Rapid Communication

Granulocyte Colony-Stimulating Factor Activation of Stat3α and Stat3β in Immature Normal and Leukemic Human Myeloid Cells

By Arup Chakraborty, Scott M. White, Timothy S. Schaefer, Edward D. Ball, Kevin F. Dyer, and David J. Tewary

Acute myelogenous leukemia (AML) is a myelo-proliferative disease characterized by arrested maturation within the myeloid lineage.1 Although the majority of AML cells, like their normal myeloid cell counterparts, are dependent on growth factors for proliferation,2 they are refractory to differentiation induction.3 Substantial effort has been made to understand the nature of this differentiation arrest and to develop therapeutic strategies to overcome it. In initial studies, granulocyte colony-stimulating factor (G-CSF) could override the differentiation block in leukemic cell lines4 and in some fresh AML cells.5 These observations suggested that this cytokine might be used as a differentiation therapy for AML. However, most responsive AML cells proliferated without differentiation on exposure to G-CSF.6,7 The molecular basis for the block in the differentiation response of AML cells to G-CSF is not known but may be caused by deviation in the G-CSF signaling pathway in AML cells.

The receptor for G-CSF (G-CSFR) is a member of the type I cytokine receptor family.8-10 Structure-function studies have determined that the C-terminal half of the cytosolic domain of the G-CSFR is essential for signaling differentiation.11,12 G-CSF activates a protein-DNA complex, G-SIF-

AML cell lines examined except HL60 and in three of six uncultured AML patient samples. In normal human CD34+ bone marrow cells and HL60 cells, both reported to differentiate in response to G-CSF, G-CSF did not activate Stat3α; rather, it activated only an 83-kD form of Stat3 that appeared to be the human homologue of a short form of Stat3, Stat3β. Because the transcriptional activity of Stat3β is distinct from Stat3α, these results suggest that the balance of the two Stat3 isoforms in myeloid cells may influence the cellular pattern of gene activation and consequently the ability of these cells to differentiate in response to G-CSF.

Supported in part by American Cancer Society grant DHP-I 11, National Institutes of Health grants AI07333 and CA31888.

The publication costs of this article were defrayed in part by charge of the author. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

From The Divisions of Infectious Diseases, and Hematology, Departments of Medicine and Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine and the University of Pittsburgh Cancer Institute, Pittsburgh, PA; Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD.

Submitted May 30, 1996; accepted July 18, 1996.

Address reprint requests to David J. Tewary, MD, W1052 Biomedical Science Tower, University of Pittsburgh Cancer Institute, 200 Lothrop St, Pittsburgh, PA 15213.

Copyright © 1996 by The American Society of Hematology.

MATERIALS AND METHODS

Cells. All factor-independent AML cell lines were maintained, as described10,21 in RPMI 1640 (Life Technologies, Gaithersburg,
MD) supplemented with heat-inactivated fetal calf serum (FCS) (10%), penicillin (100 U/mL), and streptomycin (100 U/mL). The factor-dependent cell line, AML193, was maintained, as described in Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with GM-CSF (30 ng/mL; Immunex, Seattle, WA). For CD34+ cell isolation, marrow cells were isolated from vertebral bodies of organ donors, as described. Bone marrow mononuclear cells (BMMC) were isolated on a discontinuous Ficoll-Hypaque gradient (density = 1.077; Pharmacia, Piscataway, NJ) and washed with phosphate-buffered saline (PBS). CD34+ cells were isolated from BMMC using a Ceprate LC34 biotin-avidin column (CellPro Incorp, Bothell, WA) as described by the manufacturer. CD34+ purity was 83 ± 3% assessed by flow cytometry and overall cell yield was 1% to 3%.

AML patient samples. Peripheral blood or BM aspirates were obtained from patients with AML following informed consent. Patients were newly diagnosed except for one sample taken from a patient in relapse. Mononuclear cells were isolated by discontinuous density gradient centrifugation (specific gravity < 1.077 g/mL). Samples were either studied directly or cryopreserved in RPMI 1640 (Life Technologies) containing heat-inactivated 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (Sigma, St Louis, MO) using a controlled rate freezer and stored at −160°C under the vapor phase of liquid nitrogen. Cryopreserved cells were rapidly thawed and immediately diluted in 10-fold excess of ice-cold thawing medium (RPMI/10% FBS with 100 U/mL DNase I [Sigma]). Cells were washed twice in thawing medium before removal of dead cells by density gradient centrifugation at room temperature (Ficoll-Hypaque). Microscopic examination of Wright’s stained cytospin preparation confirmed that all samples studied were >70% blasts. Cell viability was tested by dye exclusion; all samples studied had >90% viability.

Cytokines and antibodies. rhG-CSF was purchased from Amgen (Thousand Oaks, CA). Stat3 antibody, C-20 (Santa Cruz Biotechnology, Santa Cruz, CA), was generated in rabbits immunized with the C-terminal end of murine Stat3p92, amino acids 750 to 769. Stat3 antibody, K-15 (Santa Cruz Biotechnology), was generated in rabbits immunized with murine Stat3p92 amino acids 626-640. Stat3 monoclonal antibody (MoAb) (Transduction Laboratories, Lexington, KY) was developed in mice immunized with a 21.2 kD fragment of murine Stat3p92 amino acids 1-178. Stat3 antibody, Stat3c, generously provided by Dr James Darnell (Rockefeller University, New York, NY) was generated in rabbits immunized with murine Stat3/p92 amino acids 688-727. Stat3p-specific antibody was made by immunization of rabbits with a KLH-conjugated peptide consisting of fifteen residues—three alanines plus residues corresponding to the twelve C-terminal amino acid residues of Stat3β (AAAALVTPE-IADAVWK; Schaefer et al, submitted).

Electrophoretic mobility shift assay (EMSA). Cells (≥106) in suspension were incubated in 1 mL PBS with or without cytokine at 37°C. Whole-cell and nuclear extracts were prepared and EMSAs performed on 4% native polyacrylamide gels as described. Immunoblotting. Whole-cell or nuclear extracts were mixed with 2 × sodium dodecyl sulfate (SDS)-sample buffer (125 mmol/L Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol) at 1:1 ratio and were heated for 5 minutes at 100°C. Protein (40 μg/lane) were separated by 15% SDS-polyacrylamide gel electrophoresis SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Waltham, MA). Prestained molecular weight markers (Broad Range prestained molecular weight marker; Biorad, Hercules, CA) were included in each gel. Membranes were blocked for 30 minutes in Tris-buffered saline (TBS: 10 mmol/L Tris-HCl, pH 7.5 and 150 mmol/L NaCl) with 0.5% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA). After blocking membranes were incubated with primary antibody in TBS-T and 1% BSA for 1 to 2 hours. After washing the membranes three times with TBS-T (5 minutes each), they were incubated with horseradish peroxidase conjugated secondary antibody in TBS-T and 1% BSA for 30 minutes. Subsequently, membranes were washed three times with TBS-T and developed using the enhanced chemiluminescence (ECL) detection system (Amersham Life Sciences Inc, Arlington Heights, IL). All operations were performed at room temperature. Where indicated, membranes were stripped using stripping buffer (62.5 mmol/L Tris-HCl, pH 7.5; 100 nmol/L 2-mercaptoethanol; 2% SDS) for 30 minutes at 50°C, blocked, and probed with a second antibody following the same protocols as described above.

DNA binding affinity purification. Whole cell extracts (WCE) were prepared from G-CSF stimulated and unstimulated cells using a high salt buffer as described previously. The WCE were mixed with salmon sperm DNA (500 μg/mL) and incubated at 4°C for 10'. This mixture was incubated at 4°C for 40 minutes more with streptavidin conjugated paramagnetic beads (Dynabead M280 streptavidin, Dynal Inc, Lake Success, NY), to which biotinylated tandem bSHIE had previously been bound. Following this incubation, the beads were washed five times with high salt buffer containing 0.05% NP-40 and 200 μg/mL salmon sperm DNA. Proteins were eluted with high salt buffer containing 1.2 mol/L NaCl.

Reverse transcriptase-polymerase chain reactions (RT-PCR), Southern blotting, subcloning, and sequencing. Total cellular RNA was collected from cell pellets lysed with 4 mol/L guanidine isothiocyanate and isolated by centrifugation through a cesium chloride cushion. RT reaction was prepared using 4 μg of RNA, 200 U M-MLV RT (GIBCO-BRL, Gaithersburg, MD) and its supplied reaction buffer, 0.01 mol/L DTT, 1 mmol/L each dATP, dTTP, dGTP, and dCTP, 10 U human placental RNase inhibitor (GIBCO-BRL), and 10 ng oligo dT primer (Boehringer Mannheim, Indianapolis, IN) in a 10 μL reaction volume and incubated at 37°C for 1 hour. Oligodeoxynucleotide primers for human Stat3 amplification were designed to amplify both Stat3α and Stat3β and yield a fragment of 224 bp and 174 bp, respectively. The 5' primer sequence was CTGACCCAGTGCGTCGCCCCATACCC and the 3' primer sequence was TCGAATGGGGAGTGAGCGACTCCCG based on human Stat3 DNA sequence from nucleotides 2310 to 2336 and 2508 to 2533, respectively. Two microliters of the RT reaction was added to make 20 μL of PCR mixture containing 5 U Taq DNA polymerase (Perkin-Elmer Cetus, Foster City, CA) 10 mmol/L Tris-HCl pH 8.3, 50 mM KCl, 1.5 mmol/L MgCl2, 56.25 μmol/L each dATP, dTTP, dGTP, and dCTP, and 100 ng each of 5' and 3' primers. The negative control used for PCR replaced cDNA with sterile filtered water and was amplified simultaneously with experimental reactions. The thermal cycler (Perkin-Elmer Cetus) was programmed for 35 cycles with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute. The PCR products were electrophoretically separated on a 2% agarose gel in 1× TBE buffer at 100 V for 1.5 to 2 hours. The agarose gels were blotted onto nylon membranes (Zetabind; Cuno, Meridian, CT) overnight by capillary action and probed with 32P-dCTP (New England Nuclear, Boston, MA) labeled full-length murine Stat3α cDNA kindly provided by Dr James Darnell. The blots were exposed to Kodak XAR5 film (Eastman Kodak Co, Rochester, NY).

To subclone the amplified DNA fragments, the PCR products were electrophoretically separated on a 2% agarose gel. Each amplified DNA fragment was isolated by cutting out the portion of agarose containing the band and extracted using a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA). Each fragment was subcloned into pCR/SCRIPT (Stratagene, La Jolla, CA) as suggested by the manufacturer. Plasmid DNA containing each fragment was isolated from each of fifteen residues—three alanines plus residues corresponding to the十二五 amino acid residues of Stat3β (AAAALVTPE-IADAVWK; Schaefer et al, submitted).
RESULTS

G-CSF activation of Stat3α in AML cells. We previously showed that G-CSF activated a DNA-binding protein complex, G-SIF-A, composed of a distinct Stat3-like protein, StatG, in normal myeloid cells.13 In contrast to normal myeloid cells, G-CSF activated both Stat3α and StatG in the AML cell line, AML-193. AML-193 had previously been shown to proliferate but not differentiate in response to G-CSF.25 To determine the composition of G-SIF-A in a variety of AML cell lines and to determine the correlation between G-SIF-A composition and the differentiation response to G-CSF, we examined the composition of G-SIF-A in five AML cell lines (EM3, NB4, DER, THP1, and KG1) that have not been shown to differentiate in response to G-CSF either histologically26 or through induction of differentiation-specific genes such as the G-CSF receptor.20,27 We also examined one AML cell line, HL60, which had previously been shown to differentiate in response to G-CSF26,28,29 and which showed delayed upregulation of G-CSF mRNA at 48 hours (data not shown). These AML cell lines were stimulated with G-CSF (1 to 1,000 ng/mL) for 1 to 60 minutes. EMSA of whole-cell extracts was performed using the high-affinity form of the serum inducible element (hSIE) contained within the c-fos promoter.14 Each AML cell line formed a DNA-binding complex with hSIE that migrated with mobility similar to SIF-A which we previously designated G-SIF-A.13 Supershift analysis was performed using Stat3 antibody (C-20) directed against the C-terminal end of Stat3α, a region deleted in Stat3β. These studies showed that G-SIF-A contained Stat3α in all AML cell lines except HL60 (Fig 1A). G-CSF-activated G-SIF-A in each AML cell line with kinetics and dose-response curves similar to neutrophils and BMMC. In addition, no G-SIF-A complex was formed in extracts of unstimulated cells (data not shown).13 Stat3α expression in AML cells may be the result of its being abnormally expressed in these cells or may be secondary to its recruitment and activation by an abnormal G-CSFR isoform. To examine the first possibility, we performed immunoblot analysis of whole-cell extracts of each AML cell line using Stat3 antibody, C-20. Stat3α (p92) was detected in extracts of each cell line that showed Stat3α activation but not in HL60 (Fig 1B; top panel). Identical results were obtained using Stat3 MoAb directed against the N-terminal 178 amino acids (see Fig 3B) and also in immunoprecipitation studies of detergent lysates of cells using antibody C-20 (data not shown). These results indicated that Stat3α was expressed in each cell line in which it was activated and could not be detected in whole cell extracts of HL60 where it was not shown to be activated.

Expression and activation of Stat3α in leukemia cell lines may be part of their leukemic phenotype, or due to their arrest at an immature stage of differentiation, or may result from in vitro cultivation. To determine if Stat3α expression and activation is caused by their maturation arrest, we examined G-SIF-A activation and composition in extracts of G-CSF-stimulated normal CD34+ BM cells which contain the most immature population of hematopoietic precursor cells. G-SIF-A from G-CSF–activated CD34+ cells did not contain Stat3α by supershift analysis using Stat3 antibody C-20 (Fig 1C). Furthermore, immunoblotting of CD34+ whole-cell extracts with this antibody did not reveal Stat3α protein (Fig 1D). To determine if Stat3α expression and activation is due to in vitro cultivation of AML cell lines, we examined whole-cell extracts of a small number of uncultured AML patient samples stimulated with G-CSF in vitro. Six AML samples were examined from five patients; sample six was obtained from patient 1 at clinical relapse. The French-American-British classification of the AML samples included M2 (sample 2), M4 (sample 5), and M5 (samples 1, 1R, 3, and 4). Similar to normal human CD34+ cells, G-SIF-A did not contain Stat3α in 3 of 5 patient samples. However, in two of five patients and in the second sample from patient 1 in relapse (1R), G-SIF-A contained Stat3α (Fig 1C). Immunoblotting of these extracts with Stat3 antibody C-20 or with Stat3 MoAb directed against the N-terminus revealed Stat3α expression only in those samples that showed Stat3α by supershift analysis (Fig 1D; upper and lower panel).

G-CSF activation of Stat3β in normal CD34+ BM and HL60 cells. Interestingly, immunoblotting with Stat3 MoAb revealed a second Stat3 species of 83 kD in all cells examined including HL60 and normal CD34+ BM cells (Fig 1B and D; bottom panels). This 83 kD Stat3-related protein was also recognized by immunoblotting using Stat3 polyclonal antibody K-15 and antibody against human acute phase response factor, APRF/Stat317 but not by Stat3 antibody C-20 directed against the C-terminal end of Stat3α, a region deleted in Stat3β (Fig 1 and data not shown). To examine the possibility that the 83 kD protein was Stat3β we examined whether immature normal and leukemic myeloid cells expressed Stat3β mRNA. Oligodeoxynucleotide primers were designed based on human Stat3 sequence7 to amplify both Stat3α and Stat3β and to yield distinctly-sized amplified fragments for each. Using these primers for RT-PCR amplification, we observed DNA fragments corresponding to those predicted for Stat3α (band A) and Stat3β (band B) in normal CD34+ cells and in all AML cell lines tested including HL60 (Fig 2A; upper panel). Both DNA bands A and B cross-hybridized to a full-length murine Stat3 cDNA (Fig 2A; lower panel). Both DNA bands were isolated, subcloned, and sequenced (Fig 3B). Sequencing of band B revealed the identical 50 nucleotide deletion observed in murine Stat3β31 indicating that both immature normal and leukemic myeloid cells express Stat3β mRNA. The sequence of band A was completely homologous over its length to the corresponding region of human Stat3α except for nucleotide 2408, which is an A in band A instead of a G in the published sequence of human Stat3 (Fig 3B). Interestingly, there is an A at this position in the murine Stat3α sequence.32 This nucleotide change results in an amino acid substitution of threonine, a polar uncharged amino acid, for alanine, a nonpolar uncharged amino acid. The signifi-
G-CSF ACTIVATION OF Stat3 ISOFORMS

Fig 1. Stat3 activation in AML cell lines and uncultured AML patient samples. In (A), whole-cell extracts (20 μg) of the indicated G-CSF-stimulated AML cell lines were incubated with radiolabeled hSIE duplex oligonucleotide before EMSA without (−) or with (+) polyclonal Stat3 antibody C-20 directed against 29 of the 21 C-terminal amino acid residues of Stat3 which are deleted in Stat3β. The location of SIF-A, B, and C bands and the position of the SIF-A complex bound by Stat3 antibody and supershifted (SIF-A[SS]) are indicated on the left side of the panel. In the upper half of (B), whole-cell extracts (40 μg) of unstimulated AML cells were separated by SDS-PAGE, blotted onto PVDF membrane and developed with Stat3 C-20 antibody. Molecular weight markers are indicated on the right and the position of Stat3α (Stat3/p92) indicated on the left. In the lower half of (B), the identical extracts (40 μg) of unstimulated cells were separated by SDS-PAGE, blotted onto PVDF membrane and developed with Stat3 MoAb directed against the N-terminal 178 amino acid residues shared by Stat3α and Stat3β. The molecular weight markers are indicated on the right, and the positions of Stat3α (p92) and Stat3α/p83 indicated on the left. The results shown are representative of two or more experiments with each cell line. In (C), whole cell extracts (10 to 20 μg) of the indicated G-CSF-stimulated cells were incubated with radiolabeled hSIE duplex oligonucleotide before EMSA without (−) or with (+) polyclonal Stat3 antibody C-20. The location of SIF-A, B, and C bands and the position of the SIF-A complex bound by Stat3 antibody and supershifted (SIF-A[SS]) are indicated on the left side of the panel. The results shown are representative of two preparations of normal CD34+ BM cells or of two experiments with each AML patient sample. In the upper half of (D), whole-cell extracts (40 μg) of unstimulated cells were separated by SDS-PAGE, blotted onto PVDF membrane and developed with Stat3 C-20 antibody that recognizes Stat3α but not Stat3β. The position of Stat3α (Stat3/p92) is indicated. In the lower half of (D), the identical extracts (40 μg) were separated by SDS-PAGE, blotted onto PVDF membrane and developed with the Stat3 MoAb that recognizes both Stat3α and Stat3β. The positions of Stat3α (p92) and Stat3α/p83 (p83) are indicated on the left.

cance of this substitution and whether it represents a polymorphism or an artifact of PCR remains to be determined.

To confirm that the 83 kD Stat3-related protein is Statβ, we tested whether it is recognized by antibody specific for Stat3β. This antibody was capable of specifically immuno-

precipitating the 83 kD species from both HL60 cells and normal CD34+ BM cells indicating that the 83 kD Stat3-related protein is the human homologue of Stat3β (Fig 3A). These results suggested that G-SIF-A in HL60 and CD34+ cells, as well as the portion of G-SIF-A in the other AML
cell lines that did not supershift with Stat3 antibody C-20, contained Stat3α. To test this possibility, we performed DNA affinity purification of G-SIF-A from whole cell extracts of G-CSF-stimulated HL60 cells followed by immunoblotting with Stat3 MoAb directed against the N-terminus of Stat3 and a Stat3P-specific antibody. DNA-affinity purified protein from G-CSF-activated HL60 contained Stat3P (Fig 3B). Furthermore, DNA affinity purification of whole-cell extracts of unstimulated HL60 did not reveal any protein recognized by either Stat3 MoAb or by Stat3P-specific antibody indicating that ligand binding was required to activate the DNA binding capability of Stat3P.

**DISCUSSION**

G-CSF activated an hSIE-protein complex, G-SIF-A, that contained Stat3α in nearly all AML cell lines and in some uncultured AML patient samples. Activation of Stat3α in each cell line or sample corresponded to its constitutive expression determined by immunoblotting of cell extracts or immunoprecipitation of detergent lysates. The G-SIF-A complex of normal CD34+ bone marrow cells and HL60 cells, both capable of differentiating in response to G-CSF, did not contain Stat3α. Immunoblotting of extracts of these cells, using a panel of Stat3 antibodies, did not reveal Stat3α; rather, these studies showed an 83 kD protein that was recognized by all Stat3 antibodies except those directed against the C-terminal end of Stat3α, a region deleted in Stat3P. RT-PCR of RNA from normal CD34+ and HL60 cells revealed an amplified fragment corresponding to Stat3P; Southern blot analysis and sequencing of this fragment confirmed its identity as Stat3P. Using polyclonal antibody specific for Stat3β, we immunoprecipitated the 83 kD Stat3 species from both normal CD34+ and HL60 cells establishing that it is the human equivalent of Stat3β. Stat3β could
The STAT protein family currently consists of 6 members. Three STAT protein members, Stat1, Stat3, and Stat5, have more than one isoform due to either multiple genes or alternatively spliced mRNA, which can result in proteins with distinct transcriptional activities. In the case of Stat5, there are two isoforms, Stat5a and Stat5b, that arise from distinct genes. The cDNA for Stat5a encodes a protein of 91 kD whereas the cDNA of Stat5b encodes a protein of 90 kD. No information is available, yet, regarding differences in the DNA-binding and transcriptional activation properties of Stat5 isoforms. Alternative splicing of the Stat1 RNA results in two isoforms, α and β, with the result that Stat1β lacks the C-terminal 38 amino acid residues of Stat1α. Although Stat1β binds DNA with similar avidity as Stat1α, it is transcriptionally inactive and may compete with Stat1α for binding to interferon-γ activated sequence (GAS) elements within promoters and block transcriptional activation by Stat1α.

Stat3β was recently cloned from a murine fibroblast cell line cDNA library by yeast two-hybrid interaction with the N-terminus of c-Jun. The Stat3β cDNA has a 50 nucleotide deletion at the 3' end. Stat3β protein, consequently, is missing the C-terminal 55 amino acid residues of Stat3α. These 55 amino acids are replaced by 7 unique amino acid residues at its C-terminus that are contributed by 21 nucleotides of 3' sequence of Stat3α that is spliced in-frame downstream of the deletion. Stat3β cDNA encodes an 83 kD protein that has several features that are distinct from Stat3α. Unlike Stat3α, Stat3β appears to functionally interact with c-Jun. Over-expressed in COS cells, Stat3β, but not Stat3α, is constitutively active. Similar to Statα, however, additional Stat3β activity can be induced by epidermal growth factor (EGF) and to be required for maximal transcriptional activation.

We previously showed, using supershift analysis, that the hSIE-protein complex, G-SIF-A, in normal CD34+ BM cells does not contain Stat3α; rather, it contained a distinct Stat3-like protein designated StatG. The studies reported here indicate that StatG is composed, at least in part, of Stat3β. The G-SIF-A complex of HL60 cells, an AML cell line previously showed to differentiate in response to G-CSF, also contained Stat3β but not Stat3α. It is interesting to note that Stat3α could not be detected within the G-SIF-A complex of CD34+ and HL60 cells despite obtaining levels of amplified Stat3α DNA by RT-PCR similar to other AML cells in which Stat3α protein is readily detected. These findings suggest that in cells capable of differentiating in response to G-CSF there is either selective translation of the Stat3α mRNA or posttranslational instability of Stat3α protein.

Stat3α has been shown to be activated in v-Src-transformed fibroblasts and its activation was found to correlate with the transformed phenotype. In addition, overex-

---

**A**

<table>
<thead>
<tr>
<th>kDa</th>
<th>C1</th>
<th>C2</th>
<th>B</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>82</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>kDa</th>
<th>G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

---

The 83 kDa Stat3-related protein is Stat3β, which is activated to bind hSIE by G-CSF. In (A), detergent lysates of HL60 (1.5 mg, 1.5 mg, and 0.5 mg protein, respectively) were immunoprecipitated with an irrelevant antibody (rabbit IgG; C1), or with protein-A sepharose beads alone (C2), or with rabbit antibody specific for Stat3β (B). In lane 4, whole-cell extracts of CD34+ cells (250 µg) were immunoprecipitated with Stat3β-specific antibody. Immunoprecipitates were separated by SDS-PAGE, blotted onto PVDF, and developed with Stat3 MoAb. The locations of the pre-stained molecular weight markers are indicated on the left of the panels; the location of Stat3β is indicated on the right of the panels.

be DNA-affinity purified from whole-cell extracts of G-CSF-stimulated HL60 cells but not from unstimulated cells indicating that G-CSF activates DNA binding by Stat3β in these cells. Therefore, our results indicate that G-CSF activates an hSIE-binding complex, G-SIF-A, present in HL60 and CD34+ cells that is composed of Stat3β alone.
pression of Stat3α in the murine leukemic cell line, M1, blocked its terminal differentiation in response to IL-6. These results together with our findings of Stat3α activation in AML cells suggest the possibility that the presence of Stat3α within the G-SIF-A complex of some AML cells is aberrant and that activation of Stat3α in these cells may contribute to the abnormal proliferative response of AML cells to G-CSF. We previously mapped maximal activation of Stat3α and Stat3β (StatG) to the C-terminal half of the cytoplasmic portion of the G-CSFR, which is the portion of the receptor specific for controlling the differentiation program mediated by this receptor. Our finding that G-SIF-A in AML cell lines that fail to differentiate in response to G-CSF contains Stat3α, as well as Stat3β, suggests that Stat3α may be interfering with the normal transcriptional activation function of Stat3β and thereby inhibit the normal G-CSF–induced differentiation program.

ACKNOWLEDGMENT

We express our appreciation to Dr James Darnell (Rockefeller University, New York, NY) for providing the murine Stat3α cDNA and Stat3α antisera, to Dr Tadamitsu Kishimoto (Osaka University Medical School, Suita, Osaka, Japan) for the affinity-purified anti-APRF (Stat3) antibody, and to Dr Suzanne Ildstad (Allegheny University of Health Sciences, Philadelphia, PA) and her colleagues for the human vertebral body bone marrow cells.

REFERENCES


Granulocyte colony-stimulating factor activation of Stat3 alpha and Stat3 beta in immature normal and leukemic human myeloid cells

A Chakraborty, SM White, TS Schaefer, ED Ball, KF Dyer and DJ Tweardy