Lymphotoxin: Stimulation and Regulation of Colony-Stimulating Factors in Fibroblasts

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Colony-stimulating factors (CSFs) are pivotal for proliferation and function of hematopoietic cells. We found that lymphotoxin, a product of activated lymphocytes, stimulates accumulation of granulocyte-macrophage (GM)-CSF and macrophage (M)-CSF proteins and mRNAs in fibroblasts. An increase in GM- and M-CSF mRNA levels occurred within 2 hours after addition of 1,000 U/mL lymphotoxin and levels plateaued over the next 24 hours. Tumor necrosis factor alpha (TNFα) was about five times more potent than lymphotoxin at low concentrations and was nearly 1.5 to 2 times more potent at maximally stimulating concentrations of the cytokines. Stimulation by lymphotoxin did not require either new protein synthesis or protein kinase-C stimulation. Stability studies of GM- and M-CSF transcripts in fibroblasts showed that M-CSF mRNA was five times more stable (half-life [1/2t] 100 minutes) than GM-CSF mRNA (1/2t, 20 minutes). Stability of these mRNAs was unchanged after stimulation of the cells with lymphotoxin. In addition, exposure of cells to 12-0-tetradecanoylphorbol 13-acetate did not alter stability of M-CSF mRNA but markedly prolonged the stability of GM-CSF mRNA. This is consistent with data showing that the AT-rich consensus region in the 3' untranslated region of many transiently expressed cytokines including GM-CSF but not M-CSF, play a major role in their mRNA stability. Our results suggest that activated lymphocytes can affect hematopoietic cell function and growth by stimulating production of CSFs by mesenchymal cells.

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pHFB A-3' at plasmid.19 These probes were [32P]-labeled by random priming to a specific activity of 2 to 8 \times 10^{6} \text{ cpm/µg}.

**Isolation and blotteding of RNA.** For cytoplasmic RNA, WI-38 cells were suspended in hypotonic buffer (10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L KCl, 3 mmol/L MgCl}_2 and lysed with 0.3% Nonidet P-40. Cytoplasmic RNA was extracted by phenol/chloroform methods as previously described.20 After denaturation at 65°C, RNA was electrophoresed in an agarose, formaldehyde gel (1%) and transferred to a nylon-membrane filter (Biorad, ICN, Irvine, CA).21 Filters were hybridized with labeled probe for 16 to 24 hours at 42°C in 50% formamide, 2 × SSC (1 × = 150 mmol/L NaCl, 15 mmol/L sodium citrate), 5 × Denhardt's, 0.1% sodium dodecyl sulfate (SDS), 1% dextran sulfate, and 100 µg/mL salmon sperm. Filters were washed to a stringency of 0.1% SSC, 5°C for 10 minutes and exposed to Kodak film.

Blots were usually sequentially hybridized with [32P]-labeled GM- and M-CSF cDNA, and beta-actin DNA. The actin band helped confirm that similar amounts of RNA were added to each lane. Modulation in relative levels of either GM- or M-CSF RNA were quantified by standardization to the amount of beta-actin–specific transcripts. The relative density of bands of hybridization of beta-actin mRNA and either GM- or M-CSF mRNA in the different lanes was determined by laser densitometry using multiple exposures of the blot. The ratio of either GM-CSF-beta-actin or M-CSF-beta-actin in the control lane was assigned the baseline level. The fold-stimulation in control was determined by laser densitometry using multiple exposures of the blot. The ratio of hybridization of beta-actin mRNA and either GM- or M-CSF mRNA in the different lanes was calculated by multiplying the ratio of the baseline level.

**[35S] Methionine incorporation.** Fibroblasts were exposed to cycloheximide in Falcon 3001 culture dishes (Becton Dickinson Labware, Rutherford, NJ) in triplicate per experimental point for 2 hours. Cells were pulsed with 4 µCi of [35S] methionine for 1 hour at 37°C, washed twice in 1 × PBS, precipitated in 5% trichloroacetic acid (TCA; 30 mL Na_2HPO_4) at 4°C for 1 hour, filtered onto glass microfiber membranes (GF/F; Whatman, Maidstone, England), washed in 3% TCA (30 mmol/L Na_2HPO_4) and heated at 80°C for 1 hour. Each sample was counted by liquid scintillation, and results were compared with those of untreated cells that were labeled and counted.

**Protein kinase C assay.** The activity of protein kinase C was assayed as described previously.22 Briefly, soluble (cytosol) and solubilized particulate (membrane) fractions of cells were prepared, and both fractions were assayed for protein kinase C activity using histone H1 as a substrate. The amounts of protein in the cytosolic (22 µg) and solubilized membrane (5 µg) fractions were determined in the reaction mixtures (0.2 mL) containing 25 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl}_2, 5 µmol/L [32P]-labeled adenosine triphosphate (ATP), and 40 µg of histone.

**RESULTS**

**LT induction of CSF protein in fibroblasts.** LT was added to confluent cultures of WI-38 lung fibroblasts for 4 days; CM were harvested and tested for CSF activity (Table 1). LT stimulated lung fibroblasts to produce GM-CSF in a dose-dependent fashion as measured by stimulation of normal human myeloid committed stem cells from the marrow (GM-CFC). This activity was almost completely neutralized by GM-CSF antibody (Table 1). In the absence of lymphotxin, fibroblasts constitutively produced some CSF (Table 1). When LT (1,000 U/mL) was added with exogenous GM-CSF to soft agar cultures, LT (+10^4 U/mL) slightly inhibited (<30%) clonal growth of myeloid colonies (GM-CFC) (data not shown). LT alone did not stimulate colony formation (Table 1).

The fibroblast-CM were also evaluated for their ability to stimulate murine myeloid colony formation. Human granulocyte (G)- and M-CSF are potent stimuli for murine myeloid colony formation. In contrast, human interleukin-3 (IL-3) and GM-CSF are unable to stimulate murine myeloid colony formation. LT stimulated human fibroblasts in a dose-response manner to produce a murine CSF (Table 1). The colonies were composed of monocytes/macrophages as determined by morphologic examination. Furthermore, Northern blot analysis detected no hybridizing bands in LT-treated fibroblasts when blots were probed for G-CSF mRNA (data not shown).

**Dose-dependent effect of LT and TNFa on levels of GM- and M-CSF mRNA.** Fibroblasts constitutively contained low concentrations of mRNA coding for GM- and M-CSF (Fig 1). Both lymphotxin and TNFa stimulated accumulation of GM- and M-CSF mRNA in a dose-dependent manner (Fig 1). Maximal levels of GM- and M-CSF mRNA occurred at approximately 10^3 U/mL (8.3 ng/mL) of LT. TNFa was more potent than LT; 10 U/mL TNFa stimulated nearly an equivalent amount of GM- and M-CSF mRNA as did 10^4 U/mL LT as determined by densitometry. At maximally stimulating concentrations, TNFa stimulated two times more concentrations of GM-CSF mRNA as compared with LT. The incremental increase in levels of GM- and M-CSF mRNAs paralleled each other at nearly each concentration of either LT or TNFa. A very small amount of G-CSF mRNA was detected in TNFa-treated cells, but not in LT treated cells (data not shown). Endotoxin
Prior studies have shown that cells exposed for prolonged durations to TPA have markedly reduced protein kinase C activity, making them resistant to re-exposure to TPA. Fibroblasts (W138) exposed to TPA (50 nmol/L) for 4 hours had a prominent (22-fold) increase in levels of GM-CSF mRNA (Fig 4, lane 2). However, exposure of the cells for 24 hours to TPA and then re-exposure to TPA attenuated the response to TPA by 84% (Fig 4, lane 3). In contrast, response to lymphotoxin was not attenuated by prolonged exposure to TPA (Fig 4, lane 7). This lack of inhibition was seen in three separate studies (data not shown). NaF activates several G-binding proteins. We found that NaF (5 mmol/L) stimulates accumulation of GM-CSF mRNA (Fig 4, lane 4) and this stimulation also was not attenuated by prolonged exposure to TPA (Fig 4, lane 5). Reprobing of the blot with

at a dose equivalent to that found in 10⁴ units of TNFα or LT (<1 pg/mL) failed to stimulate accumulation of CSF mRNAs (data not shown).

**Time-dependent effect of LT on levels of GM- and M-CSF mRNAs.** Fibroblasts were exposed to 1,000 U/mL LT for different durations; cytoplasmic RNA was extracted and analyzed by Northern blot (Fig 2). Accumulation of GM-CSF mRNA was prominent by 2 hours; levels plateaued for 24 hours and began to decrease by 48 hours of exposure. Maximal levels of M-CSF mRNA occurred at about 8 hours and levels returned to baseline at 48 hours of exposure.

**Effect of prolonged exposure of a phorbol diester on expression of CSF mRNA stimulated by LT.** The potent phorbol diester, known as TPA, stimulates activity of protein kinase C in a variety of cells. Various phorbol derivatives were examined for their ability to stimulate accumulation of GM- and M-CSF mRNA. TPA, PDD, and PDB (Fig 3, lanes 1, 3, and 5, respectively) are potent activators of protein kinase C; their derivatives 4-O-methyl TPA and 4-α-PDD (Fig 3, lanes 2 and 4, respectively) are poor stimulators of protein kinase C. Potency of each of the phorbol diesters to stimulate accumulation of GM- and M-CSF mRNA paralleled their known abilities to activate protein kinase C.
Effect of an inhibitor of protein synthesis on expression of CSF mRNA in fibroblasts stimulated by LT. Exposure of fibroblasts to the protein synthesis inhibitor cyclohexamide (CHX; 20 μg/mL) stimulated the accumulation of GM-CSF mRNA 24-fold (Fig 5, lane 2). Protein synthesis in these cells was less than 10% of untreated fibroblasts as measured by 35S-methionine incorporation (data not shown). Fibroblasts exposed to LT stimulated at least fivefold greater levels of GM-CSF mRNA as compared with untreated fibroblasts (Fig 5, lane 1). Cells cultured initially with CHX followed by the addition of LT had a 30-fold enhanced accumulation of GM-CSF mRNA as compared with untreated control cells (Fig 5, lane 3). Levels of M-CSF mRNA increased after culture with either LT or CHX (threetifold), but little change occurred when the cells were precultured with CHX and then cultured with both compounds as compared with either agent alone.

Stability of steady-state GM- and M-CSF mRNA in fibroblasts exposed to either LT or TPA. To examine for post-transcriptional regulation of expression of GM- and M-CSF mRNA, fibroblasts were cultured initially with either LT or TPA for 4 hours, and then with actinomycin D. Cells were harvested at different times and examined for levels of GM- and M-CSF mRNA as well as β-actin mRNA (Fig 6). GM-CSF mRNA was less stable than M-CSF mRNA in untreated cells with the half-life (t1/2) of GM- and M-CSF mRNA of approximately 20 and 100 minutes, respectively. After stimulation with LT, the stability of both mRNAs remained unchanged. After stimulation with TPA, t1/2 of M-CSF mRNA remained unchanged, but the stability of GM-CSF mRNA was markedly increased (t1/2 > 2 hours).

DISCUSSION

We investigated the ability of LT to regulate production of CSFs in fibroblasts. This lymphokine rapidly stimulated accumulation of GM-CSF mRNA with near maximal levels occurring at 2 hours; maximal levels of M-CSF mRNA
Fig 5. Accumulation of M- and GM-CSF mRNA in fibroblasts cultured with LT in absence of new protein synthesis. Northern blot analysis of mRNA levels for M-CSF (4.0 kb), GM-CSF (0.9 kb), and β-actin (2.1 kb) in human lung fibroblasts after initial exposure to the protein synthesis inhibitor. CHX (20 μg/ml, for 0.5 hours); LT (3,000 U/ml) was then added to these cultures for 2 hours. As controls, cells were treated with either LT (2 hours) or CHX (2.5 hours). Analysis was performed by blotting total RNA (15 μg per lane) and hybridizing sequentially with 32P-labeled probes coding for either M-CSF, GM-CSF, and β-actin as described in Materials and Methods. lane 1. IT; lane 2, CHX; lane 3, CHX and IT; lane 4, untreated control; lane 5, positive control (Lu-CSF-1).

appeared at 8 hours. These levels of M- and GM-CSF mRNA were approximately 8 to 16 times greater than in untreated fibroblasts. Within 48 hours of exposure to LT, levels of both CSF mRNAs began to decrease.

LT is a glycoprotein mainly produced by activated lymphocytes; TNFα is synthesized by activated macrophages as well as many other kinds of cells including lymphocytes and tumor cell lines. Many of the actions of both of these cytokines are similar or identical, including their inhibition of growth of many types of cells transformed by chemicals or viruses. Both share 30% homology in amino acid sequences, and both probably use the same cellular receptors. Nevertheless, several quantitative and qualitative differences in biological effects of the two cytokines have been reported. TNFα is more potent than LT in stimulating release of IL-1 from endothelial cells and in inducing expression of neutrophil adhesion molecules. TNFα also is more potent than LT in inhibiting clonogenic growth of certain tumor cells. Moreover, TNFα was reported to stimulate the accumulation of M-CSF mRNA in human monocytes, but lymphotxin did not.

We found that TNFα was more potent than LT in stimulating the accumulation of GM- and M-CSF mRNA.
Ten units per milliliter of TNFα had nearly the same stimulatory activity as 10² to 10³ U/mL of LT. However, at maximally stimulating concentrations, TNFα was only two-fold more potent than LT. Why differences in potency and range of activity exist between the two cytokines is not clear. Although both bind to the same cellular receptor, this binding may not be identical. Also, natural LT is glycosylated, but TNFα is not. Our recombiant LT is not glycosylated, which may decrease activity of the compound. However, evidence in other cell systems suggested that LT does not require glycosylation for complete activity.

Previously, we showed that TNFα increased accumulation of GM-CSF mRNA in fibroblasts (W138) by increasing both the rate of transcription of the gene and the stability of the mRNA. In contrast, we find that LT has no effect on the stability of either GM- or M-CSF mRNA in W138. LT may be, therefore, increasing the rate of transcription of these CSFs; further studies are in progress to confirm this hypothesis. This, however, may explain why TNFα is more potent than LT in causing the accumulation of CSF mRNA.

Previous studies have reported that recombiant human LT was a very weak stimulator of CSF production as compared with the present investigation. The reason for this discrepancy in results is unclear, but may be due to differences in LT preparations.

Earlier studies by us found that TNFα increased levels of GM-CSF mRNA in the absence of new protein synthesis. Our present study finds that LT also increases levels of GM-CSF mRNA in the absence of protein synthesis. LT therefore might have either a direct effect on transcription of GM-CSF or could be modifying an already existing transactivating protein that interacts with the DNA sequences in the region of the GM-CSF gene.

LT presumably binds to its receptor on the cell surface, but how the signal is transduced in order to increase levels of CSF mRNA is not understood. Phorbol diesters including TPA activates protein kinase C. We found that phorbol compounds which activate protein kinase C (TPA, PDB, PDB) markedly increased levels of GM- and M-CSF mRNA. In contrast, phorbol esters incapable of activating protein kinase C were unable to increase the concentration of CSF mRNAs. Protein kinase C is involved in signal transduction by coupling receptor-mediated inositol phospholipid turnover with a variety of cellular functions. For example, one study suggested that IL-1 utilizes both calcium and protein kinase C–dependent signal pathways to induce expression of IL-2 receptor and facilitate release of IL-2.

We provide indirect evidence that the accumulation of CSF mRNAs by LT probably does not occur through the protein kinase C pathway. Fibroblasts that became refractory to stimulation by TPA continued to be stimulated by LT.

We also showed that NaF stimulates accumulation of CSF mRNA and this occurs probably without the help of protein kinase C. Pertussis toxin was unable to block the effect of either NaF or LT (data not shown). NaF is able to activate G-binding proteins suggesting that levels of CSF mRNA in fibroblasts possibly may be modulated through a G-binding protein pathway that is not inhibitable by pertussis toxin. Another report which studied myeloid leukemia cells suggested that TNFα mediates several effects through G-binding proteins. Further studies are required to determine if a G-binding protein tranduces the effect of LT.

M-CSF enhances the microbicidal activity of macrophages as well as perhaps increasing production of monocytes/macrophages. We show for the first time that both LT and TNFα can increase in vitro levels of M-CSF mRNA in fibroblasts. In addition we found that stability of M-CSF mRNA (t½, 100 minutes) is five times greater than stability of GM-CSF mRNA (20 minutes). GM-CSF mRNA has an 8-nucleotide (nt) consensus sequence (UUAAUUUAU) which is repeated a number of times in the 3’ untranslated region.

Earlier studies have shown that these sequences are critical for the regulation of stability of these mRNAs. M-CSF mRNA does not have the 8-nt consensus sequences, but has four groups having 6 or 7 nt sequences (UUAUUUAU, UUAUUU, or UAUUAU) in the 3’ untranslated region. Previous studies showed that the instability imparted by the eight base-pair AU rich sequence element is abrogated by protein kinase C activation. Consistent with these results we found that TPA increased the stability of mRNA coding for GM-CSF but not M-CSF. Our studies provide further support for the importance of the AU-rich 8-nt consensus sequence in the regulation of transiently expressed genes such as many cytokines and nuclear-located oncogenes.

We have shown that human embryonic fibroblasts (W138) constitutively transcribe GM-CSF as detected by transcriptional run-on analysis. Zucali et al reported that the CM of human lung fibroblasts (CCL 202) had no detectable CSF-activity when cultured in the absence of TNF or LT. On the other hand, Yang et al suggested nontransformed fibroblasts constitutively produced GM-CSF. Perhaps steady-state hematopoiesis is, in part, regulated by the constant short-range interactions of CSF produced by mesenchymal cells; these studies do not rule out, however, an artificial effect of in vitro culture.

Fibroblasts constitute a major element of the bone marrow stroma, as well as submucosal and subcutaneous tissue. Our data demonstrate that LT, which is produced by normal activated lymphocytes, can enhance the level of CSF in human fibroblasts. The CSFs not only stimulate proliferation and differentiation, they also enhance the function of granulocytes, macrophages, and eosinophils. Therefore, lymphocytes can indirectly affect growth, differentiation, and function of other hematopoietic cells through stimulation of local production of CSF by mesenchymal cells.

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