Enhanced Expression of Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor Receptor Subunits in Murine Hematopoietic Cells Stimulated With Hematopoietic Growth Factors

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AIC2A and AIC2B are closely related genes encoding components of the receptors for murine interleukin-3 (IL-3) (AIC2A) and granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 (AIC2B). We have studied the parallel regulation of expression of these genes in erythroid and myeloid progenitor cell lines. AIC2A and AIC2B transcription was transiently induced in these cells in response to a variety of hematopoietic growth factors, including erythropoietin (EPO), monocyte-CSF, IL-3, GM-CSF, and stem cell factor (SCF or kit ligand). Run-on assays established that the increase occurred mainly at the transcriptional level. Immunoprecipitation experiments confirmed that the increase in messenger RNA expression resulted in augmented synthesis of both AIC2A and AIC2B proteins, and binding studies further showed these proteins to be functional. We observed a fourfold increase in low-affinity IL-3 sites in an erythroid precursor cell line stimulated with EPO, and a threefold increase in GM-CSF high-affinity sites in a myeloid cell line stimulated with IL-3. In addition, we showed that the increase in the IL-3 receptor chain AIC2A in the erythroid precursor cell line correlated with the ability of IL-3 to exert a cooperative effect with EPO in the induction of β-globin in these cells.

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PROLIFERATION and differentiation of hematopoietic cells are mediated by a hierarchy of general and specialized hematopoietic growth factors (HGFs). Among these, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to act primarily on early progenitors, whereas erythropoietin (EPO), granulocyte-CSF (G-CSF), and monocyte-CSF (M-CSF) appear to act at later stages of differentiation. As multiple HGF receptors are displayed on the surface of progenitor cells, ordered differentiation requires that expression and activation of the receptors be specific and tightly regulated. Walker et al. reported that IL-3 could downmodulate receptors for GM-CSF, M-CSF, or G-CSF on mouse bone marrow cells. Likewise, GM-CSF could downmodulate M-CSF and G-CSF receptors. The later-acting growth factors M-CSF and G-CSF modulated the expression of GM-CSF and M-CSF receptors only when present in high concentrations. This hierarchical pattern of HGF-induced receptor modulation suggested a model for growth factor regulation of differentiation. Although it provided an explanation for the capacity for IL-3 to promote differentiation in diverse lineages, it did not fully explain the synergism of action that early and late-acting factors have on progenitor cells. Indeed, G-CSF and IL-6, in combination with IL-3, have been shown to enhance the ability of progenitor cells to form colonies in vitro. Similarly, EPO plus IL-3, and G-CSF plus IL-3 or plus GM-CSF act synergistically in erythroid colonies and granulocyte colony assays, respectively. Little is known about the mechanisms mediating these synergistic effects, but it is believed that HGFs can influence the expression and activation of nonisolated receptors.

We have investigated whether expression of components of the IL-3 and GM-CSF receptors (AIC2A and AIC2B, respectively) could be regulated by the action of the later-acting HGFs EPO and M-CSF, as well as by the earlier acting HGFs IL-3 and GM-CSF and by kit ligand, which appears to function in both the early and late stages of hematopoiesis. AIC2A encodes the low-affinity binding chain of the mouse IL-3 receptor, whereas the 95% homologous AIC2B gene encodes a shared subunit that combines with the low-affinity binding chains of GM-CSF and IL-5 receptors and confers high-affinity binding. We examined expression of AIC2A and AIC2B in a murine erythroid precursor cell line, J2E, and a phagocytic precursor cell line, 32D. We found that messenger RNA (mRNA) expression of both AIC2A and AIC2B transiently increased when cells were stimulated by EPO and M-CSF, as well as by several other growth factors. This transcriptional induction directly affected HGF receptor populations on the cell surface. In J2E cells, upmodulation of expression led to an increase in low-affinity receptors to IL-3, attributable to AIC2A expression. Analogously, in 32D cells, stimulated AIC2B gene expression resulted in an increased number of GM-CSF high-affinity binding sites. Furthermore, costimulation of J2E cells with EPO and IL-3 enhanced their ability to synthesize β-globin, indicating that these cells were pushed down a pathway towards erythroid differentiation. Interestingly, longer treatment of J2E cells with EPO eventually led to destabilization of AIC2A and AIC2B transcripts, consistent with the notion that multipotential growth factor receptors were no longer needed.

We suggest that regulation of AIC2A and AIC2B genes by HGFs may be central to the synergistic effects of IL-3 and GM-CSF in combination with later-acting growth factors, such as EPO, M-CSF, and G-CSF, and may operate in the regulation of normal hematopoietic differentiation.
MATERIALS AND METHODS

Cell Culture, Serum-Free Conditions, and Growth Factors

Cells. J2E cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) in 5% CO2 at 37°C. BaF3-EPOR cells (kindly provided by Dr A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA) and the phagocytic precursor clone 32D c12314 were grown in RPMI/10% FCS supplemented with growth factors as indicated in the text. BaF3-EPOR cells (kindly provided by Dr A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA) and the phagocytic medium (DMEM) with 10% fetal calf serum (FCS) in 5% CO2 at 37°C. BaF3-EPOR cells (kindly provided by Dr A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA) and the phagocytic medium (DMEM) with 10% fetal calf serum (FCS) in 5% CO2 at 37°C. BaF3-EPOR cells (kindly provided by Dr A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA) and the phagocytic medium (DMEM) with 10% fetal calf serum (FCS) in 5% CO2 at 37°C. BaF3-EPOR cells (kindly provided by Dr A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA) and the phagocytic medium (DMEM) with 10% fetal calf serum (FCS) in 5% CO2 at 37°C.

DNA and RNA Probes

AIC2A DNA. Total RNA was isolated from BaF3 cells, and polyA+ RNA was prepared by guanidinium extraction. Two micrograms of polyA+ mRNA was subjected to first- and second-strand cDNA synthesis according to the manufacturer's instructions (GIBCO-BRL, Grand Island, NY). An AIC2A-specific 2.67-kb fragment was amplified by PCR from total BaF3 cDNA. This fragment starts 27 nucleotides upstream of the initiating ATG and extends 6 nucleotides past the termination codon.8

The 5' primer (5'-GGGCTCGAGTCCTGTGCAGCCAC-CA A A A G-3') and 3' primer (5'-GGGCTCGAGGGTAGTCTTAAATCTTTAAGG-3') included Xho I restriction sites for subcloning into the expression vector, pXM. The identity of the amplified fragment was confirmed by partial sequencing and IL-3 binding experiments with COS cells transfected with this DNA.

v-myc and EPO receptor (EPO-R) DNAs. The v-myc (OK10) DNA was a 2.5-kb BamHI fragment isolated from MMCV virus DNA.15 The EPO-R cDNA was a kind gift of Dr Alan D'Andrea (Dana-Farber Cancer Institute).16

RNase protection probes. For AIC2, a SacI-Sau3AI fragment (position 410 to 536 in AIC2A CDNA) was isolated and inserted into the BamHI-Sac sites of the SP72 polylinker. Antisense probe was synthesized with SP6 polymerase after linearization with EcoRI. DNA for the β-globin probe was kindly provided by Richard Mulligan (Whitehead Institute, Cambridge, MA).17 The DNA was linearized with EcoRI and antisense RNA was obtained with SP6 polymerase.

Cell Stimulation, RNA Extraction, and Analysis

RNA extraction. Total RNA was obtained by the guanidinium isothiocyanate (GITC) method after centrifugation through cesium chloride.18

Northern blot. Fifteen micrograms of total RNA was fractionated on a 1% agarose formaldehyde gel, transferred to nitrocellulose filter, and hybridized as previously described.19

RNase protection assay. Twenty-five micrograms of total RNA was resuspended in 31 μL of hybridization buffer (80% [vol/vol] formamide, 40 mmol/L PIPEs, pH 6.4, 400 mmol/L NaCl, 1 mmol/L EDTA) containing the indicated 32P-labeled RNA probe. Hybridization, digestion with RNase A and T1, and digestion with proteinase K were performed as described.20 Samples were resuspended in formamide-dye mix, denatured at 90°C, and loaded on a 6% Tris borate EDTA (TBE) urea/acylamide gel.

Nuclear Run-On Assay

J2E cells (106) were incubated for 0, 4, and 24 hours with EPO and then lysed in 30 mL of lysis buffer (50 mmol/L Tris, pH 7.5, 100 mmol/L KCl, 5 mmol/L MgCl2, 15% glycerol, 1 mmol/L diithiothreitol [DTT], and 0.5% NP-40). Nuclei were resuspended in 200 μL of lysis buffer that lacked NP-40, and were used immediately for RNA extraction in the presence of α-32P-UTP as previously described.21 Equal counts of the labeled RNAs were used for hybridization to filters carrying 0.5 μg of denatured AIC2A, MMCV (v-myc),15 pUC, and EPO-R16 DNA. Hybridization was performed at 47°C for 48 hours. Filters were washed, treated for 20 minutes at 25°C with 10 μg/mL of RNase A in 2X SSC, and autoradiographed for 2 days.

Metabolic Labeling and Immunoprecipitation

J2E cells were placed in methionine-free medium for 1 hour before labeling for 2 hours with 250 μCi of [35S]methionine (New England Nuclear [NEN], Boston, MA). Cells extracts were obtained as described.22 Aliquots were immunoprecipitated with a rat monoclonal antibody (MoAb), 18H6-14,23 that recognizes both AIC2A and AIC2B proteins (18H6-14 was a generous gift of Dr R. Devos, Roche Research, Gent, Belgium). Goat antirat antibody was added to improve binding of the immune complex to protein A-Sepharose beads. Bound immune complexes were washed and separated on a 6% sodium dodecyl sulfate (SDS) polyacrylamide gel. Gels were dried and treated with EnHance (NEN) and autoradiographed for 2 days.

RESULTS

The effect of hematopoietic growth factors on IL-3 receptor (IL-3R) gene expression was studied initially in the murine erythroid progenitor cell line J2E, which expresses a functional EPO-R.13 Although these cells are not factor dependent, their growth under serum-free conditions is enhanced if IL-3 is added to the medium, showing that these cells expressed a functional IL-3R.

To investigate whether EPO treatment would affect expression of the IL-3R gene, cells were treated with EPO and steady-state AIC2A and AIC2B mRNA was measured after various intervals by an RNase protection assay. A probe, complementary to AIC2A and derived from a region where the AIC2A and AIC2B genes differed at several contiguous residues, was used to discriminate between transcripts of the two genes. As shown in Fig 1, J2E cells expressed constitutively higher levels of AIC2B mRNA than of AIC2A. However, there was a marked increase in both AIC2A mRNA (127-nt protected fragment) and AIC2B mRNA (90-nt protected fragment) after 5 hours of...
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Nuclear run-on experiments were performed to determine whether the changes in AIC2A and AIC2B mRNA expression in J2E cells treated with EPO were transcriptional or posttranscriptional. As shown in Fig 2, the transcription rate of the AIC2A/AIC2B gene was significantly increased after 5 hours of EPO treatment, while little change in the rate of v-myc or EPO-R transcription was observed. Interestingly, the rate of transcription for the AIC2A/B gene was still elevated after 24 hours of EPO treatment. This contrasts with the barely detectable level of steady-state mRNA detected at 24 hours for these genes (Fig 1).

![Figure 1. Induction of AIC2A and AIC2B gene transcription in J2E cells stimulated with EPO. Determination of the AIC2A or AIC2B transcripts by RNase protection analysis. RNA extracted from J2E cells unstimulated or stimulated with EPO for 5, 24, or 48 hours was hybridized with a riboprobe (Materials and Methods) that discriminates between the two AIC2 transcripts. AIC2A (127n) represents the fully AIC2A-protected fragment. mRNA from AIC2B protects a smaller size fragment (90n). The 76nt band may result from an existing single base change between AIC2A and AIC2B or may be the product of an alternative splice. As a control, tRNA was hybridized with the same riboprobe and processed identically to the other samples.](image1)

As shown in Fig 2, the transcription rate of the AIC2A/AIC2B gene was significantly increased after 5 hours of EPO treatment, while little change in the rate of v-myc or EPO-R transcription was observed. Interestingly, the rate of transcription for the AIC2A/B gene was still elevated after 24 hours of EPO treatment. This contrasts with the barely detectable level of steady-state mRNA detected at 24 hours for these genes (Fig 1).

We next examined whether the increase in transcription led to an increase in AIC2A and AIC2B proteins. J2E cells, either unstimulated or stimulated with EPO for 6 hours, were metabolically labeled with 35S methionine. Cell extracts were immunoprecipitated with an MoAb reacting with both AIC2A and AIC2B proteins. As shown in Fig 3B, EPO treatment resulted in increased synthesis of both AIC2A and AIC2B proteins. Consistent with the mRNA data, the AIC2B gene product was more abundant than that of AIC2A. The identity of the upper band as AIC2B protein was inferred from the immunoprecipitation pattern obtained with a Ba/F3 cell extract that had been treated with an MoAb specific for AIC2A protein or with the same antibody used for the J2E cell extracts (Fig 3A).

![Figure 2. Nuclear run-on transcriptional analysis. Nuclei from untreated J2E cells and from J2E cells treated for 5 and 24 hours with EPO (1 U/mL) were isolated and labeled with [α-35S]UTP. Equal amounts of the radiolabeled RNA were used to hybridize nitrocellulose filters on which EPO-R, pUC, v-myc (which is expressed constitutively in these cells), and AIC2A/B DNAs had been previously immobilized. Filters were hybridized for 48 hours at 47°C and washed under stringent conditions. Filters were autoradiographed at -70°C.](image2)
We next tested whether AIC2 gene expression in J2E cells was under the specific regulation of EPO, or whether a similar effect could be observed with other hematopoietic growth factors such as SCF (kit ligand) or IL-3. To ensure that trace amounts of EPO (present in FCS) did not interfere with our analysis, the cells were switched to a defined medium that lacked serum. Under these conditions, cells could proliferate for up to 3 days without any adverse effect (data not shown). For these experiments, the cells were maintained for 1 day in defined medium, and were subsequently stimulated with SCF or IL-3 for 5 hours. After stimulation, RNA was isolated and analyzed by Northern blot with a probe recognizing both AIC2A and AIC2B. As shown in Fig 5, the effect was not restricted to EPO, and could be achieved with either SCF or IL-3.

To examine regulation of AIC2A and AIC2B by M-CSF or GM-CSF, we studied the effect of HGF stimulation on 32D cells. These cells can differentiate into phagocytic lineages and express M-CSF and GM-CSF receptors. Accordingly, 32D cells were washed free of IL-3 and resuspended for 2 hours in the absence of all growth factors. Under these conditions, cells could proliferate for up to 3 days without any adverse effect (data not shown). For these experiments, the cells were maintained for 1 day in defined medium, and were subsequently stimulated with SCF or IL-3 for 5 hours. After stimulation, RNA was isolated and analyzed by Northern blot with a probe recognizing both AIC2A and AIC2B. As shown in Fig 5, the effect was not restricted to EPO, and could be achieved with either SCF or IL-3.

Table 1. IL-3 Binding in J2E Cells Stimulated With EPO

<table>
<thead>
<tr>
<th></th>
<th>Low Affinity Sites per Cell</th>
<th>High Affinity Sites per Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>270</td>
<td>520</td>
</tr>
<tr>
<td>EPO</td>
<td>1,000</td>
<td>750</td>
</tr>
</tbody>
</table>

J2E cells were treated with EPO for 12 hours. Cells were then incubated with iodinated IL-3 in RPMI for 5 hours at 4°C. After labeling, cells were centrifuged through FCS and the pellet counted. Nonspecific binding was determined by incubating the sample in a 100-fold excess of unlabeled ligand for 30 minutes before adding the iodinated ligand. Representative data from one experiment are shown. Although slight differences in the calculated number of sites were found in three other experiments, the same overall stimulation fold in receptor number and similar affinities were observed after treatment with EPO. Low affinity kd was 10⁻¹⁰; high affinity kd was 10⁻¹².
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Cells were factor-deprived for 2 hours, and were subsequently stimulated with IL-3 for 12 hours. Cells were harvested and binding at equilibrium with 125I-GM-CSF was measured in the presence or absence of cold competitor (Table 2). High-affinity GM-CSF sites increased 2.4-fold after stimulation. Low-affinity GM-CSF sites were not determined.

Table 2. GM-CSF Binding in 32D Cells Stimulated With IL-3

<table>
<thead>
<tr>
<th>HGF</th>
<th>Sites/Cells</th>
<th>Affinity (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3,350</td>
<td>1.9 x 10^{-10}</td>
</tr>
<tr>
<td>IL-3</td>
<td>8,100</td>
<td>1.9 x 10^{-10}</td>
</tr>
</tbody>
</table>

32D cells were treated with IL-3 for 12 hours. After this period, cells were incubated with labeled GM-CSF in RPMI for 5 hours at 4°C. Cells were then centrifuged through FCS and the pellet counted. A representative experiment is shown. Although slight variations in the number of sites were found in another experiment, the same overall stimulation fold in high-affinity receptors and similar-affinity constants were observed after treatment with IL-3.

125I-GM-CSF were performed after IL-3 stimulation. 32D cells were factor-deprived for 2 hours, and were subsequently stimulated with IL-3 for 12 hours. Cells were harvested and binding at equilibrium with 125I-GM-CSF was measured in the presence or absence of cold competitor (Table 2). High-affinity GM-CSF sites increased 2.4-fold after stimulation. Low-affinity GM-CSF sites were not determined.

Because both phagocytic and erythroid precursor cells transiently increase the expression of components of the IL-3 and GM-CSF receptors in response to appropriate HGFs, we next investigated whether this effect might have functional significance. To do so, we studied β-globin induction in J2E cells. The previous studies of Klinken et al10 had shown that J2E cells gradually increase β-globin expression when incubated in the presence of EPO. Because our studies show that EPO increases the number of low-affinity IL-3 receptors and IL-3 is known to enhance differentiation of erythroid precursors, we tested whether addition of IL-3 to EPO during the incubation would augment β-globin synthesis (Fig 6). The results show that, although EPO or IL-3 alone could induce β-globin mRNA (Fig 6, lanes 3 and 4), greater activation was observed when EPO and IL-3 were present together (Fig 6, lane 5).

DISCUSSION

Walker et al3 showed that IL-3 downregulates the receptors for the lineage-specific HGFs, M-CSF and G-CSF. In this study, we investigated the reverse aspect: expression of homologous components of the murine IL-3 and GM-CSF receptors in response to later-acting HGFs. The murine AIC2A gene encodes a low-affinity binding chain of the IL-3R,8 whereas the 95% homologous AIC2B gene9 encodes a subunit that converts the low-affinity GM-CSF or IL-5R to high affinity.10–12 J2E cells were selected as our initial model system, as this erythroid precursor cell line was described to undergo limited differentiation in response to EPO.13 We reasoned that as J2E cells acquired a more differentiated phenotype, expression of the IL-3R AIC2A gene might be lost.

Although our expectations turned out to be correct, we observed that the initial response was a transient increase in AIC2A gene expression in response to EPO. The increase in steady-state mRNA correlated with an increased rate of transcription of the AIC2A gene, was dose-dependent, did not require new protein synthesis, and occurred when S phase was prevented by hydroxyurea. Furthermore, the transient activation was seen with EPO, SCF, or IL-3 and was not restricted to the J2E cell line. Similar increases were observed in the murine cell line BaF3-EPOR in response to EPO, and in the phagocytic progenitor clone 32D-c123 in response to M-CSF, GM-CSF, or IL-3. The increase in AIC2A expression was accompanied by an increase in AIC2B expression in all cells tested. This is not unexpected as expression of AIC2A and AIC2B genes has been reported to be tightly linked.9

The influence of these factors on AIC2A and AIC2B
gene transcription has functional significance. Metabolic labeling of J2E cells stimulated for 6 hours with EPO showed that the increase in AIC2A and AIC2B gene expression was accompanied by a functional increase in protein synthesis. EPO-stimulated J2E cells expressed a higher number of IL-3 low-affinity binding sites on their cell surface, and IL-3-stimulated 32D cells displayed an increased number of GM-CSF high-affinity binding sites.

Upmodulation of AIC2A and AIC2B gene expression by later-acting HGFs in precursor cells may be part of a more general phenomenon. Expression of several other HGF receptors has been shown to be affected by binding of noncognate HGFs. However, there is some underlying specificity in the response. In contrast to what we observed for AIC2A and AIC2B expression, EPO or IL-3 treatment had no effect on the expression of the EPO receptor in J2E cells.

The remarkable crossregulation between HGFs and their receptors is intriguing in the light of the recent finding that the human GM-CSF, IL-3, and IL-5 receptors share a common subunit that combines with specific low-affinity binding chains to convert them into high-affinity receptors. As already mentioned, AIC2A encodes the low-affinity binding chain for IL-3 and AIC2B encodes the common subunit conferring high affinity to the GM-CSF and IL-5 receptors. Increasing AIC2B protein thus affects the number of the two high-affinity receptors if their respective low-affinity binding chains are expressed. This may have important implications for progenitor cells, as occupancy of a small number of high-affinity receptors is sufficient for the biologic effect of GM-CSF. In addition, upmodulation of receptors after HGF exposure may contribute to the synergism of action between different HGFs. Cotreatment of J2E cells with EPO and IL-3 leads to maximal activation of β-globin gene expression. It is possible that the increase in AIC2A expression in response to EPO may be related to this observation. Although the number of IL-3 high-affinity sites was only marginally affected in J2E cells treated with EPO, the increase in AIC2A protein expression alone might have been sufficient to enhance the probability for each cell to be simultaneously activated by both factors.

In contrast to the increase in AIC2A and AIC2B mRNAs that followed a short incubation time with EPO, we observed that prolonged treatment of J2E cells with EPO, the increase in AIC2A protein expression alone might have been sufficient to enhance the probability for each cell to be simultaneously activated by both factors.

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Enhanced expression of interleukin-3 and granulocyte-macrophage colony-stimulating factor receptor subunits in murine hematopoietic cells stimulated with hematopoietic growth factors

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