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MODULATION OF GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTORS ON MURINE PERITONEAL EXUDATE MACROPHAGES BY TUMOR NECROSIS FACTOR-α[1]

JAE-HUNG SHIEH,[2] ROBERT H. F. PETERSON, AND MALCOLM A. S. MOORE

From The James Ewing Laboratory of Developmental Hematopoiesis, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

Modulation of granulocyte CSF (G-CSF) receptors on murine peritoneal exudate macrophages (PEM) by various cytokines was investigated. At 4°C, ^{125}I-G-CSF receptor binding on PEM reached a plateau after 6 h and was specifically competed by unlabeled human rG-CSF but not by other cytokines, including human rCSF-1, murine recombinant granulocyte-macrophage CSF, murine rIFN-γ, human rIL-1β, and murine rTNF-α. ^{125}I-G-CSF bound to PEM has a half-life of 30 min at 37°C. Preincubation of PEM with murine rTNF, murine recombinant granulocyte-macrophage CSF, CSF-1, or G-CSF for 30 min at 37°C resulted in partial reduction of ^{125}I-G-CSF binding capacity, whereas IL-1 or IFN-γ did not inhibit G-CSF binding. Further studies indicated that reduction of G-CSF binding caused by TNF was a dose- and time-dependent process and did not require FCS. The reduction was transient, and receptor binding was recovered by incubation at 37°C for 8 h. The recovery of G-CSF binding was inhibited in the presence of cycloheximide. In addition, G-CSF binding studies suggested that the TNF-induced decrease in G-CSF binding to PEM was probably due to a reduction in receptor number rather than receptor affinity. Modulation of G-CSFR by TNF was also observed on nonelicited macrophages from various strains of mice. Our results demonstrate a physiologic response of G-CSFR on macrophages that is modulated by TNF. This phenomenon may play an important, as yet unknown, role in the macrophage inflammatory response.

G-CSF[3] is a glycoprotein growth factor required for proliferation, differentiation, and survival of progenitors and for activation of various factors of mature neutrophils (1, 2). This growth factor is synthesized by activated macrophages, endothelial cells, fibroblast cells, placental tissue, and various tumor cell lines (2–4). Human G-CSF is also known to cross-react with the murine G-CSFR, and vice versa (1, 2). G-CSFR have been identified on neutrophils, monocytes, bone marrow cells, human myeloid leukemia cells, small cell lung cancer cell lines, endothelial cells, and trophoblastic cells (5–8). Recently, a receptor for murine G-CSF has been cloned (9). Besides the biologic action of G-CSF on the granulocytic lineage in vivo and in vitro, G-CSF also elicits a chemotatic response in monocytes (10), induces the differentiation of myeloid leukemic cell lines (WEHI-3D$^+$ and HL-60) and blast cells from acute myeloid leukemia patients (1, 11), and enhances GM-CSF induction of monocyte/macrophage differentiation of U-937 cells in vitro (12).

Monocytes/macrophages, a major source of G-CSF, display receptors for various cytokines, such as CSF-1, IFN-γ, GM-CSF, and TNF, and respond to each factor with a spectrum of biologic responses (13–17). Available data also indicate that monocytes/macrophages display G-CSFR and may also respond to G-CSF with various biologic functions (10, 11). In this study, we examined the effect of various cytokines on G-CSFR expression on murine peritoneal macrophages.

MATERIALS AND METHODS

Growth factors. Murine rTNF and rIFN-γ were gifts from Dr. M. A. Palladino, Genentech Inc. (San Francisco, CA). Human rCSF-1 was kindly supplied by Dr. P. Ralph, Cetus Corp. (Emeryville, CA). Human rIL-1α, human rIL-1β, and murine rGM-CSF were provided by Immunex Co. (Seattle, WA). *Escherichia coli*-derived human rG-CSF was from Amgen Inc. (Thousand Oaks, CA).

Animals. C3H/HeJ, BALB/cBy, and B6D2F$_1$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in laminar air-flow rooms under specific pathogen-free conditions and were used at ages ranging from 8 to 12 weeks.

Tissue culture media. RPMI 1640 medium (GIBCO, Grand Island, NY) was supplemented with 2.2 g/ml $NaHCO_3$ and 20 mM HEPES, pH 7.2. A single lot of FCS (KC Biologicals, Lenoxa, KS) was used throughout the present study. Dissociation buffer contained 120 mM NaCl and 100 mM acetic acid, pH 4.0 (13).

Cell cultures and cell preparations. NFS-60 cells were obtained from Dr. Ihle (NCI-Frederick Cancer Research Facility, Frederick, MD) and cultured in RPMI 1640 medium supplemented with 10% FCS and 10% WEHI-3 conditioned medium.

PEM were harvested 2 days after i.p. injection of 0.5 ml of thioglycollate medium (3 mg/ml) (Difco Laboratories, Detroit, MI) into C3H/HeJ mice. Cells were washed twice with cold PBS, resuspended in cold RPMI 1640 medium with 15% FCS, and adjusted to a cell density of 4×10^5 cells/ml. One-milliliter aliquots of the cell suspension were seeded in 24-well plates. After a 1-h incubation, nonadherent cells were removed by two washes with 400 μl of cold PBS. Before binding assays were performed, adherent cells were cultured overnight in RPMI 1640 medium with 10% FCS. The procedure for obtaining nonelicited macrophages was similar to that for PEM, except that nonadherent cells were removed after a 2-h incubation. More than 95% of the adherent cells were identified as mononuclear

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[2] Address all correspondence and requests for reprints to Jae-Hung Shieh, The James Ewing Laboratory of Developmental Hematopoiesis, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021.
[3] Abbreviations used in this paper: G-CSF, granulocyte CSF; B_{max}, maximal binding sites; CSF-1, macrophage CSF; K_d, dissociation constant; GM-CSF, granulocyte-macrophage CSF; PEM, peritoneal exudate macrophages.

phagocytes by esterase stain and morphology. The cell number was determined by the cetrimide counting technique, as described by Stewart et al. (18). Briefly, cultures were depleted of medium by aspiration, washed twice with 400 μl of PBS, and replenished with 1 ml of warm cetrimide solution (30 g of cetrimide, 0.37 g of disodium EDTA, and 8.5 g of NaCl, in 1 liter of water), at 37°C for 5 min. Another 2 ml of cetrimide solution were used to completely remove and lyse the adherent cells. The intact nuclei were then counted in a hemocytometer.

Blood was collected from normal donors, and neutrophils were purified by mono-poly resolving medium (Flow Laboratory), as suggested by the manufacturer. This method yielded more than 97% neutrophils, as judged by morphology. Cell viability was greater than 98%, by trypan blue exclusion.

Iodination of G-CSF. *E. coli*-derived human rG-CSF was iodinated by the chloramine T method, as previous described, with minor modifications (19). Briefly, 35 μl of a reaction mixture containing 0.5 μg of human rG-CSF, 10% DMSO, 0.02% polyethylene glycol, 6 nmol of chloramine, 1 mCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston, MA), and 0.25 M sodium phosphate buffer, pH 6.8, were incubated on ice for 5 min. Thereafter, 6 μl of 1.1 nmol/μl cysteine and 10 μl of 0.2 M KI were added, and the reaction mixture remained on ice for 10 min. The reaction mixture was then desalted by passage over a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ), which was conditioned with 5 ml of 10 mg/ml BSA and 25 ml of eluting buffer containing 0.1 mg/ml BSA and 20 mM Tris-HCl, pH 6.8. The crude human ^{125}I-rG-CSF was further fractionated on a Sephracryl S-200 column, with a buffer containing 0.5 M NaCl, 0.02% NaN_3, 0.1 mg/ml BSA, and 20 mM Tris-HCl, pH 8.0. The purity of ^{125}I-G-CSF was determined by SDS-PAGE, as described by Laemmli (20). The specific activity of human ^{125}I-rG-CSF, which was usually between 150 and 330 μCi/μg, was determined by self-displacement analysis, and as described by Calvo et al. (21). The biologic activity of labeled G-CSF was measured by NFS-60 proliferation assay, with minor modifications (22). Briefly, NFS-60 cells in log phase were harvested, washed twice with RPMI 1640 medium, resuspended in RPMI 1640 medium supplemented with 0.2% FCS, and adjusted to 5×10^4 cells/ml. One-tenth milliliter of cell suspension/well, in a 96-well plate, was incubated in the presence or absence of various amounts of G-CSF (or labeled G-CSF) at 37°C for 2 days. The cultures were then pulsed with 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) for 6 h. A cell harvester (Cambridge Technology, Watertown, MA) was used to harvest DNA, with five washes with 250 μl of water. [^{3}H]Thymidine incorporation was measured in a liquid scintillation counter (LS-250; Beckman Instruments). All ^{125}I-G-CSF preparations were used within 2 weeks.

Receptor binding assays. Receptor binding assays on adherent macrophages were performed as described previously (23). To determine total binding, adherent cells in 24-well plates were washed twice with 400 μl of cold PBS, followed by the addition of 140 μl of cold binding buffer containing RPMI 1640 medium (without sodium bicarbonate), 2 mg/ml BSA, 0.02% NaN_3, 20 mM HEPES, pH 7.3, and 10 μl of ^{125}I-G-CSF (approximately 50,000 cpm). The reaction mixtures were then incubated at 4°C for 6 h with gentle shaking. After incubation, the unbound ^{125}I-G-CSF was aspirated, and the cells were washed twice with 400 μl of cold PBS and solubilized with 400 μl of membrane buffer containing 10% glycerol, 1% Triton X-100, and 20 mM HEPES, pH 6.8 (24), at room temperature for 2 h. Total binding was determined by measurement of the radioactivity of the resulting lysates in a γ counter (Gamma 310; Beckman Instrument Inc., Fullerton, CA), with counting efficiency of 70%. Nonspecific binding (<15% of total binding) was determined by preincubation of the cells with a 100-fold excess of unlabeled G-CSF at 4°C for 30 min before addition of ^{125}I-labeled growth factor. Specific binding was defined as total binding minus nonspecific binding. Receptor binding on nonadherent cells was assayed as described by Nicola and Metcalf (6). Briefly, NFS-60 cells or neutrophils were incubated with ^{125}I-G-CSF, in the presence or absence of unlabeled G-CSF, at 4°C for 8 h. After incubation, the reaction mixtures were layered onto 200 μl of cold FCS in a 400-μl centrifuge tube and centrifuged at 400 × *g* for 5 min. The tips were excised and counted in a γ counter. Specific binding activity was defined as described above. All binding data were corrected for nonspecific binding and presented as mean ± SD ($n = 3$).

Internalization and degradation studies of G-CSF on PEM surfaces. PEM were incubated with ^{125}I-G-CSF at 4°C for 8 h and then washed twice with 400 μl of cold PBS, to remove unbound ^{125}I-G-CSF. The PEM bound with labeled G-CSF were further incubated at 37°C for various time periods, depleted of medium, and washed twice with 400 μl of cold PBS. The medium and the washes were saved, pooled, adjusted to a final concentration of 10% TCA, and incubated at 4°C overnight. These mixtures were further fractionated into TCA-insoluble fractions, representing intact ^{125}I-G-CSF, and TCA-soluble fractions, representing degraded ^{125}I-G-CSF. The PEM were treated with dissociation buffer at 4°C for 30 min and resolved into acid-dissociable fractions, representing ^{125}I-G-CSF bound to PEM surfaces, and acid-nondissociable fractions, representing ^{125}I-G-CSF embedded in membranes and/or internalized into the cells.

Data analysis. Equilibrium binding data were analyzed by LIGAND, as modified for the IBM PC by McPherson (25).

RESULTS

Homogeneity, receptor binding activity, and biologic activity of ^{125}I-G-CSF. Because Uzumaki et al. (26) and Nicola and Metcalf (6) reported difficulty in obtaining ^{125}I-G-CSF that retained full biologic activities and receptor binding capacity, the quality of ^{125}I-G-CSF prepared in this study was tested for homogeneity, receptor binding activity, and biologic activity. The ^{125}I-G-CSF appeared as a single band in autoradiograms of SDS-PAGE gels, suggesting a homogeneous preparation of iodinated human rG-CSF (data not shown). To determine whether iodinated G-CSF retained receptor binding capacity, the binding assay was performed with murine NFS-60 cells and human neutrophils, which display G-CSFR (26–28). ^{125}I-G-CSF binding to both NFS-60 cells and human neutrophils was competed for by unlabeled G-CSF, in a dose-dependent manner (Fig. 1).

From self-displacement analysis (21), the radiospecific activity of ^{125}I-G-CSF was approximately 3.6×10^8 cpm/μg (Fig. 2*A*). The recovery of bioactivity of labeled G-CSF was further measured by the NFS-60 proliferation assay (22). As shown in Fig. 2*B*, the ability of 1.9×10^3 cpm of ^{125}I-G-CSF to induce a half-maximal proliferation in NFS-60 cells was equivalent to that of 4 pg of unlabeled G-CSF, suggesting that ^{125}I-G-CSF retained more than 75% of the biologic activity. These results indicated that ^{125}I-G-CSF was homogeneous and retained biologic activity, as well as receptor binding capacity.

Time course of G-CSF receptor binding at 4°C and 37°C. To study the kinetics of G-CSF binding on PEM, PEM were incubated with ^{125}I-G-CSF (5×10^4 cpm), in the presence or absence of unlabeled G-CSF (20 ng/ml), at 4°C and 37°C. As shown in Fig. 3, G-CSF binding on PEM reached an equilibrium within 6 h and >50% equi-

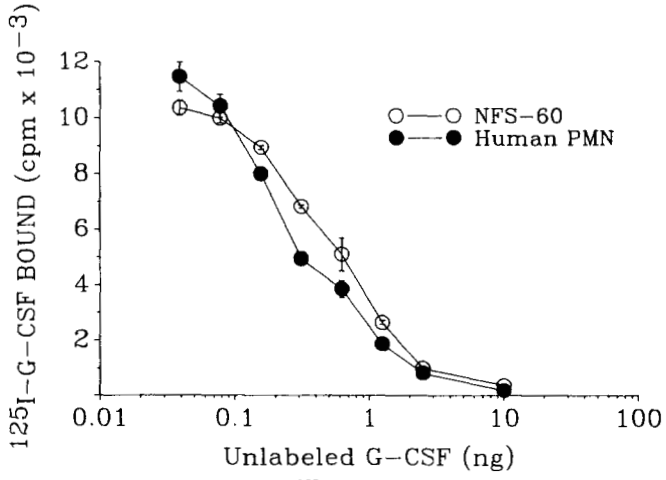

Figure 1. Competition with ^{125}I-G-CSF binding to NFS-60 cells and human neutrophils by unlabeled G-CSF. Human neutrophils and NFS-60 cells were prepared as described in *Materials and Methods*. Two million cells in 120 μl of binding buffer were incubated with ^{125}I-G-CSF (10^5 cpm), in the absence or presence of various amounts of unlabeled G-CSF, at 4°C for 8 h. After incubation, the unbound and the bound ^{125}I-G-CSF were separated as described in *Materials and Methods*.

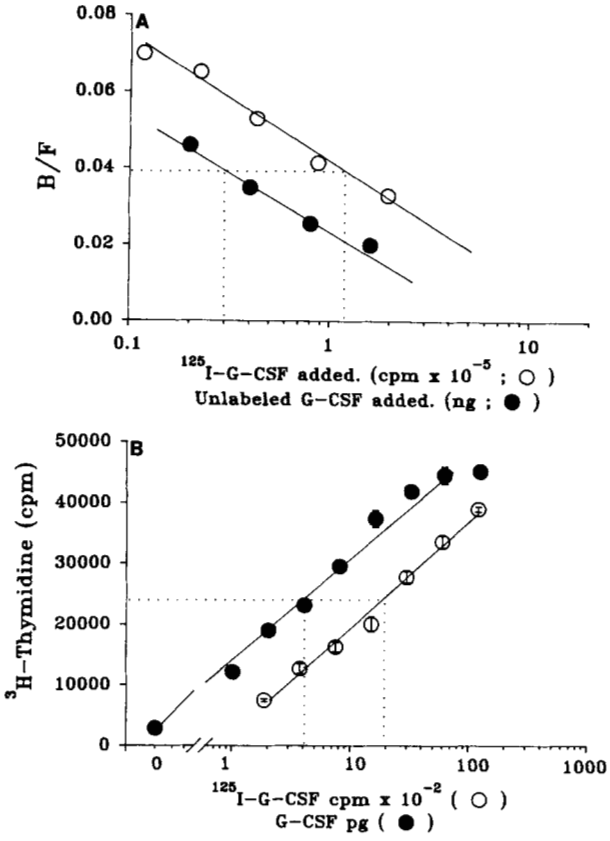

Figure 2. Characteristics of iodinated G-CSF. *A*, determination of the specific radioactivity of ^{125}I-G-CSF. Either various amounts of ^{125}I-G-CSF (12,000 to 192,000 cpm) or ^{125}I-G-CSF (45,000 cpm) in the presence of increasing amounts of unlabeled G-CSF (up to 1.6 ng) were added to 10^6 NFS-60 cells and incubated for 8 h at 4°C. Specific binding was determined and plotted against the bound/free (*B/F*) ratio. The specific radioactivity was determined as the amount of radioactivity divided by the amount of unlabeled G-CSF added to obtain the same bound/free value. *B*, ability of iodinated G-CSF and unlabeled G-CSF to stimulate proliferation in NFS-60 cells. Proliferation assays were carried out as described in *Materials and Methods*.

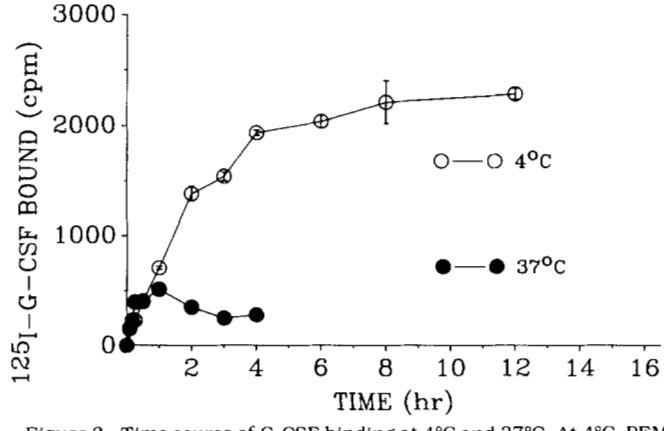

Figure 3. Time course of G-CSF binding at 4°C and 37°C. At 4°C, PEM in 140 μl of receptor binding buffer were incubated with ^{125}I-G-CSF (5×10^4 cpm), in the presence or absence of a 100-fold excess of unlabeled G-CSF, for various time periods. After incubation, ^{125}I-G-CSF bound to PEM was determined as described in *Materials and Methods*. At 37°C, the binding assay was performed as described for that at 4°C, except that the receptor binding buffer was RPMI 1640 medium with 5% FCS, 0.2% NaN_3, 2.2 g/liter $NaHCO_3$, and 20 mM HEPES, pH 7.2.

librium binding within 2 h at 4°C. In contrast, G-CSF receptor binding on PEM at 37°C reached a maximum (approximately 500 cpm) within 1 h and gradually decreased to about 300 cpm. The mechanism for the discrepancy in binding between 4°C and 37°C remains unclear. Because ^{125}I-G-CSF receptor binding on PEM at 4°C was 3.5-fold higher than that at 37°C, binding assays in this study were performed at 4°C.

Specificity of G-CSF binding on PEM. To examine the specificity of G-CSF binding on PEM at 4°C, PEM in 0.11 ml of binding buffer were incubated in the presence or absence of various cytokines (100 ng/ml) at 4°C for 30 min before addition of 10 μl of ^{125}I-G-CSF (5×10^4 cpm), and the binding assay was performed as described in *Materials and Methods*. The results showed that >90% of ^{125}I-G-CSF binding to PEM could be competed for by unlabeled G-CSF but not by other cytokines tested, including CSF-1, murine rGM-CSF, murine rIFN-γ, murine rTNF, and IL-1α (data not shown). To titrate ^{125}I-G-CSF binding to PEM by using unlabeled G-CSF, PEM in 0.12 ml of binding buffer were incubated with ^{125}I-G-CSF (5×10^4 cpm), in the absence or presence of various doses of unlabeled G-CSF, at 4°C for 8 h. The results showed that 100 ng/ml G-CSF inhibited >89% of ^{125}I-G-CSF binding to PEM; 25 ng/ml, >74%; 12.5 ng/ml, >60%; 6.25 ng/ml, 40%; 3.125 ng/ml, >28%; and 1.56 ng/ml, 16%, suggesting that this competition is a dose-dependent process. These two studies indicated the specificity of ^{125}I-G-CSF binding to PEM.

Internalization and degradation of G-CSF by PEM. The fate of G-CSF after binding to PEM was examined by analysis of the distribution of radioactivity in the medium, on the cell surface, and inside the cells. PEM were incubated with ^{125}I-G-CSF at 4°C for 8 h, washed to remove unbound ^{125}I-G-CSF, and further incubated at 37°C for various time periods. The radioactivity of various fractions was analyzed as described in *Materials and Methods*. The results (Fig. 4) showed that approximately 50% of bound G-CSF disappeared from the cell surface within 30 min, and more than 90% after 8 hr. Intact G-CSF in the medium increased from 400 to 850 cpm within 15 min and then gradually reached a plateau (approximately 1000 cpm), suggesting that some ^{125}I-G-CSF was rapidly dissociated from the cell surface in the first 15 min. The degraded ^{125}I-labeled material was identified in the medium after 30 min and reached a plateau after 8 h. On the other hand, the ^{125}I radioactivity associated

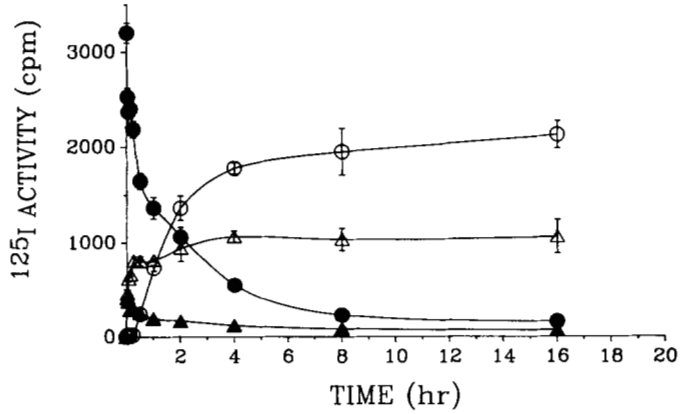

Figure 4. Time course of internalization and degradation of ^{125}I-G-CSF on PEM. PEM (2×10^5 cells/well) were incubated with ^{125}I-G-CSF (10^5 cpm) at 4°C for 8 h. After incubation, cultures were washed twice with 400 μl of cold PBS and incubated in 400 μl of RPMI 1640 medium supplemented with 10% FCS, at 37°C. The distribution of ^{125}I-G-CSF and its metabolites was analyzed at the indicated times, as described in *Materials and Methods*. All data are presented as mean ± SE ($n = 2$). ○, TCA-soluble fractions; ●, acid-dissociable fractions; △, TCA-insoluble fractions; ▲, acid-nondissociable fractions.

with PEM was approximately 7–8% of total bound G-CSF and gradually decreased.

Effect of various cytokines on G-CSF receptor binding to PEM at 37°C. To investigate the modulation of G-CSFR on PEM by various cytokines, PEM were incubated in the presence or absence of various cytokines at 37°C for 30 min. Subsequently, receptor binding assays were carried out as described in *Materials and Methods*. The data (Fig. 5) show that 20 ng/ml G-CSF, TNF, CSF-1, and GM-CSF caused approximately 94, 75, 45, and 32% reduction in G-CSF binding, respectively. This reduction was also a dose-dependent process. In contrast, IL-α and IFN-γ did not modulate the G-CSF binding. Among the cytokines tested, TNF incubation resulted in a greater reduction in ^{125}I-G-CSF binding on PEM other than G-CSF incubations. The incubation of cytokines with PEM did not cause detachment of cells from the plates; therefore, the modulation of G-CSF binding capacity on PEM by TNF was further analyzed.

Serum contains a multiplicity of activities, which could synergize with TNF to modulate G-CSFR. To resolve this question, PEM were preincubated in the presence (control PEM) or absence (FCS-depleted PEM) of 10% FCS at 37°C for 1 h before TNF treatment. The data (Table I) showed that FCS did not affect, nor was it necessary for, the action of TNF.

Time course of G-CSFR modulation by TNF. In order to study the kinetics of G-CSFR modulation by TNF at 37°C, PEM were incubated in the absence (control PEM) or presence of TNF. After a 15-min incubation, the PEM were either maintained in the presence of TNF (TNF-treated PEM) or washed and incubated with RPMI 1640 medium supplemented with 10% FCS (TNF-preincubated PEM). As shown in Fig. 6, more than 70% of ^{125}I-G-CSF binding on TNF-treated PEM was eliminated within 15 min, relative to that of control PEM. After 8 h, more than 84 and 95% of G-CSF binding was restored on TNF-treated and TNF-preincubated PEM, relative to that of control PEM, respectively. The G-CSF binding on TNF-treated PEM was 70% of that on TNF-preincubated PEM after a 24-h incubation.

Comparison of binding on control PEM and TNF-treated PEM. The reduction of G-CSF receptor binding on PEM by TNF may be due to a reduction in receptor number or a change in receptor affinity. To resolve this question, the receptor binding of G-CSF on TNF-treated PEM and control PEM was compared. The binding results (Fig. 7) revealed that G-CSFR on control PEM showed a B_{max} of 900/cell, with an apparent K_d of 40 pM, and receptors on TNF-treated PEM showed a B_{max} of 290 sites/cell, with a K_d of 65 pM. Therefore, the decrease in G-CSF binding was probably due to reduction in receptor number rather than a change in receptor affinity.

Effect of cycloheximide on the recovery of G-CSF

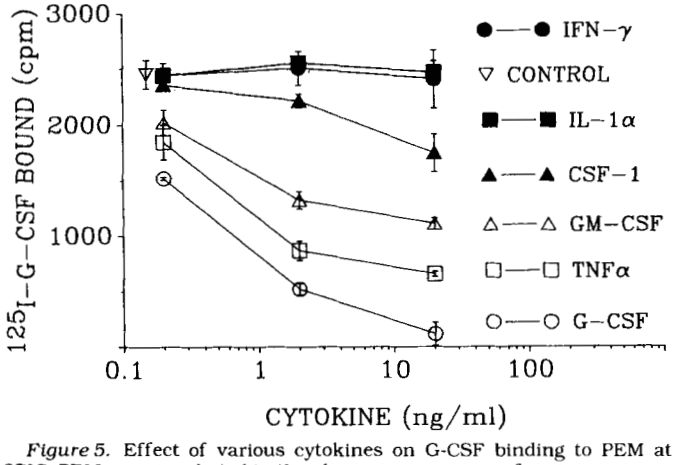

Figure 5. Effect of various cytokines on G-CSF binding to PEM at 37°C. PEM were incubated in the absence or presence of various concentrations of cytokines at 37°C for 30 min. After incubation, receptor binding was assayed at 4°C, as described in *Materials and Methods*.

TABLE I

Effect of serum on modulation of G-CSF binding by TNF[a]

TNF (ng/ml)	^{125}I-G-CSF bound (cpm)	
	Control PEM	FCS-depleted PEM
0	3905 ± 77	3807 ± 107
0.02	3887 ± 19	3816 ± 44
0.2	2697 ± 19	2878 ± 144
2	1637 ± 17	1965 ± 114
20	1398 ± 128	1465 ± 63

[a] PEM were depleted of medium, washed twice with 400 μl of cold PBS, and replenished with RPMI 1640 medium, in the presence (control PEM) or absence 10% of FCS (FCS-depleted PEM), at 37°C for 1 h. The PEM were then incubated, in the absence or presence of various concentrations of TNF, at 37°C for 30 min. Receptor binding assays were performed as described in *Materials and Methods*.

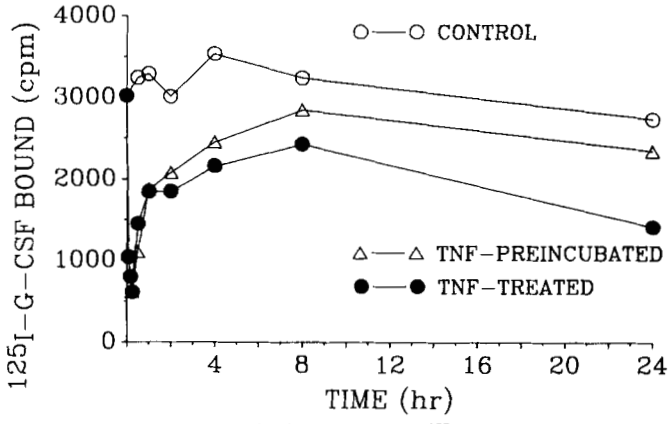

Figure 6. Time course of effects of TNF on ^{125}I-G-CSF binding to PEM. After incubation with TNF at 37°C for 15 min, PEM were continuously exposed to TNF (TNF-treated PEM) or washed free of residual TNF (TNF-preincubated PEM). The ^{125}I-G-CSF binding activities of control, TNF-treated, and TNF-preincubated PEM were compared at the indicated times. Receptor binding assays were performed at 4°C, as described in *Materials and Methods*.

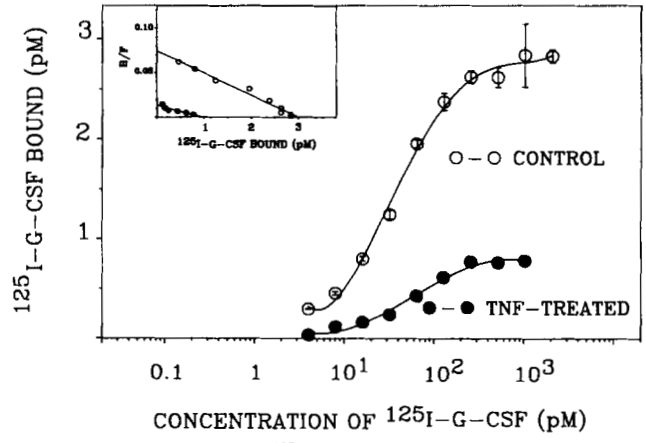

Figure 7. Comparison of ^{125}I-G-CSF binding to control and TNF-treated PEM. After preincubation in the presence or absence of TNF (20 ng/ml) at 37°C for 30 min, PEM (2.8×10^5 cells/well) were washed twice with 400 μl of cold PBS. Binding assays were performed at the indicated ^{125}I-G-CSF concentrations. *Inset*, Scatchard analysis of binding data.

binding. To examine the role of protein synthesis in the recovery of G-CSF binding on TNF-treated PEM, the PEM were preexposed to TNF for 20 min and then incubated in the presence or absence of cycloheximide for 8 h at 37°C. The results (Table II) showed that 0.2 μg/ml cycloheximide suppressed more than 64% of the recovery of G-CSF binding on TNF-treated PEM. At 0.2 μg/ml cycloheximide, PEM did not detach from the plates and more than 95% of PEM were viable, as judged by trypan blue exclusion.

Modulation of G-CSFR on nonelicited macrophages. In the preceding studies, PEM were elicited by i.p. injection of thiolglycollate into C_3H/HeJ mice, an endotoxin-hyporesponsive strain of mice. This eliciting agent may introduce a nonphysiologic artifact in the macrophage population. Alternatively, the observed response may be influenced by the C3H/HeJ genotype. To clarify these points, the modulation of G-CSFR by TNF was also investigated with nonelicited peritoneal macrophages obtained from BDF, BALB/c, and C3H/HeJ mice. The results (Table III) indicated that 20 ng/ml TNF resulted in more than 70% reduction in G-CSF receptor binding to nonelicited peritoneal macrophages.

DISCUSSION

Our results showed that the binding between G-CSF and PEM is specific. After binding to PEM, ^{125}I-G-CSF is internalized, subsequently degraded to small molecules, and released into the medium (Fig. 4). We also demonstrated, for the first time, the modulation of G-CSFR on macrophages by TNF (Fig. 5 and Table III). This modulation was a temperature-, time-, and dose-dependent process (Figs. 5 and 6) and did not require FCS (Table I). The reduction of G-CSF binding caused by TNF was due to a decrease in receptor number rather than in receptor affinity (Fig. 7).

Possible mechanisms for this receptor modulation include 1) TNF occupying G-CSFR, 2) TNF directly transmodulating G-CSFR, or 3) TNF inducing PEM to secrete a component(s) that indirectly modulates G-CSFR. Because ^{125}I-G-CSF binding on PEM is specific at 4°C, direct receptor competition is excluded. Recently, several studies have shown that TNF induces macrophages to produce various cytokines, such as IL-1, CSF-1, G-CSF, and GM-CSF (14, 15, 29, 30). Neither IFN-γ nor IL-1 modulated the G-CSFR, suggesting that these two cytokines are not involved in this down-modulation. Conditioned medium from TNF-treated PEM at cell densities used in this study contained less than 0.1 ng of CSF-1, as judged by the receptor binding assay (23), and it requires 20 ng/ml CSF-1 to produce a 35% reduction in G-CSF binding. Thus, it is unlikely that modulation of G-CSFR is due to TNF-induced PEM secretion of CSF-1. Likewise, 20 ng/ml GM-CSF caused approximately a 45% reduction in G-CSF binding, and TNF induction of GM-CSF secretion is not evident at this level. As shown in Fig. 5, 1 ng/ml G-CSF may result in an approximately >75% reduction in ^{125}I-G-CSF binding. Conditioned medium from TNF-treated PEM contained less than 0.2 ng of G-CSF, as judged by receptor binding assays in neutrophils and NFS-60 (Fig. 1). In addition, incubation of TNF-treated PEM with dissociation buffer (pH 4) at 4°C for 30 min, to strip the surface cytokines, did not affect G-CSF binding (data not shown). It seems unlikely that TNF-induced PEM secret G-CSF to interfere with subsequent ^{125}I-G-CSF binding. However, one cannot totally rule out the possibility that TNF induction of PEM to secrete cytokine(s) is responsible for this modulation. Antibodies against various cytokines may help to resolve whether a direct or an indirect mechanism is operating. In this study, the possibility of TNF inducing PEM to secrete other metabolites, such as prostaglandins, remains unresolved.

Reduction of G-CSF binding on PEM by TNF was transient, and binding recovered after prolonged incubation at 37°C, even in the presence of TNF (Fig. 6). This recovery could be accelerated by removal of TNF. In contrast, cycloheximide, an inhibitor of protein synthesis, suppressed this recovery (Table II). Whether cycloheximide inhibits the synthesis of receptor or the synthesis of protein(s) required for receptor recycling remains unknown. We also observed that the incubation of PEM with G-CSF dramatically reduced G-CSF binding (Fig. 5). Recently, Nicola et al. (31) showed that both internalized ^{125}I-materials and surface-bound ^{125}I-G-CSF can be detected after a 30-min incubation of various cells with labeled G-CSF at 37°C. Therefore, G-CSF suppression of ^{125}I-G-CSF binding to PEM may be due to the combination of receptor down-modulation, receptor turnover, and occupancy.

TNF transmodulates both G-CSFR (Fig. 6) and CSF-1R on macrophages (23, 32). On the other hand, neither G-CSF nor CSF-1 modulates TNFR on PEM (23). It seems that TNF acts as a cytokine modulating its own receptors, G-CSFR, and CSF-1R on macrophages. In addition, i.v. injection of endotoxin into animals results in the secretion of TNF (360 ng/ml) after 1 h (33). A similar study conducted in humans showed that TNF levels in serum

TABLE II

Effect of cycloheximide on recovery of G-CSF binding[a]

Cycloheximide (μg/ml)	^{125}I-G-CSF bound (cpm)	
	Control PEM	TNF-treated PEM
0	2303 ± 212	2035 ± 112
0.00002	2316 ± 89	1932 ± 28
0.0002	2109 ± 103	1967 ± 51
0.002	2099 ± 159	1995 ± 186
0.02	2013 ± 10	1243 ± 23
0.2	1639 ± 21	732 ± 37
2	1429 ± 80	734 ± 63
20	1370 ± 35	531 ± 1
200	588 ± 27	ND

[a] PEM were preincubated in the absence (control PEM) or presence of 20 ng/ml murine rTNF-α (TNF-treated PEM) at 37°C for 15 min, depleted of medium, washed twice with 400 μl of cold PBS, replenished with RPMI 1640 medium supplemented with 10% FCS, in the absence or presence of various concentrations of cycloheximide, and incubated at 37°C for 8 h. Receptor binding assays were performed as described in *Materials and Methods*.

TABLE III

Effect of TNF on G-CSF binding to nonelicited macrophages from various strains of mice[a]

TNF (ng/ml)	^{125}I-G-CSF bound (cpm)		
	BDF	BALB/C	C3H/HeJ
0	1181 ± 77	1183 ± 9	1567 ± 103
0.2	1075 ± 9	1144 ± 53	1145 ± 19
2	594 ± 11	539 ± 37	601 ± 52
20	338 ± 58	379 ± 27	491 ± 15

[a] Cells were prepared as described in *Materials and Methods*. After preincubation with 20 ng/ml murine rTNF-α at 37°C for 20 min, the cells were depleted of medium and washed twice with cold PBS. Receptor binding assays were performed as described in *Materials and Methods*.

were 240 ± 70 pg/ml 1 to 2 h after LPS injection (34). Therefore, the modulation of G-CSFR and CSF-1R on macrophages by TNF is an immediate event during bacterial infection in vivo.

In addition to the modulation of G-CSFR on macrophages by TNF and GM-CSF (Fig. 4), Nicola et al. (28) showed that G-CSFR on human neutrophils are modulated by GM-CSF, bacterial LPS, and *N*-formyl-methionine-leucine-phenylalanine. Recently, modulation of G-CSFR on murine bone marrow cells by murine GM-CSF and murine IL-3 was reported (35). We also observed that TNF modulates G-CSFR on bone marrow cells (BALB/c mice) and NFS-60 cells at 37°C in vitro (data not shown). The modulation of G-CSFR on hematopoietic cells by other cytokines may play a significant, as yet unknown, role in the hematopoietic system and the inflammatory response.

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