Regulation of c-myc Expression by Granulocyte-Macrophage Colony-Stimulating Factor in Human Leukemia Cells

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates both the proliferation and functional properties of normal and leukemic myeloid cells via cell surface receptors. The postreceptor mechanisms for these two actions, and the extent to which they represent overlapping biochemical pathways, have not been fully clarified. We have examined the actions of GM-CSF on the expression of c-myc, an early response oncogene associated with the proliferative stimulus of growth factors. GM-CSF reduced the population doubling time of HL-60 leukemia cells from 32 hours to 25 hours, and, at concentrations that were correlated with mitogenicity, induced a rapid twofold increase in the level of c-myc mRNA. Nuclear runoff studies indicated that GM-CSF approximately doubled the transcription rate of c-myc by reversing the transcription attenuation that occurs at the exon 1-intron 1 junction. GM-CSF had no effect on the half-life of c-myc messenger RNA. The biochemical basis for the modulation of c-myc expression by GM-CSF was explored. GM-CSF treatment caused intracellular alkalinization.

The recombinant human polypeptide granulocyte-macrophage colony-stimulating factor (GM-CSF) has several distinctive biologic effects on normal and transformed hematopoietic cells. GM-CSF is a growth factor for committed myeloid stem cells both in vitro and in vivo, it potentiates the proliferative activity of other factors, and it maintains cell viability, induces the differentiation, and activates the functional properties of mature granulocytes and macrophages. A role for GM-CSF in leukemia is suggested by its ability to stimulate colony formation from blast cells from patients with acute and chronic myeloid leukemia, and the identification of several patients whose leukemic blast cells produce autocrine GM-CSF. These biologic actions of recombinant GM-CSF mimic those of its natural counterpart, and are also observed on the treatment of human leukemia cell lines in vitro.

The cellular mechanisms by which GM-CSF mediates its multiple actions have not been determined. Both high- and low-affinity receptors have been detected on human hematopoietic cells, and a low-affinity receptor from human placenta has been cloned and characterized. The majority of studies evaluating the cellular actions of GM-CSF, including postreceptor events, have examined the mechanism by which GM-CSF primes normal granulocytes to the actions of chemotactic peptides. GM-CSF does not directly affect phagocytosis or superoxide production in neutrophils, but rather increases the cellular response to stimulants such as formyl-methionyl-leucyl-phenylalanine (fMLP). GM-CSF presumably acts on the cascade of events subsequent to fMLP binding to the cell surface, and modulation of several signal transduction pathways has been reported. The signal transduction pathway that modulates the growth stimulatory actions of GM-CSF has also been investigated.

GM-CSF stimulates the proliferation and clonogenicity of HL-60 human leukemia cells, under certain conditions can promote the differentiation of the cells, and also can potentiate the functional properties of the cells in response to fMLP (E.L. Schwartz, unpublished observation). There is a fivefold increase in the number of cell surface GM-CSF receptors as the HL-60 cells undergo granulocytic differentiation that is accompanied by an increase in mitogenic responsiveness to the growth factor. HL-60 cells have an amplified copy number of the c-myc oncogene, and c-myc expression has been closely linked to HL-60 growth and differentiation. Treatment of HL-60 cells with inducers of differentiation leads to a rapid reduction in c-myc expression, and inhibition of c-myc with antisense oligonucleotides causes growth arrest and differentiation of the cells. We have investigated the mechanisms of GM-CSF signal transduction in the context of the mitogenic actions of the growth factor. In this study, we report that GM-CSF stimulates c-myc expression in HL-60 cells; furthermore, the transcriptional regulation of c-myc by GM-CSF appears to be directly opposite to the attenuating actions of the inducers of differentiation.

MATERIALS AND METHODS

Reagents. Human recombinant GM-CSF was a gift of Genetics Institute (Cambridge, MA) and was judged to be 100% pure by gel electrophoresis and high pressure liquid chromatography. It was stored in aliquots at -20°C. Actinomycin D, isoproterenol, cholera...
toxin, prostaglandin E₂ (PGE₂), and amiloride were obtained from Sigma (St Louis, MO). Pertussis toxin was obtained from List Biological Labs (Campbell, CA), and the acetoxyethyl ester of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) from Molecular Probes (Eugene, OR).

Cells. HL-60 human leukemia cells were obtained from Dr R.C. Gallo (National Cancer Institute) and were cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (GIBCO, Grand Island, NY). Cells were resuspended weekly at 10⁶/mL and mid-log phase cells, between passages 25 and 50, were used for all experiments. For experiments using defined medium, cells were washed twice and resuspended in RPMI 1640 supplemented with ITS+ (Collaborative Research, Bedford, MA), which contains insulin, transferrin, selenium, bovine serum albumin, and linoleic acid. Cell concentration was determined with a Coulter Counter (Hialeah, FL).

RNA extraction and analysis. Cells were resuspended at 10⁶/mL and were treated with GM-CSF for, except when indicated, 5 hours. RNA was extracted and guanidinium thiocyanate and purified by centrifugation through CsCl, as has been described. The RNA was fractionated on formaldehyde-containing agarose gels, transferred to nitrocellulose, and probed with ³²P-labeled c-myc plasmid (pHSR; obtained from American Type Culture Collection, Rockville, MD). Blots were washed at 55°C in 0.1X SSC and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at 85°C with intensifying screens. Blots were stripped and reprobed with ³²P-labeled actin cDNA. Autoradiographs were quantitated by scanning on a Hoeffer (San Francisco, CA) densitometer equipped with a Hewlett-Packard integrator.

Nuclear runoff assay. Nuclear runoff labeling of nascent RNA transcripts was performed using previously described procedures with some modifications. Cells were lysed in buffer A (10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L NaCl, 5 mmol/L MgCl₂) containing 0.5% NP-40, and nuclei were isolated by centrifugation (15,000 rpm for 10 minutes) through buffer A containing 1.5 mol/L sucrose. Nuclei were resuspended in buffer A containing 1 mmol/L dithiothreitol and 40% glycerol, and were stored in liquid nitrogen. Labeling and purification of RNA was performed exactly as previously described using 2 × 10⁶ nuclei and 0.1 mCi ³²P-UTP (800 Ci/mmol; New England Nuclear, Boston, MA).

Combinant M13 phage containing human c-myc fragments were kindly provided by Drs R. Dalla Favera and M. Groudine. The DNA inserts were as follows: a 535-bp Nsi I/SmaI fragment upstream from the c-myc transcription initiation site; a 443-bp Xho I/Pvu II fragment from exon 1; a 430-bp Sst I fragment from intron 1; and a 414-bp Sst I/Pvu II fragment from exon 2. M13 single-stranded c-myc sense DNA was isolated from culture supernatants and 2.5 µg was immobilized on nitrocellulose filters using a slot blot apparatus (Schleicher & Schuell, Keene, NH), as has been described.

The filters were prehybridized in 5X SSC with 0.5% nonfat dry milk for 2 hours. Purified labeled RNA (equal cpm from control and GM-CSF-treated cells) in the same buffer were then added, and hybridization was continued for 48 hours at 65°C. Filters were washed in 0.1X SSC, 0.1% sodium dodecyl sulfate (SDS) at 60°C, followed by incubation with RNase (10 µg/mL in 2X SSC for 30 minutes at 37°C). Filters were exposed to Kodak XAR film at −85°C with intensifying screens, and autoradiograms scanned with a densitometer.

Intracellular pH measurement. Intracellular pH was measured based on the fluorescence of the pH-sensitive probe BCECF, using methods similar to those previously described. HL-60 cells were incubated with 5 µmol/L BCECF-AM in Dulbecco’s phosphate-buffered saline (D-PBS) for 5 minutes at 37°C. Cells were centrifuged and incubated in D-PBS for 15 minutes at 25°C. Fluorescence emissions at 530 nm with excitation alternatively at 500 nm and 440 nm were measured in cell aliquots, and were expressed as the 500/440 ratio. Measurements were performed for at least 10 minutes before the addition of GM-CSF to the cuvettes; the effect of the GM-CSF was assayed for 30 minutes.

Cyclic nucleotide measurements. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels in cell extracts were measured by radiolimunosay using commercially available kits (Amersham, Arlington Heights, IL).

The limit of sensitivity of the assays was 2 fmol. HL-60 cells were suspended at 5 × 10⁶/mL, and were incubated in fresh medium (RPMI 1640/10% FBS) for 3 hours. GM-CSF (2.5 nmol/L) was added and aliquots of cells were removed at the specified time intervals. Boiling hot assay buffer (0.05 mol/L sodium acetate, pH 5.8) was immediately added, and the samples were boiled for an additional 5 minutes, cooled on ice, and centrifuged. Duplicate supernatant aliquots were acetylated and assayed according to the manufacturer’s directions.

RESULTS

GM-CSF stimulates cell proliferation and c-myc expression. HL-60 cells exhibited exponential growth in suspension culture that was enhanced, but not dependent on, the presence of FBS. Recombinant human GM-CSF increased the rate of HL-60 cell proliferation in FBS, reducing the population doubling time during log phase growth from 32 to 25 hours (Fig 1). In contrast, the effect of GM-CSF on doubling time was substantially blunted in serum-free defined medium (DM).

HL-60 cells contain amplified copies of the c-myc gene and express relatively high levels of a 2.5 kb c-myc messenger RNA (mRNA). GM-CSF increased the cellular levels of c-myc mRNA in a concentration and time-dependent manner (Figs 2 and 3), with a twofold increase in mRNA levels at 2.5 nmol/L GM-CSF that was first observed after 1
I c-myc

Fig 2

Northern RNA blot of c-myc and actin levels in GM-CSF-treated HL-60 cells. HL-60 cells were suspended at 10^6/mL and treated with the indicated concentrations of recombinant human GM-CSF (0 to 2.5 nmol/L) for 3 or 5 hours. RNA was extracted by the guanidinium thiocyanate method as described in Materials and Methods, and 8 µg of RNA was fractionated by electrophoresis on formaldehyde-containing agarose gels. After transfer to nitrocellulose, the blots were successively probed with nick-translated c-myc and actin DNA, as indicated, and exposed to Kodak XOMat film at -85°C.

Fig 3. Quantitative analysis of autoradiographs of c-myc mRNA after GM-CSF treatment. RNA was extracted from HL-60 cells and analyzed for c-myc and actin levels as described in Fig 2. Band densities on the autoradiographs were determined with a densitometer and recording integrator. All values for c-myc were first corrected for actin, and then expressed relative to the c-myc level of control cells in RPMI 1640/10% FBS medium equal to 1.00. (A) Cells in medium with 10% FBS (■) or serum-free DM (□) were treated for 3 hours with the indicated concentrations of GM-CSF. (B) Time course of cells in FBS-containing medium treated with 2.5 nmol/L GM-CSF. Values are means ± SEM of three experiments.

The elevation in c-myc RNA is due to transcriptional activation. The cellular levels of c-myc mRNA are a function of the processes of gene transcription, RNA processing, and mRNA degradation. Hence, agents such as GM-CSF can modulate c-myc mRNA levels by a number of potential mechanisms. To examine the effect of GM-CSF on c-myc stability, RNA was isolated from control and GM-CSF–treated cells at 15-minute intervals after the addition of a concentration of actinomycin D that was sufficient to inhibit RNA synthesis. As has been previously reported for this and other cell lines, c-myc mRNA has a relatively short half-life of approximately 0.5 hours (Fig 4). GM-CSF had no effect on the stability of c-myc mRNA, nor did it affect the half-life of the actin mRNA (Fig 4).

Transcription was assessed in HL-60 cell nuclei by synthesizing radiolabeled RNA under conditions in which transcription elongation, but not initiation, occur. RNA synthesized in this manner was then hybridized to immobilized single-stranded DNA fragments representing defined regions of the c-myc sense strand (Fig 5A). These 414 to 535 base DNA fragments had been cloned in M13, were roughly equally spaced along the c-myc gene, and contained sequences from exon 1, intron 1, exon 2, and upstream from the transcription initiation site (P0). Results from a representative experiment, with nuclear runoff RNA isolated from cells treated for 0.5 or 1 hour with or without GM-CSF, are shown in Fig 5B and C.

Under control conditions, the majority of runoff transcription occurs within the region containing the exon 1 sequences, with a large decrease in transcription in downstream regions and little or no transcription on the upstream sense region. GM-CSF caused an overall increase in c-myc transcription; however, runoff transcription on exon 1 remained constant. GM-CSF treatment did stimulate a progressive increase in transcription on intron 1 and exon 2 at 0.5 and 1 hour, with a greater than 2.5-fold increase at 1 hour. In contrast to the control nuclei, there was an approximately equal degree of transcription on exon 1,
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![Graph A](image)

**Fig 4.** Measurement of c-myc mRNA half-life by disappearance after actinomycin D. (A) HL-60 cells were incubated without (lanes 1 through 5) or with 2.5 nM GM-CSF (lanes 6 through 10) for 3 hours. Actinomycin D (5 μg/mL) was then added, and RNA was extracted from the cells at 15-minute intervals, as indicated. RNA blots were prepared as described in Fig 2, and were sequentially probed with 32P-labeled c-myc and actin. (B) Quantitative analysis of c-myc RNA levels on Northern blots. The half-life of c-myc mRNA was calculated as described in Materials and Methods. The nuclei were isolated from control (C) or 2.5 nM GM-CSF-treated (G) cells. The actin mRNA levels were used as an internal control for comparison between the two cultures. The data are representative of three experiments.

intron 1, and exon 2 in the nuclei of cells treated with GM-CSF for 1 hour.

**Signal transduction and c-myc expression.** The role of second messengers and potential signal transduction pathways in the control of c-myc expression was explored. Intracellular pH (pH_i) was measured based on the fluorescence of the pH-sensitive probe BCECF, after preloading of the cells with BCECF-AM. In all experiments, control and GM-CSF-treated cells were analyzed simultaneously and the difference in fluorescence calculated. Data from three experiments provide evidence for an increase in pH_i in HL-60 cells treated with GM-CSF (Fig 6). The antibiotic nigericin permits equalization of the pH_i concentration across the cell membrane, and, as has been described, can be used as a positive control.

Incubation of the cells with nigericin in buffers with pH ranging from 6.25 to 7.75 resulted in the expected increase in fluorescence (data not shown).

The role of the observed changes in pH_i in modulating the stimulation of c-myc expression was further explored using the inhibitor of Na^+ transport, amiloride. Sodium influx has been shown to be coupled to H^+ efflux via a plasma membrane antiporter. This exchange is a major pathway for Na^+ entry into cells and likely plays an important role in regulating intracellular pH. Amiloride is a sodium channel inhibitor that at high concentrations has a wide range of cellular effects, including inhibition of Na^+/H^+ and Na^+/Ca^{2+} exchange, Na^+K^+ ATPase, protein kinase C, protein tyrosine kinases, and adenylyl cyclase. Amiloride, at a concentration that inhibits sodium channels (0.3 mmol/L), blocked the change in pH_i observed with GM-

**Fig 5.** Nuclear runoff analysis of c-myc transcription with GM-CSF treatment. Nuclear runoff transcripts from control and GM-CSF-treated cells were hybridized to distinct single-stranded sense segments of the c-myc gene. (A) The four DNA segments used are shown relative to a map of the c-myc locus, with the three c-myc exons shown as open boxes. (B) Autoradiographs of 32P-labeled runoff RNA after hybridization to 2.5 μg immobilized DNA. The immobilized, single-stranded phage DNA was hybridized to sense transcripts. Nuclei were isolated from control (C) or 2.5 nM GM-CSF-treated (G) cells after 0.5 or 1 hour. Transcription from 2 x 10^7 nuclei and RNA purification and hybridization were performed as described in Materials and Methods. (C) Densitometric analysis of autoradiograph in (B) with transcription of nuclei from GM-CSF-treated cells expressed relative to nuclei of control cells. Abscissa is length of GM-CSF treatment. There were no detectable transcripts hybridizing to the P1 segment of c-myc.
Fig 6. Effect of GM-CSF on pH. HL-60 cells were incubated with 5 μmol/L BCECF-AM in D-PBS for 5 minutes at 37°C. Cells were centrifuged and incubated in D-PBS for 15 minutes at 25°C. Fluorescence emissions at 530 nm with excitation alternatively at 500 nm and 440 nm were measured in cell aliquots, and were expressed as the F/F0 ratio. At the indicated time, GM-CSF (2.5 nmol/L) was added to the cuvettes; fluorescence was measured alternatively in the control and GM-CSF–treated cells for an additional 30 minutes. The F/F0 ratios for GM-CSF–treated cells relative to control cells are shown for three experiments (A). In some experiments, amiloride (0.3 mmol/L) was added at time −10, and the difference in the F ratio between amiloride + GM-CSF and amiloride-alone treated cells is shown (V).

Amiloride 0 0.3 1.0 0 0.3 1.0
GM-CSF − − − + + +

Amiloride treatment (Fig 6), but had no effect on c-myc levels in control or GM-CSF–treated cells (Fig 7). Higher concentrations of amiloride also did not block the actions of GM-CSF on c-myc.

The ability of a variety of agents that can increase cellular cAMP levels to modulate the actions of GM-CSF was examined. Three compounds, cholera toxin, isoproterenol, and PGE2, substantially lowered c-myc levels in GM-CSF–treated cells (Fig 8). However, a proportional decrease in c-myc was observed under control conditions in the absence of GM-CSF. Interestingly, pertussis toxin had no effect on c-myc mRNA levels either in control or GM-CSF–treated cells (Fig 8). The possibility that cyclic nucleotides might be involved in the GM-CSF signalling pathway was further explored by measuring cellular levels of cAMP and cGMP by radiomunoassay. Transient decreases of 35% and 45% in cGMP and cAMP levels, respectively, occurred upon treatment with GM-CSF (Fig 9). cGMP declined rapidly, reaching a trough at 5 minutes after GM-CSF and returning to control levels by 20 minutes. In contrast, the decline in cAMP occurred over 30 minutes, and did not return to control levels until 180 minutes.

DISCUSSION

The HL-60 leukemia cell has a relatively high level of c-myc expression that is further increased by GM-CSF. GM-CSF also stimulates the proliferation of the HL-60 cells both in suspension culture and in semisolid medium.3,8 In addition to its mitogenic activity, HL-60 cells that have been induced to undergo differentiation can be “primed” by GM-CSF for an enhanced response on stimulation with fMLP (E.L. Schwartz, unpublished observation). Hence, the cell line combines two of the responses to GM-CSF observed in either normal myeloid stem cells or fully mature human neutrophils.13,27 Although the induction of HL-60 cell differentiation causes a fivefold increase in the number of cell surface GM-CSF receptors,6,9 it is not known what the role of this increase, other changes in receptor characteristics, or varying signal transduction mechanisms might be in the two distinctive cellular actions of GM-CSF.

Amplification and high expression of c-myc appears to be critical to the neoplastic phenotype of the HL-60 cell. The amplification was present in the original leukemia cell isolate,10 and antisense oligonucleotides to c-myc inhibit cell proliferation and induce differentiation of the cell line.8,15 The level of c-myc mRNA declines with a half-life of less than 0.5 hours on treatment of HL-60 cells with dimethyl sulfoxide (DMSO) and other inducers of differentiation.13,14,15 This rapid decrease in c-myc transcription is due to a reversible block to elongation that occurs in the region of the exon 1-intron 1 border.16,17 After approximately 48 hours with differentiation inducers, an additional, apparently irreversible inhibition of transcription initiation oc-
The control of *c-myc* mRNA expression by GM-CSF appears to be similar in nature but opposite in direction from that of the inducers of differentiation. The increase in *c-myc* mRNA expression with GM-CSF occurs over a period of 2 hours and is due to transcriptional rather than posttranscriptional events. The transcriptional activation is due primarily to the reversal of the *c-myc* elongation block; inducers of differentiation inhibit *c-myc* expression by enhancing the elongation block at this same loci. These observations are consistent with the hypothesis that *c-myc* stimulation by GM-CSF is mechanistically related to the actions of the hematopoietic growth factor.

Recent studies have provided some insight into the mechanisms of GM-CSF signal transduction. In human neutrophils, GM-CSF potentiated the membrane depolarizing action of fMLP without having a direct effect of its own on membrane potential; however, this action was not sensitive to inhibitors of Na⁺ or K⁺ channels, nor was it required for the augmentation of superoxide production. GM-CSF increased the sensitivity of the cells to intracellular Ca²⁺ for activation of superoxide production, but did not directly elevate cytosolic free Ca²⁺ nor affect the release of Ca²⁺ when used with fMLP. The actions of GM-CSF appear not to be due to effects on diacylglycerol production, phosphatidyl-inositol levels, or protein kinase C activity, but were associated with an increased release of arachidonic acid.

Although some of the biochemical actions of GM-CSF in neutrophils were blocked by pertussis toxin, it had no effect on *c-myc* expression in our studies with HL-60 cells, indicating that this action of GM-CSF is not mediated either directly or indirectly by a pertussis toxin-sensitive step. In agreement with our studies in HL-60 cells, GM-CSF was previously found to cause the intracellular alkalization of human neutrophils and AML-193 leukemia cells; the change in pH in all three cell types develops slowly, reaching a maximal increase over 20 to 30 minutes. Although amiloride did not affect the expression of *c-myc* in response to GM-CSF in our studies, an amiloride analog did inhibit GM-CSF-stimulated proliferation of the AML-193 cells. In contrast to the AML-193 cells, which are strictly dependent on GM-CSF for proliferation, the HL-60 cells have circumvented the requirement for exogenous growth factors for proliferation; hence, they may also have developed alternative pathways for some of the biochemical components of the GM-CSF signal transduction pathway. Alternatively, there are qualitative and quantitative differences in the Na⁺/H⁺ exchanger that occur with HL-60 differentiation, which suggests that the stage of differentia-
tion of the cells could influence their sensitivity to Na+ channel blockers.44

Agents that elevate cAMP or cGMP inhibit proliferation and induce and potentiate the differentiation of HL-60 cells.5,47 Stimulation of cAMP also causes a large decline in the basal level of c-myc expression56,69 (Fig 8). These observations render problematic the interpretation of the observed effect of cholera toxin, isoproterenol, and PGE2 on GM-CSF-induced changes in c-myc expression. The reduction seen in cAMP and cGMP levels with GM-CSF treatment are consistent with, but not proof of, a role for these second messengers in the actions of GM-CSF. GM-CSF also causes a reduction in adenylate cyclase activity, but an increase in guanylate cyclase activity in normal human neutrophils.21 Recent studies have shown that an antisense oligonucleotide to the mRNA of the type II regulatory subunit of the cAMP-dependent protein kinase substantially reduced the level of this cAMP receptor in HL-60 cells.50 The antisense oligonucleotide had no direct effect on cell proliferation, but blocked the anti-proliferative actions of cAMP and potentiated the proliferative actions of GM-CSF.52 In contrast, GM-CSF caused an elevation in the levels of the RIIa subunit of the protein kinase, and an antisense oligonucleotide to the RIIa subunit antagonized the actions of GM-CSF.31 These studies provide direct evidence for the role of cAMP and cAMP-dependent protein kinase in mediating the actions of GM-CSF, but also illustrate the complexity of the system. Further studies are required to establish the role of cyclic nucleotides in GM-CSF signal coupling pathways.

REFERENCES

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Regulation of c-myc expression by granulocyte-macrophage colony-stimulating factor in human leukemia cells

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