Transcriptional and Posttranscriptional Regulation of Macrophage-specific Colony Stimulating Factor Gene Expression by Tumor Necrosis Factor

Involvement of Arachidonic Acid Metabolites

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Abstract

The effects of tumor necrosis factor (TNF) on the regulation of macrophage-specific colony stimulating factor (CSF-1) gene expression have been studied in HL-60 cells during monocytic differentiation. CSF-1 transcripts were undetectable in uninduced HL-60 cells, reached maximal levels by 3 h of exposure to TNF, and returned to that of control cells by 24 h. Transcriptional run-on analysis demonstrated that exposure to TNF stimulated the rate of CSF-1 gene transcription by 6.4-fold. The combination of a protein synthesis inhibitor, cycloheximide, and TNF increased levels of CSF-1 mRNA compared with treatment by TNF alone. We also studied the signal transduction mechanisms responsible for regulating TNF-induced CSF-1 mRNA levels. Both 4-bromophenacyl bromide and quinacrine, inhibitors of phospholipase A_2 activity, blocked TNF-induced increases in CSF-1 transcripts in a concentration-dependent manner, while caffeic acid and nordihydroguaiaretic acid, inhibitors of the 5-lipoxygenase pathway, had no detectable effect on induction of CSF-1 RNA. PGE_2 or dibutyryl cAMP treatment of HL-60 cells in the presence of TNF blocked the expression of CSF-1 mRNA in a dose-dependent manner. These findings suggest that the increase in CSF-1 RNA observed during TNF treatment is regulated, at least in part, by both transcriptional and posttranscriptional mechanisms, and that PGE_2 and cAMP regulate transcriptional activation of the CSF-1 gene by TNF. (*J. Clin. Invest.* 1990. 85:442–447.) colony stimulating factors • cycloheximide • phospholipase A_2 • prostaglandin E_2 • cyclic AMP

Introduction

The macrophage-specific colony stimulating factor (CSF-1, also referred to as M-CSF)[1] stimulates hematopoietic stem cells to form colonies containing monocytes and macrophages (1, 2). Semipurified CSF-1 from mouse L929 cell-conditioned media also regulates mouse macrophage production of biologic factors such as prostaglandin E (3), plasminogen activator (4), interleukin 1 (5), and granulocyte-specific CSF (6). Furthermore, mouse CSF-1 enhances the induction of interferon by lipopolysaccharide or polyinosinic/polycytidylic acid (7), whereas human CSF-1 purified from the pancreatic carcinoma cell line MIA-PaCa stimulates the production of interferon, myeloid colony stimulating activity and tumor necrosis factor (TNF) (8).

In contrast to the known multiple effects of CSF-1 protein, the regulation of CSF-1 gene expression by biologic mediators has not been extensively examined. Phorbol esters and the granulocyte/CSF-1 have been shown to induce CSF-1 expression in MIA-PaCa cells, normal human monocytes, and during monocytic differentiation of human promyelocytic leukemia cells (9–12). Furthermore, recent studies demonstrate that the CSF-1 gene is constitutively expressed in a variety of human ovarian, breast, and lung carcinoma cell lines (13).

TNF is a regulatory cytokine which binds to a specific, high-affinity plasma-membrane receptor. Initially, in vitro studies demonstrated that TNF is cytotoxic or cytostatic to a variety of human and murine tumor cells but has no antiproliferative effects against normal cells. TNF also has pleiotropic effects on hematopoietic cell growth and differentiation. TNF inhibits colony growth of BFU-E, CFU-GM, and CFU-GEMM (14–16). TNF also induces monocytic differentiation of HL-60 cells (17) and is associated with rapid down-regulation of c-myc proto-oncogene expression at the transcriptional level (18, 19). Recent studies have demonstrated that both transcriptional and posttranscriptional mechanisms regulate granulocyte-specific CSF and granulocyte/CSF-1 expression by TNF in fibroblasts (20). Furthermore, TNF has been shown to induce secretion of CSF-1 by human monocytes (21).

In the present study, we examined the effects of TNF on the regulation of gene expression of CSF-1 in HL-60 cells during monocytic differentiation. Our data show that TNF transcriptionally regulates CSF-1 expression and that CSF-1 transcripts are degraded posttranscriptionally by a labile protein. Furthermore, the induction of CSF-1 by TNF is inhibited by the cyclooxygenase metabolite PGE_2, as well as cAMP.

Methods

Cell culture. The HL-60 promyelocytic cells were grown in RPMI 1640 media containing 15% FBS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM nonessential amino acids. Cell cultures were maintained in a humidified 5% CO_2 in air atmosphere at 37°C. Human recombinant TNF (Asahi Chemical Industry Co., New York) had a specific activity of 2.3×10^6 U/mg and contained < 10 pg endotoxin/mg protein by the Limulus lysate assay.

Preparation of RNA and Northern blot hybridization. Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride technique as described (22). Total cellular RNA (20 μg) was subjected

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1. *Abbreviations used in this paper:* BPB, 4-bromophenacyl bromide; Bt_2cAMP, dibutyryl cAMP; CSF-1, macrophage-specific colony stimulating factor; 5-LO and 15-LO, 5- and 15-lipoxygenase, respectively; NDGA, nordihydroguaiaretic acid; TNF, tumor necrosis factor.

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to electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose paper, and hybridized to one of the following ^{32}P-labeled DNA probes: (*a*) the 0.57-kb Acc I/Eco RI fragment of a human CSF-1 cDNA purified from the pc–CSF-12 plasmid (23); (*b*) the 4.0-kb Eco RI fragment of the human c-fms gene purified from the pc-fms 102 plasmid (24); and (*c*) the pA1 plasmid containing a 2.0-kb Pst I insert of the chicken beta-actin gene (25).

Hybridization reactions were carried out for 16–24 h at 42°C in 50% (vol/vol) formamide, 2× SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 1× Denhardt's solution, 0.1% (wt/vol) SDS and 200 μg/ml salmon sperm DNA. Filters were washed and exposed to Kodak X-Omat XAR film using an intensifying screen. The autoradiograms were scanned using a laser densitometer (UltroScan XL; LKB Instruments, Inc., Gaithersburg, MD) and analyzed using the Gelscan XL software package (LKB Instruments, Inc.). The intensity of CSF-1 or c-fms hybridization was normalized against beta-actin expression.

Run-on transcriptional analyses. HL-60 cells (10^8 cells per treatment) were washed with ice-cold PBS and the nuclei isolated by lysis in 0.5% NP-40 buffer as described (22). Nuclei were resuspended in glycerol buffer and incubated in an equal volume of reaction buffer containing 100 mM KCl, 0.5 mM each of ATP, GTP, and CTP, and 200 μCi [alpha-^{32}P]UTP (800 Ci/mmol; New England Nuclear, Boston, MA) at 26°C for 30 min. The reaction was terminated by the addition of 100 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 20 mM $MgCl_2$, 150 U/ml RNasin, and 40 μg/ml DNase and allowed to incubate at 28°C for 15 min. After proteinase K digestion, the RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in ethanol and 2.5 M ammonium acetate. The RNA was further purified by Sephadex G-50 column separation.

Plasmid DNAs containing various cloned inserts were digested with restriction endonucleases as follows: (*a*) the 2.0-kb Pst I fragment of the chicken beta-actin pA1 plasmid; and (*b*) the 1.6-kb Sal I/Eco RI fragment of the pc–CSF-12 plasmid. The digested DNA was run in a 1% agarose gel and transferred to nitrocellulose filters by the method of Southern. The filters were prehybridized in 5× Denhardt's solution, 40% formamide, 4× SSC, 5 mM EDTA, 0.4% SDS, and 100 μg/ml yeast tRNA for 2 h. Hybridization was performed with 10^7 cpm of ^{32}P-labeled RNA/ml hybridization buffer for 72 h at 42°C. The filters were then washed in 2× SSC and 0.1% SDS at 37°C for 30 min, 10 μg/ml RNase A in 2× SSC at 37°C for 30 min, and 0.1× SSC and 0.1% SDS at 42°C for 30 min. Autoradiography was performed for 3–10 d.

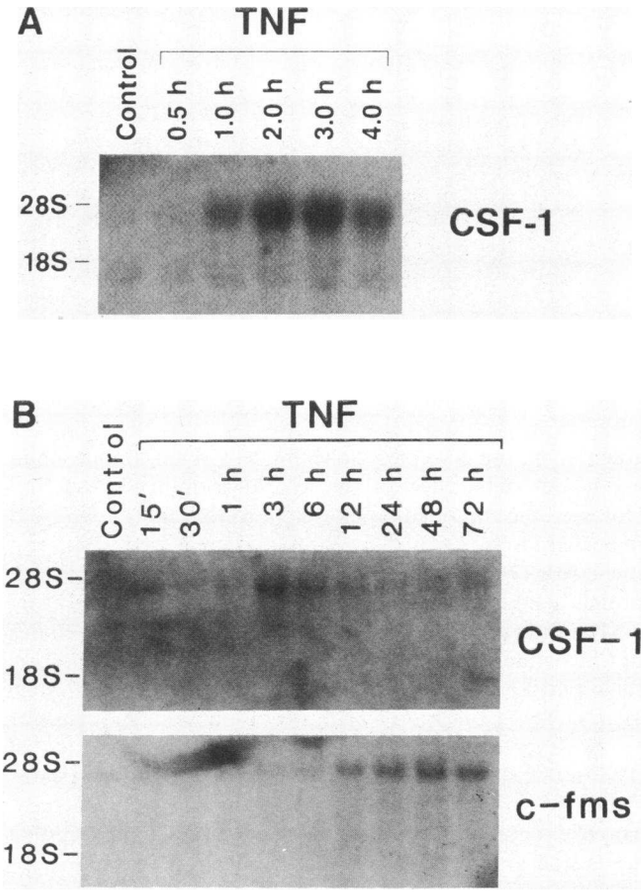


Figure 1. Effects of TNF on CSF-1 and c-fms mRNA levels in HL-60 cells. Northern blot analysis of RNA levels was performed in HL-60 cells after treatment with TNF (200 U/ml). (*A*) Total cellular RNA (20 μg/lane) was hybridized to a 0.57-kb ^{32}P-labeled CSF-1 DNA probe. (*B*) Hybridization was performed using ^{32}P-labeled CSF-1 and c-fms DNA probes. The control lane represents RNA from untreated HL-60 cells.

Results

CSF-1 transcripts are rapidly and transiently induced by TNF. We first determined the effects of TNF on the expression of CSF-1 gene transcripts during induction of monocytic differentiation. Northern blot analysis of HL-60 cellular RNA using a CSF-1 cDNA probe is shown in Fig. 1. The level of CSF-1 transcripts increased 20-fold after 3 h of exposure to TNF (Fig. 1 *A*). This increase was transient and the level of CSF-1 transcripts returned to that of control cells by 24 h (Fig. 1 *B*). TNF treatment was also associated with the appearance of c-fms transcripts that code for the CSF-1 receptor. However, in contrast to the induction of CSF-1 gene expression, maximal c-fms mRNA levels were detected at 48 h of TNF treatment (Fig. 1 *B*). TNF had no effect on levels of actin transcripts.

CSF-1 expression is controlled at both the transcriptional and posttranscriptional levels. Run-on transcriptional assays in isolated nuclei were performed to determine the mechanisms responsible for the regulation of CSF-1 gene expression by TNF. A low level of CSF-1 gene transcription was detectable in untreated HL-60 cells (Fig. 2). In contrast, exposure to TNF for 1 and 2 h increased CSF-1 gene transcription by 4.0- and 6.4-fold, respectively (Fig. 2).

Increased levels of CSF-1 mRNA in HL-60 cells exposed to TNF could also result from enhanced stabilization of the CSF-1 transcript. In order to study posttranscriptional regulation of CSF-1 RNA, HL-60 cells were treated with TNF in the

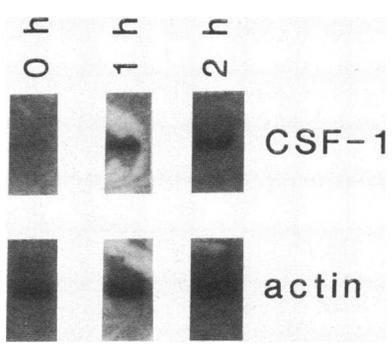


Figure 2. Effects of TNF on rates of CSF-1 gene transcription. HL-60 cell nuclei were isolated at the indicated times as described in Methods. Newly elongated ^{32}P-labeled RNA transcripts were hybridized to 2 μg CSF-1 or beta-actin DNA insert after restriction enzyme digestion and Southern blotting.

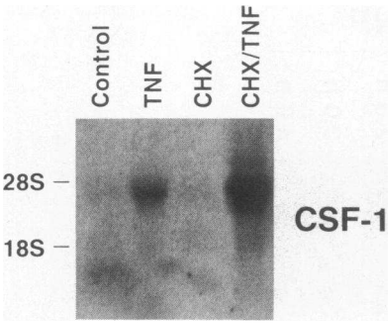


Figure 3. Effects of cycloheximide on CSF-1 mRNA levels in untreated and TNF-treated HL-60 cells. HL-60 cells were treated with 200 U/ml TNF and/or 1 μg/ml cycloheximide for 3 h. Total cellular RNA (20 μg/lane) was isolated and analyzed by hybridization to a ^{32}P-labeled CSF-1 probe. The control lane represents RNA from untreated HL-60 cells.

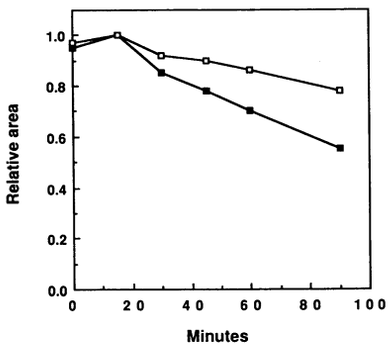
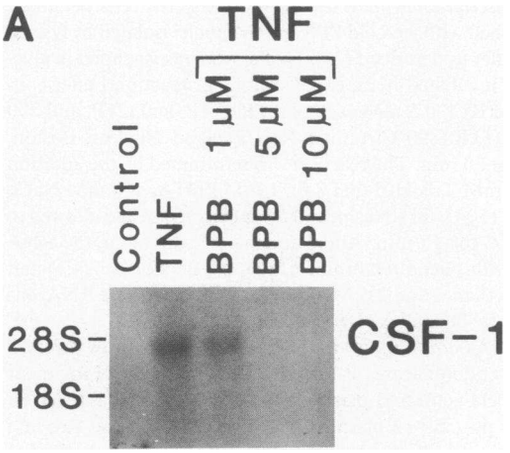


Figure 4. Effects of cycloheximide on CSF-1 mRNA half-life in TNF-treated HL-60 cells. HL-60 cells were treated with TNF (200 U/ml) for 3 h followed by 5 μg/ml actinomycin-D (■) or actinomycin-D and cycloheximide (□). Cells were harvested at the indicated times (after the addition of actinomycin-D) and analyzed as described.

absence and presence of cycloheximide. Cycloheximide alone had no detectable effect on the accumulation of CSF-1 RNA. However, the combination of cycloheximide and TNF for 3 h increased levels of CSF-1 by 3.4-fold as compared with treatment with TNF alone (Fig. 3). These results also indicated that protein synthesis is not required for the induction of CSF-1 by TNF. Furthermore, cycloheximide had no effect on the rates of CSF-1 transcription as monitored by nuclear run-on assays (data not shown). These results suggested that cycloheximide affects CSF-1 expression by a posttranscriptional mechanism.

In order to study the posttranscriptional regulation of TNF-induced CSF-1 mRNA levels, HL-60 cells were treated with TNF for 3 h to induce CSF-1 expression and then exposed to actinomycin-D for various times to inhibit further transcription. The half-life of CSF-1 mRNA as determined by densitometric scanning was 97 min (Fig. 4). In contrast, inhibition of protein synthesis with cycloheximide in the absence of transcription increased the half-life of CSF-1 mRNA in TNF-treated HL-60 cells to 220 min (Fig. 4). Taken together, these findings suggested that the increase in CSF-1 RNA observed during TNF treatment is also mediated, at least in part, by posttranscriptional mechanisms involving the synthesis of a labile protein that affects the turnover of CSF-1 RNA.

CSF-1 expression is blocked by inhibitors of the arachidonic acid cascade. Recent work in our laboratory has demonstrated that the autoinduction of TNF mRNA by TNF protein in HL-60 cells is associated with increased phospholipase A_2 activity and arachidonic acid release.[2] In order to determine whether arachidonic acid metabolism is also involved in CSF-1 expression, we studied the signal transduction mechanisms responsible for regulating TNF-induced CSF-1 transcripts. The induction of CSF-1 RNA by TNF was examined after treatment of HL-60 cells with 4-bromophenacyl bromide (BPB) and quinacrine, inhibitors of phospholipase A_2, and eicosanoid production (26, 27). Both BPB and quinacrine inhibited TNF-induced increases in CSF-1 transcripts in a concentration-dependent manner. For example, 1 μM BPB inhibited TNF-induced increases in CSF-1 transcripts by 57%, whereas 5 and 10 μM BPB completely blocked this induction (Fig. 5 *A*). Similarly, 2 and 5 μM quinacrine inhibited the induction of CSF-1 mRNA by 38 and 64%, respectively,



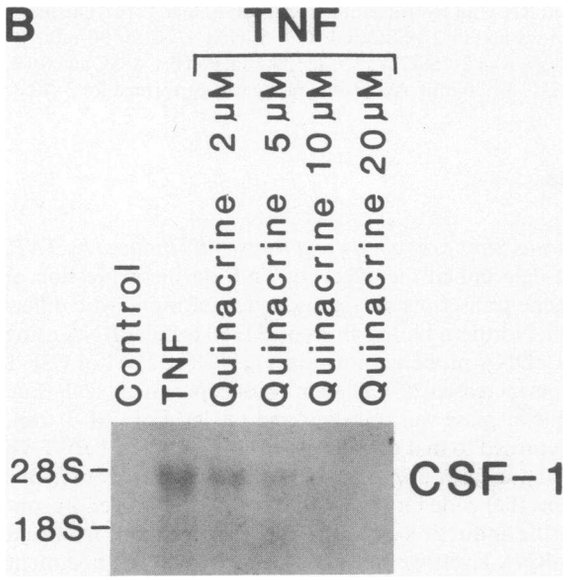


Figure 5. Effects of BPB and quinacrine on CSF-1 gene expression in TNF-treated HL-60 cells. (*A*) HL-60 cells were treated with TNF (100 U/ml) for 3 h in the presence of varying concentrations of BPB and monitored for CSF-1 RNA. (*B*) Graded doses of quinacrine were added concomitantly with 100 U/ml TNF for 3 h. Hybridizations were performed with ^{32}P-labeled CSF-1 probe. Control indicates RNA from untreated HL-60 cells.

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whereas 10 μM quinacrine completely inhibited the effects of TNF on CSF-1 gene expression (Fig. 5 *B*).

Arachidonic acid is the major substrate for 5-lipoxygenase (5-LO) and cyclooxygenase with formation of leukotrienes and prostaglandins, respectively. Thus, it was also of interest to determine the effects of these metabolites on CSF-1 expression. Leukotriene production is inhibited by the 5-LO pathway inhibitor, caffeic acid (28). Treatment of HL-60 cells with caffeic acid in the presence of TNF had no detectable effect on the induction of CSF-1 RNA (Fig. 6). Similar results were obtained with another 5-LO pathway inhibitor, nordihydroguaiaretic acid (NDGA) (29) (Fig. 6). Furthermore, treatment of HL-60 cells with the 5-LO metabolite leukotriene B_4 (LTB_4) failed to induce CSF-1 transcripts (data not shown).

Previous studies had shown that the cyclooxygenase metabolite PGE_2 is a potent regulator of monokine production (30). Thus, we first studied the effects of the cyclooxygenase inhibitor indomethacin (31) on CSF-1 gene expression. Indomethacin alone had no effect on CSF-1 expression, and in combination with TNF had no detectable effect on the induction of CSF-1 RNA (Fig. 7). However, PGE_2 treatment of TNF-induced HL-60 cells blocked the expression of CSF-1 RNA in a dose-dependent manner (Fig. 8 *A*). PGE_2 decreased the accumulation of CSF-1 transcripts by 78 and 89% at concentrations of 1 and 10 nM, respectively. Furthermore, treatment of HL-60 cells with 200 U/ml TNF and 1 μM PGE_2 inhibited CSF-1 gene transcription by 89% as compared with cells treated with TNF alone. Since we had previously shown (32) that TNF treatment of HL-60 cells had no significant effect on the production of cAMP and that PGE_2 increased cAMP levels in both untreated and TNF-stimulated HL-60 cells, it was also of interest to determine if the effects of PGE_2 on CSF-1 transcripts could be mimicked by the addition of exogenous cAMP. Indeed, decreases in the induction of CSF-1 transcripts by TNF were observed in HL-60 cells treated with dibutyryl cAMP (Bt_2cAMP) (Fig. 8 *B*).

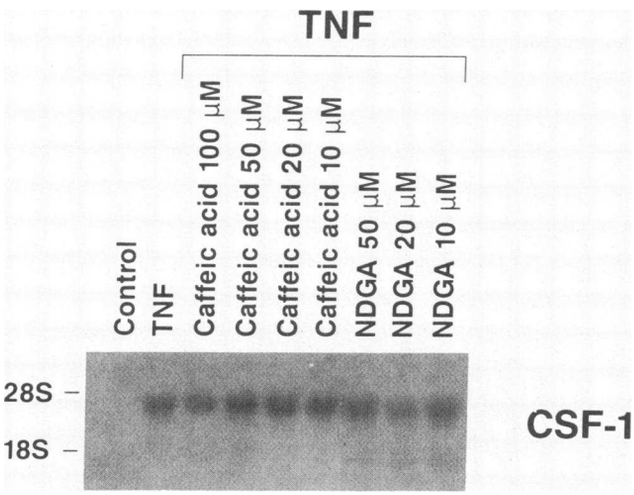

Figure 6. Effects of lipoxygenase inhibitors on the induction of CSF-1 transcripts by TNF. HL-60 cells were treated with TNF (200 U/ml) for 3 h and in the presence of varying doses of caffeic acid or NDGA. Total cellular RNA (20 μg) was isolated for Northern blot analysis. The control lane represents RNA from untreated HL-60 cells.

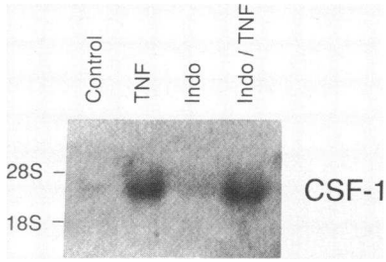

Figure 7. Effects of cyclooxygenase inhibition on the induction of CSF-1 transcripts by TNF. HL-60 cells were treated with TNF (200 U/ml) for 3 h and in the presence of 1 μM indomethacin (Indo). Hybridizations were performed with ^{32}P-labeled CSF-1 probe. Control indicates RNA from untreated HL-60 cells.

Discussion

TNF induces HL-60 cells to undergo monocytic differentiation. In this regard, HL-60 cells treated with TNF express histochemical, morphological, and functional characteristics of the monocytic lineage (17, 33). The effects of TNF on the regulation of gene expression during HL-60 cell differentiation have been less clearly defined. TNF treatment of HL-60 cells is associated with a down regulation of c-myc gene expression at the transcriptional level (18, 19). Recent studies have also demonstrated the autoinduction of TNF gene expression by TNF in HL-60 cells.[2]

The present results indicate that TNF induces transcriptional activation of the CSF-1 gene and appearance of CSF-1 transcripts in HL-60 cells. This induction of CSF-1 transcripts was also associated with expression of the CSF-1 receptor gene. Recent studies have demonstrated that phorbol diesters regulate CSF-1 gene expression by transcriptional and posttranscriptional mechanisms (9). The present findings suggest that similar effects may play a role in the regulation of this gene by TNF. TNF treatment of HL-60 cells was associated with transcriptional activation of the CSF-1 gene. Furthermore, the results indicate that posttranscriptional mechanisms requiring de novo protein synthesis regulate CSF-1 gene expression in TNF-treated cells. In this regard, inhibition of protein synthesis by cycloheximide had no effect on CSF-1 expression in untreated HL-60 cells, but increased levels of CSF-1 transcripts by 3.4-fold during TNF treatment. Furthermore, although cycloheximide had no effect on CSF-1 gene transcription, this agent did prolong the half-life of the CSF-1 transcript. This posttranscriptional regulation of CSF-1 mRNA levels may be related to the presence of several short AU-rich sequences in the 3′ untranslated region of its mRNA (23). These sequences appear to mediate selective processing and degradation of mRNAs for certain other cytokines and proto-oncogenes (34, 35).

The interaction of growth factors with their receptors is associated with the generation of second messengers. Our previous work has shown that autoinduction of TNF gene expression is mediated by phospholipase A_2 activity and arachidonic acid metabolites.[2] In this regard, TNF mRNA is induced by the 5-LO metabolite LTB_4 in HL-60 cells. The present results demonstrate that induction of CSF-1 transcripts by TNF is also mediated by the arachidonic acid cascade. Inhibition of phospholipase A_2 activity by BPB or quinacrine blocked TNF-induced expression of CSF-1 RNA. However, in contrast to the autoinduction of TNF mRNA, the present

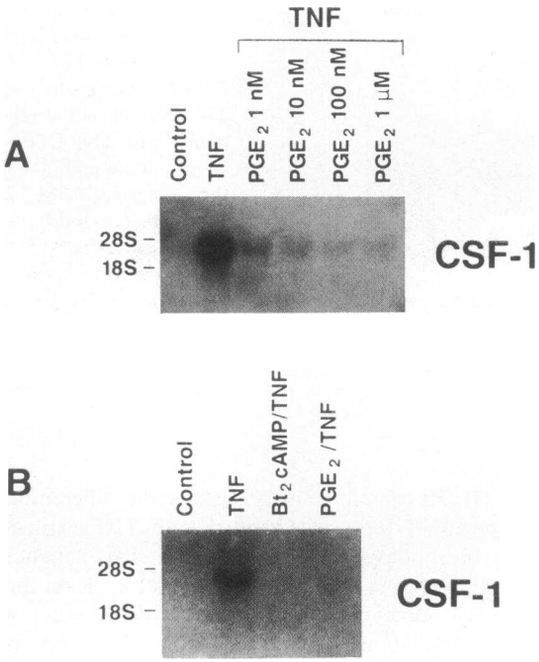

Figure 8. Effects of PGE_2 on TNF induction of gene expression. (*A*) HL-60 cells were treated with TNF (200 U/ml) for 3 h alone and in the presence of varying concentrations of PGE_2, and monitored for CSF-1 RNA by Northern blot analysis. (*B*) HL-60 cells were treated with TNF (200 U/ml) for 3 h alone and in the presence of 500 μM Bt_2cAMP or 1 μM PGE_2. Total cellular RNA (20 μg) was isolated for Northern blot analysis. The control lane represents RNA from untreated HL-60 cells.

studies indicate that inhibition of 5-LO activity with caffeic acid or NDGA had no detectable effect on the induction of CSF-1 transcripts. Furthermore, while the 5-LO metabolite LTB_4 induced TNF expression, there was no increase in CSF-1 transcripts detected after treatment of HL-60 cells with this leukotriene. Taken together, these findings would suggest that in contrast to the activation of TNF gene expression, other eicosanoids are probably required for inducing CSF-1 transcripts in these cells. For example, arachidonic acid is also a substrate for the 15-lipoxygenase (15-LO) enzyme with production of lipoxins A_4 and B_4. The lipoxins are potent activators of protein kinase C (36) and in view of our findings that phorbol esters induce CSF-1 transcripts (9), these 15-LO metabolites may be involved in the regulation of CSF-1 expression.

PGE_2 generated by cyclooxygenase metabolism of arachidonic acid is a potent immunomodulatory agent which mediates cell function by transducing information via the second messenger cAMP. Murine macrophages possess PGE_2-specific receptors and prostaglandin-sensitive adenylate cyclase. TNF treatment of resting macrophages or synovial cells releases PGE_2 into the supernatant (37, 38). Furthermore, PGE_2 treatment of murine peritoneal macrophages blocks LPS-induced expression of TNF mRNA (30). Previous studies in a murine osteoblast-like cell line have demonstrated that TNF stimulates production of PGE_2 and macrophage-colony stimulating activity (39). However, while indomethacin abolished TNF-induced PGE_2 production in these cells, there was little, if any, effect on induction of colony stimulating activity (39).

In the present studies, inhibition of the cyclooxygenase pathway had no effect on induction of CSF-1 mRNA. However, PGE_2 was a potent inhibitory signal for the induction of CSF-1 transcripts by TNF. Bt_2cAMP also inhibited the induction of CSF-1 transcripts. These findings suggested that the effects of PGE_2 may be due, at least in part, to cAMP metabolism. Recent results have demonstrated that the induction of TNF mRNA is also inhibited by the cyclooxygenase metabolite PGE_2 (40). Thus, although the early events that induce CSF-1 mRNA transcription appear to be distinct from that for the induction of TNF transcripts, cAMP represents a common inhibitory signal for induction of both the CSF-1 and TNF genes.

TNF-induced stimulation of phospholipase A_2 and production of arachidonic acid is thus associated with induction of several genes involved in monocytic differentiation. Arachidonic acid formation is the rate-limiting step in the synthesis of multiple metabolites including leukotrienes, lipoxins and prostaglandins. This cascade of secondary messengers appears to be involved in the regulation of genes required for the proliferation, differentiation, and activation of monocytes.

Acknowledgments

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