect on PEM. These findings further suggest that TGF-β acts directly on PEM in transducing its biologic effects.

Similar to other cytokines, TGF-β1 acts on the target cells by binding to specific receptors, which have been identified on essentially all cell types including macrophages. Three types of TGF-β receptors have been identified based on their ability to interact with radiolabeled ligand.22-24 The underlying mechanisms by which TGF-β1 upregulates GM-CSF receptors are not known. Several lines of evidence suggest that both human and mouse GM-CSF receptors are composed of at least two subunits, i.e., α- and β-subunits.25,26 We found that TGF-β1 only affects the number of GM-CSF receptor sites but not their affinity, similar to that reported by Keller et al20 using normal bone marrow cells. It is likely that TGF-β1 promotes α-subunit gene expression instead of that of β-subunit. Unlike human GM-CSF receptors, no direct competition for receptor binding between IL-3 and GM-CSF has been reported in the mouse system, suggesting that these two receptor systems do not share a common β unit and that the latter is present in abundance. This notion is also supported by the fact that over 6,000 high-affinity GM-CSF receptors per cell have been detected in PEM. Experiments are in progress to determine whether TGF-β regulates gene expression through cis-regulation or by stabilizing the existing cytoplasmic GM-CSF receptor mRNA in this process.

REFERENCES

5. Wilcox JN, Derynick R: Developmental expression of transforming growth factors α and β in mouse fetus. Mol Cell Biol 8:3415, 1988


Transforming growth factor-beta 1 bifunctionally regulates murine macrophage proliferation

K Fan, Q Ruan, L Sensenbrenner and B Chen