

Independent Regulation of M-CSF and G-CSF Gene Expression in Human Monocytes

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The macrophage and granulocyte colony-stimulating factors, M-CSF and G-CSF, act in vitro to induce proliferation and differentiation of monocyte and granulocyte progenitor cells, respectively. We show here that both of these CSFs can be produced by stimulated human blood monocytes, but the M-CSF and G-CSF genes are independently regulated. Recombinant human interleukin-3 (IL-3) and GM-CSF primarily induce expression of the M-CSF gene

and secretion of M-CSF, whereas bacterial lipopolysaccharide primarily induces expression of the G-CSF gene and secretion of G-CSF. These results suggest that under different conditions of in vitro stimulation the monocyte secretes factors that could lead selectively to either granulocyte or monocyte production.

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GRANULOCYTES and monocytes are derived from common bone marrow progenitor cells that will proliferate in vitro in semisolid media to form colonies of granulocytes, monocytes, or both.¹ Proliferation of these colony-forming cells (CFU-GM) is dependent on addition of one or more glycoprotein growth factors termed colony-stimulating factors (CSFs).^{2,3} The four known human CSFs include interleukin-3 (IL-3), granulocyte/macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), and macrophage-CSF (M-CSF).^{2,3} IL-3 and GM-CSF induce proliferation in vitro of erythroid burst-forming cells and multipotent progenitor cells as well as CFU-GM. In contrast, the effects of G-CSF and M-CSF are much more restricted: G-CSF selectively induces formation of granulocyte colonies in vitro and granulocytosis in vivo, whereas M-CSF selectively supports proliferation of monocyte progenitor cells.^{2,3}

Several types of human cells produce one or more CSFs, including fibroblasts,⁴ endothelial cells,⁵ monocytes,⁶ and T lymphocytes.⁷ However, the factors that regulate secretion of CSFs are little understood. Despite the "opposite" effects of G-CSF and M-CSF on the CFU-GM, we noted that treatment of blood monocytes with phorbol 12-myristate 13-acetate (PMA) induces mRNA for both M-CSF and G-CSF with minimal induction of GM-CSF transcripts. We investigated the effects of a series of potentially physiologic regulatory factors on induction of G-CSF and M-CSF gene expression in normal human monocytes in an effort to determine if the M-CSF and G-CSF genes are coordinately or independently regulated. The results show that each CSF gene can be selectively induced.

MATERIALS AND METHODS

Monocyte preparation. Peripheral blood cells were obtained from volunteer platelet donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque density-gradient centrifugation. T lymphocytes were removed by E rosetting. Monocytes were further enriched by plastic adherence (1 hour, 37°C). In some experiments, the E- fraction was not adhered and was further cultured only in polypropylene flasks (Corning 25350, Corning Glass Works, Corning, NY), which do not support monocyte adherence. The percentage of monocytes in all cell preparations used in this study was monitored by indirect immunofluorescence staining with the CD14 monoclonal antibody anti-MY4.⁸ After an adherence step, 90% to 95% of cells were MY4+. Without adherence, 60% to 85% of cells were MY4+. T-cell contamination was <1% in all cases. Monocytes were then cultured for various times at a concentration of 1 to 2 × 10⁶/mL in RPMI 1640 with 10% fetal bovine serum (FBS), glutamine, and antibiotics (GIBCO, Grand Island, NY). The con-

centration of endotoxin in this medium was <0.1 ng/mL as determined by the limulus amoebocyte assay.

Reagents. Lipopolysaccharide (LPS), *Escherichia coli* 0111:B4, and PMA were obtained from Sigma Chemical, St Louis. Recombinant human IL-3, GM-CSF, G-CSF, and M-CSF were provided by Dr Steven Clark, Genetics Institute, Cambridge, MA. The CSFs were in the form of media conditioned by Chinese hamster ovary (CHO) cells transfected with vectors containing full-length cDNAs for each CSF.^{9,12} The media also contained 10% FBS and was used at a final dilution of 1:1,000 to 1:2,000 (twofold in excess of the maximum stimulatory concentration for marrow CFU-GM). Medium from "mock"-transfected CHO cells contained no CSF activity. All CSFs contained <0.1 ng/mL endotoxin at the concentrations used.

Northern blot analysis for CSF gene expression. Total cellular RNA was isolated as previously described¹³ using the guanidium isothiocyanate/cesium chloride method. Adherent monocytes were lysed in situ without prior removal from plastic dishes. Twenty-microgram mRNA samples were fractionated on 1.2% agarose gels with 6% formaldehyde and blotted onto nylon membranes (Gene Screen Plus, New England Nuclear, Boston). Plasmids containing CSF probes were supplied by Dr Steven Clark, Genetics Institute.^{9,12} M-CSF mRNA was detected by using a 3.5-kilobase (kb) segment of the human M-CSF cDNA inserted in the *Xho* site of the pXMT2 vector. G-CSF, GM-CSF, and IL-3 mRNA were detected with near full-length cDNA clones in the same vector.⁹ Probes were excised from their vectors, purified by gel electrophoresis, and labeled with hexanucleotide primers and ³²P-dCTP as previously described.¹³ Hybridization and washing were performed as previously described.^{6,13} Actin mRNA was detected by hybridizing a washed membrane with a similarly labeled mouse actin cDNA probe.

Bioassays for human M-CSF and G-CSF. Supernatants were prepared by incubating purified cell populations in RPMI 1640 medium with 10% FBS at 1 × 10⁶ cells/mL. CSF activity was then

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measured in a murine CFU-GM assay as previously described⁶ with endotoxin-resistant C3H/HeJ mice. Macrophage colonies (M-CSF activity) were distinguished from granulocyte colonies (G-CSF activity) by staining of whole agar cultures for α -naphthyl acetate esterase activity (staining monocyte colonies brown and not staining granulocyte colonies).⁶ Quadruplicate cultures were counted on day 7.

RESULTS

Expression of CSF transcripts in human monocytes exposed to phorbol ester. Peripheral blood monocytes were purified by E rosetting and plastic adherence and then exposed to PMA (10^{-10} mol/L) for 2 to 18 hours, or to IL-3 (1:1,000 dilution) for 18 hours at 37°C. Total cellular RNA was extracted, and M-CSF, G-CSF, GM-CSF, and actin transcripts were detected by Northern hybridization (Fig 1). Human umbilical vein endothelial cells stimulated for 12 hours with tumor necrosis factor (TNF) were used as a positive control. CSF transcripts were undetectable in control (unstimulated) monocytes (Fig 1) and endothelial cells (data not shown). TNF-stimulated endothelial cells contained transcripts for all three CSFs. Following exposure to PMA, monocytes expressed M-CSF and G-CSF transcripts at 2 and 18 hours, but GM-CSF transcripts remained at very low levels and were not further studied in these experiments. In experiments not shown, IL-3 transcripts were not detected in monocyte RNA by this technique, nor were IL-3 transcripts detected following poly A selection of mRNA. In contrast to PMA, exposure of monocytes to IL-3 (1:1,000 dilution for 18 hours) resulted in expression of only M-CSF transcripts (Fig 1).

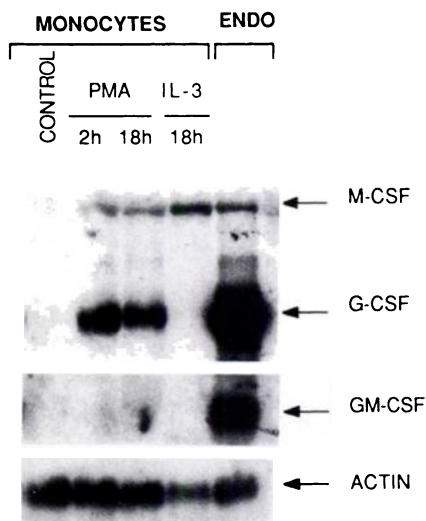


Fig 1. Induction of CSF mRNA by phorbol ester treatment of normal monocytes. Aliquots of 50×10^6 E⁻ adherent blood cells were exposed to 10^{-10} mol/L PMA for 2 to 18 hours or to IL-3 (1:2,000) for 18 hours. RNA was extracted, blotted, and hybridized simultaneously with cDNA probes for M-CSF (detecting a 4.0-kb message) and G-CSF (1.6-kb message). The same blot was later washed and rehybridized with a cDNA probe for GM-CSF (1.0-kb message) and finally with an actin probe to control for RNA loading in each lane. Human umbilical vein endothelial cells cultured with TNF^{4,5} were used as a positive control.

Differential induction of the M-CSF and G-CSF genes by IL-3 and LPS. Peripheral blood monocytes were purified by E rosetting and plastic adherence and exposed to control medium, IL-3, or LPS for 0, 2, or 18 hours at 37°C. As we previously reported, adherence of monocytes to plastic induces low-level expression of M-CSF transcripts (Fig 2, control).⁶ The addition of interleukin-3 (1:2,000 dilution of CHO supernatant) augmented M-CSF expression severalfold. An increase in M-CSF transcripts was detectable as early as 2 hours and further increased at 18 hours of culture. Expression of the G-CSF gene was not detected in either control or IL-3-treated cells (Fig 2). In contrast, treatment of monocytes with *E coli* LPS induced rapid expression of G-CSF transcripts, with a lesser effect on M-CSF transcripts. The effects were maximal at 2 hours, and G-CSF transcripts were considerably diminished at 18 hours of culture. The effects of recombinant GM-CSF were also tested and were identical to those of IL-3 (induction of M-CSF with little accumulation of G-CSF mRNA) (data not shown). These results show that during in vitro culture the ratio of M-CSF mRNA to G-CSF can be altered by different stimuli.

Differential secretion of M-CSF and G-CSF by human monocytes. Secretion of M-CSF and G-CSF by human monocytes was evaluated by collecting culture supernatants at 2, 14, 24, and 48 hours of culture and assaying for CSF activity in a murine CFU-GM assay. This assay was selected because human M-CSF and G-CSF are highly active in promoting growth of murine CFU-GM, but human GM-CSF and IL-3 have no effects on murine cells and thus do not interfere with the assay.⁶ Figure 3 shows one of five representative experiments. Control (unstimulated) monocytes released virtually no CSF activity for up to 48 hours of

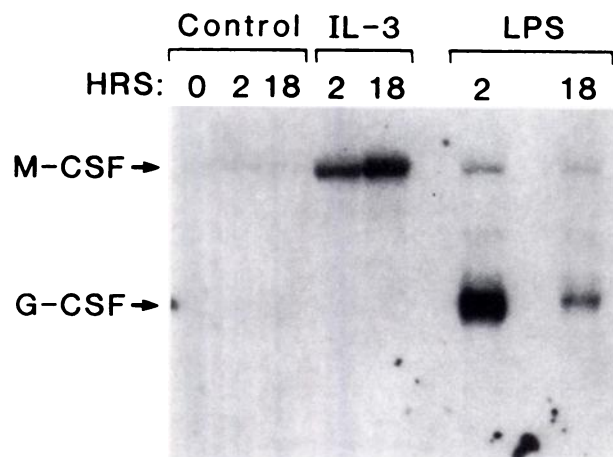


Fig 2. Induction of M-CSF and G-CSF transcripts in human monocytes. Blood monocytes were prepared by E rosetting and adherence, and aliquots of 50×10^6 cells were cultured for 0 to 18 hours in control media, IL-3 (1:2,000) or LPS (100 ng/mL). RNA was extracted, blotted, and hybridized simultaneously with probes for M-CSF and G-CSF. The M-CSF transcripts are 4.0 kb and the G-CSF transcripts are 1.6 kb. Rehybridization of the blot with an actin probe showed equivalent amounts of RNA in each lane (data not shown).

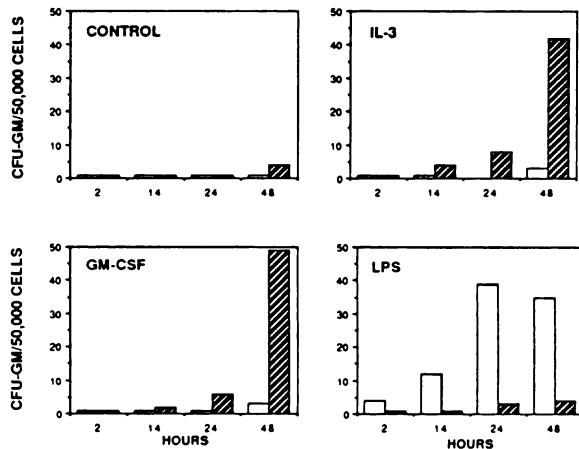


Fig 3. Secretion of M-CSF and G-CSF by monocytes. Monocytes enriched by E rosetting and plastic adherence were cultured at 1×10^6 cells/mL for 48 hours with LPS (100 ng/mL), IL-3 (1:2,000 dilution of CHO supernatant), GM-CSF (1:1,000 dilution of CHO supernatant), or control medium. Aliquots were removed as indicated, stored at 4°C and then assayed for M-CSF and G-CSF content by determining ability to induce murine monocyte or granulocyte colony formation. One of five similar experiments is shown. SEM was $\pm 25\%$ for each point and is not shown. In other controls not shown, LPS, human IL-3 or human GM-CSF had no effect by themselves on CFU-GM from C3H/HeJ mice.

culture. In contrast, IL-3 or GM-CSF-treated monocytes released primarily M-CSF, which was detectable at 24 hours of culture but not maximal until 48 hours of culture. LPS-treated monocytes released primarily G-CSF activity detectable variably at 14 hours of culture and maximal at 24 hours of culture (Fig 3). These results show that secretion of M-CSF or G-CSF is closely correlated with the gene expression studies shown in Fig 1, except that detection of the protein in the bioassay used was delayed for 12 to 24 hours.

Effects of adherence on induction of the M-CSF and G-CSF genes by IL-3 and LPS. Because adherence of monocytes to plastic can induce certain monocyte functions, including low-level expression of the M-CSF gene as shown in Fig 2, we attempted to determine the requirement for adherence in the CSF gene induction by IL-3 and LPS. Blood monocytes were enriched by E rosetting followed by adherence or without adherence by just E rosetting. Adherent cells were then exposed to control medium, IL-3, or LPS while adherent to plastic. Nonadherent cells were exposed to the same factors in polypropylene flasks. Total cellular RNA was harvested at 0, 2, or 18 hours of culture from equal aliquots of cells and analyzed for M-CSF and G-CSF transcripts. Induction of M-CSF transcripts was similar in IL-3-treated nonadherent and adherent monocytes (data not shown). Treatment of either adhered or nonadherent monocytes with LPS induced expression of the G-CSF gene without detectable accumulation of M-CSF transcripts. These results suggest that adherence is not required for the induction of M-CSF and G-CSF genes by IL-3 and LPS. However, the possibility that adherence might accelerate or augment CSF gene expression induced by other factors was not addressed in these experiments.

DISCUSSION

The results show that a degree of differential regulation of the M-CSF and G-CSF genes exists in normal human monocytes in vitro. The T-cell lymphokine IL-3 induces expression of the M-CSF gene at the mRNA level as well as secretion of M-CSF but has much less effect under these experimental conditions on expression of the G-CSF gene. In contrast, LPS induces expression of the G-CSF gene and secretion of G-CSF, with a considerably lesser effect on expression of the M-CSF gene. This ability of monocytes to produce either M-CSF or G-CSF in response to different induction stimuli is potentially important in the regulation of granulocyte and monocyte production. There are abundant clinical examples of granulocytosis (ie, bacterial infections and certain inflammatory disorders)¹⁴ and monocytosis (ie, tuberculosis, malignancies, and some collagen vascular diseases)¹⁵ but possible mechanisms whereby selective granulocyte or monocyte production might occur have never been elucidated. Differential granulocyte or monocyte production from the same progenitor cell could occur through several different mechanisms, including differential regulation of CSF receptor expression at the level of the progenitor cell, or by differential supply to the progenitor cell of factors that will induce production of either monocytes or granulocytes. Differential supply of these two CSFs might promote production of either granulocytes or monocytes in vivo. Our results with murine bone marrow CFU-GM (Fig 3) show that such differential production of granulocytes or monocytes is possible in vitro. The differential production of M-CSF and G-CSF by the monocyte may also be important in the inflammatory response. Both CSFs exert "priming" effects on mature cells,^{16,17} and the production of the CSFs locally in response to different stimuli may be important in augmenting granulocyte or macrophage function in many tissues.

The types of factors that induce expression of the M-CSF and G-CSF genes in monocytes may be physiologically relevant. This report describes the induction of M-CSF by IL-3, and previous studies from our laboratory⁶ and those of other researchers¹⁸ have shown that two other T-cell lymphokines, γ -interferon and GM-CSF, also induce M-CSF expression in monocytes. In addition, we observed that GM-CSF, like IL-3, fails to induce expression of G-CSF in monocytes. In contrast to the effects of these lymphokines from activated T cells, exposure of monocytes to endotoxin (LPS), as would occur during an acute bacterial infection, rapidly induced expression of the G-CSF gene and release of G-CSF by monocytes. Thus, an acute bacterial infection might promote selective granulocytosis through this mechanism, whereas activation of the T-cell antigen receptor (the major primary stimulus for lymphokine release) would not.

The kinetics of transcript accumulation and release of G-CSF and M-CSF by monocytes were different. Although M-CSF transcripts were readily detectable at 2 hours of exposure to IL-3, peak accumulation of mRNA and secretion of M-CSF did not occur until ≥ 18 hours. In contrast, LPS induced accumulation of G-CSF transcripts that peaked at 2 hours and were almost gone at 18 hours.

Thus, both the factors inducing expression of these two genes and the kinetics of gene expression are different. We have not determined whether single monocytes are capable of producing both M-CSF and G-CSF or whether distinct populations of monocytes exist that are capable of secreting only one of these CSFs. Future experiments with *in situ* hybridization should answer this question.

We did not readily detect GM-CSF or IL-3 transcripts in PMA-stimulated monocytes (Fig 1), and regulation of these CSFs was not further studied in these experiments. Murine peritoneal macrophages induced by thioglycollate secrete GM-CSF, and GM-CSF transcripts may be inducible in human monocytes with other culture conditions that we did not study.

The regulation of granulocyte and monocyte production *in vivo* is probably complex. Many cells have the capacity to produce CSFs, and considerable interaction clearly exists among these various cells with many levels of regulation. For example, interleukin-1 or TNF from the monocyte promotes release of GM-CSF from T cells, endothelial cells, and fibroblasts.^{4,5} GM-CSF and IL-3 act back on the monocyte

to induce TNF secretion,^{19,20} thus forming an activation "loop," and further promoting release of M-CSF by the monocyte as shown here. As the control of the CSF genes in other cells is studied, these interactions are likely to become even more complex. The net effect of any stimulus on the production of granulocytes and monocytes will be determined by all of the interacting cell systems involved in hematopoietic regulation, and it is not possible to conclude that the independent regulation of M-CSF and G-CSF genes in a single cell type *in vitro* as described here would be relevant *in vivo*. Nonetheless, independent regulation of CSF genes in accessory cells could be important in many pathologic situations (such as bacterial infections) both in promoting production and/or augmenting function of mature myeloid cells, and further examples of independent regulation in monocytes and other accessory cell types²¹ should be sought.

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