Inhibition of Src family kinases enhances retinoic acid–induced gene expression and myeloid differentiation

Michelle B. Miranda, 1 Robert L. Redner, 1 and Daniel E. Johnson 1,2

Departments of 1 Medicine and 2 Pharmacology, University of Pittsburgh and the University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Abstract

Treatment of acute promyelocytic leukemia with retinoic acid (RA) results in differentiation of the leukemic cells and clinical remission. However, the cellular factors that regulate RA-induced myeloid differentiation are largely unknown, and other forms of acute myelogenous leukemia (AML) do not respond to this differentiation therapy. A greater understanding of the molecules that positively or negatively regulate RA-induced differentiation should facilitate the development of more effective differentiation therapies. In this study, we investigated the potential role of Src family kinases (SFK) in the regulation of RA-induced gene expression and myeloid differentiation. We report that inhibition of SFKs markedly enhanced RA-induced differentiation in myeloid cell lines and primary AML cells, as assessed by flow-cytometric analysis of cell surface markers, morphologic analysis, and nitroblue tetrazolium reduction. In addition, inhibition of SFKs enhanced expression from retinoic acid receptor (RAR) target genes encoding CCAAT/enhancer binding protein α (C/EBPα), PU.1, intercellular adhesion molecule-1 (ICAM-1), and cathepsin D. Moreover, a constitutively active Src inhibited RAR-dependent transcription, whereas a kinase-dead Src exerted little effect. These studies provide the first demonstration that SFKs act as negative regulators of RA-induced gene expression and myeloid differentiation and suggest that the combination of SFK inhibition and RA treatment may be therapeutically beneficial in AML. [Mol Cancer Ther 2007;6(12):3081–90]

Introduction

Acute myelogenous leukemias (AML) are characterized by defective differentiation and excessive accumulation of proliferative progenitor cells. A variety of molecular abnormalities have been shown to be associated with differentiation blockades in AML and include aberrant expression of (a) fusion oncoproteins such as AML1/ETO (1, 2), PML/RARα (3–5), and CBFβ/MYH11 (6); (b) mutant myeloid transcription factors such as mutant PU.1 (7) and CCAAT/enhancer binding protein α (C/EBPα; ref. 8); and (c) mutant cytokine receptors such as Flt3-ITD (9) and truncated granulocyte colony-stimulating factor (G-CSF) receptor (10). At the same time, the molecular bases for defective differentiation in many cases of AML remain unknown. A promising strategy for treating AML involves the use of differentiation therapy. Indeed, in the case of acute promyelocytic leukemia (APL), characterized by expression of fusion oncoproteins incorporating the retinoic acid receptor (RAR; ref. 11), treatment with retinoic acid (RA) results in the induction of myeloid differentiation and clinical remission (12–14). However, despite the success of RA in the treatment of APL, other subtypes of AML fail to respond to this agent, and effective differentiation strategies for these malignancies do not exist. Moreover, the cellular factors that regulate RA-induced myeloid differentiation and may contribute to responsiveness to RA are poorly understood.

Emerging evidence has implicated Src family kinases (SFK) as regulators of proliferation and survival of myeloid lineage cells and regulators of cytokine-induced myelopoi-esis (15). The SFK family comprises nine different members, with the primary SFKs expressed in myeloid cells being Hck, Lyn, and Fgr. Early studies revealed that expression of v-Src or constitutively active Hck abrogated cytokine dependence and inhibited granulocyte colony-stimulating factor (G-CSF)–induced granulocytic differentiation in the murine myeloid progenitor cell line 32Dcl3 (16, 17). Subsequent studies have shown that Lyn plays an important role in G-CSF–induced production of reactive oxygen species and myeloid cell growth (18, 19). Experiments involving knock-out mice have provided evidence for the regulation of cytokine-stimulated myelopoiesis by SFKs. Granulocytic precursor cells derived from mice deficient in Hck exhibit enhanced proliferation in response to G-CSF (20). Mice lacking Lyn manifest an increase in myeloid progenitors, an enhancement of G-CSF–stimulated granulopoiesis, and development of a myeloproliferative disorder leading to monocyte/macrophage tumors (20, 21). Similar findings were reported in triple knock-out mice lacking Lyn, Fgr, and Hck (20). Together, these studies imply that myeloid SFKs as negative regulators of myelopoiesis.
Although a role for SFKs in the regulation of cytokine-induced myelopoiesis is becoming clearer, the importance of SFKs in RA-induced myeloid differentiation has not been addressed. In this report, we examined the role of SFKs in RA-induced gene transcription and myeloid differentiation. We find that inhibition of SFKs significantly enhanced RA-induced differentiation in myeloid cell lines and primary AML cells. Inhibition of SFKs also enhanced RAR-mediated gene transcription, whereas expression of constitutively active Src markedly attenuated RAR-mediated gene transcription. These results suggest that inhibition of SFKs, in combination with RA treatment, may have therapeutic benefit in the treatment of AML.

Materials and Methods

Cells and Reagents

NB-4, HL-60, and U937 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. HeLa cells were grown in DMEM supplemented with 10% FBS and antibiotics. All-trans-retinoic acid (RA) was purchased from Sigma, and a stock solution of 5 mmol/L was prepared in ethanol and stored at −20°C. The Src family kinase inhibitors PP1 and PP2 were obtained from Biomol International, and stock solutions were prepared in DMSO and stored at −20°C. Rabbit polyclonal antibody against Src phosphotyrosine 418 (α-phosphoSrc Y418) was obtained from Invitrogen. Antibodies against C/EBPα, PU.1, and intercellular adhesion molecule-1 (ICAM-1) were purchased from Santa Cruz Biotechnology, whereas antibody against cathepsin D was from EMD Biosciences. Antibody against HA tag was purchased from Covance, and antibody against β-actin was purchased from Sigma. Secondary anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibodies were from Promega. Peripheral blood from a patient with AML was obtained and following institutional guidelines after informed consent. Mononuclear cells were separated using Histopaque (Sigma), and CD33+ cells were subsequently selected using a CD3 Easy Sep positive selection kit from Stem Cell Technologies.

Cell Lysis and Immunoblotting

For immunoblotting experiments, cells were pelleted, washed in cold PBS, then lysed in cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPI, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride]. The lysates were incubated on ice for 30 min, followed by centrifugation at 11,000 × g for 10 min at 4°C. Protein concentrations in the lysate samples were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Equal amounts of total protein (40 µg/sample) were separated on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes. The nitrocellulose filters were blocked for 1 h with 5% nonfat dry milk in 10 mmol/L Tris (pH, 8.0), 150 mmol/L NaCl, and 0.1% Tween 20 (TBS-T), followed by incubation with primary antibody overnight at 4°C. After several washes with TBS-T, filters were incubated with secondary antibody for 1 h at room temperature. Following another series of washes in TBS-T, the membranes were developed using enhanced chemiluminescence (ECL, Perkin-Elmer) according to the manufacturer’s guidelines.

Flow Cytometry

Flow cytometry was used to assess cell surface CD11b expression following treatment of NB-4, HL-60, U937, and primary AML CD33+ cells with various combinations of RA and inhibitors, as previously described (22). Briefly, harvested cells were blocked with 10% serum and then incubated with anti–CD11b-PE antibody or isotype-matched antibody (BD Biosciences) for 30 min at 4°C. The labeled cells were then washed with PBS/1% FBS and fixed in 1% formaldehyde. A minimum of 10,000 cells per sample were analyzed for CD11b expression on an Epics Coulter XL flow cytometer (Beckman Coulter).

Assays for Differentiation

Cells were induced to differentiate by treatment with RA in the absence or presence of PP1 or PP2. Although both RA and the inhibitors were dissolved in DMSO, the concentration of DMSO added to cells never exceeded 0.1%. Control experiments revealed that 0.1% DMSO does not affect the differentiation of the NB-4, HL-60, and U937 cell lines (data not shown).

Granulocytic differentiation was assessed by nitroblue tetrazolium (NBT) reduction as previously reported (23, 24). Briefly, 1 × 10⁶ cells were resuspended in 50 µL of Krebs Ringer phosphate buffer containing 4.4 mmol/L glucose. To this, an equal volume of NBT (1 mg/mL) in 0.9% saline was added. After 5 min at 37°C, phosphol 12-myristate 13-acetate (100 µg/mL) was added, and incubation was continued for 15 min at 37°C. Paraformaldehyde was then added to a final concentration of 1%, and the tubes were incubated at room temperature for an additional 10 min. The cells were then centrifuged at 6,000 × g for 3 min, and cytospin preparations were generated using a Cytospin 4 apparatus (Thermo Shandon). Slides were dipped in methanol for 30 s and then stained with 0.1% saffrin.

For each data point, a total of 300 cells were counted from three different fields.

For morphologic assessment of differentiation, cytospin preparations of treated cells were stained using the Hema 3 stain set (Fisher Scientific). For each data point, a total of 300 cells were scored for morphologic features of granulocytic or monocytic differentiation.

 Luciferase Reporter Assays

Before transfection, HeLa cells were plated in 24-well plates (5 × 10⁴ cells per well). Following recovery for 24 h, cells were transfected with 200 ng of pRAR-Luc (Panomics) alone or in combination with constitutively active Src construct (CA-Src; Upstate Biotechnology) or kinase-dead Src construct (KD-Src; Upstate Biotechnology), using FuGENE transfection reagent (Roche Applied Science). The total amount of DNA used per transfection was adjusted to 1 µg using pcDNA 3.1 empty vector. All cells were co-transfected with the phRL-TK vector (Promega).
encoding Renilla luciferase, and the activity of Renilla luciferase was used to normalize samples for transfection efficiency. Twenty-four hours after transfection, cells were treated with 1 μmol/L RA, in the absence or presence of SFK inhibitor, for 16 h in serum-free media. The cells were then harvested, and luciferase activities were determined using the dual-luciferase reporter assay system (Promega). The ratio of firefly to Renilla luciferase activity was determined for each transfection, and results were reported as the fold increase in firefly luciferase activity relative to the untreated control. Results represent the mean ± SD from three identical wells.

Results

Inhibition of SFKs Enhances RA-Induced Differentiation of NB4 Cells

To determine the role of SFKs in RA-induced myeloid differentiation, we examined the impact of pharmacologic SFK inhibitors on RA-induced granulocytic differentiation of NB4 cells, an acute promyelocytic cell line (25). Because preliminary findings suggested that SFK inhibition may enhance RA-induced differentiation, we first sought to define an appropriate suboptimal dose of RA for use in combination experiments with SFK inhibitors. A dose-response analysis was done wherein NB4 cells were treated with varying

Figure 1. Inhibition of SFKs enhances RA-induced differentiation of NB-4 cells. A, SFK inhibitors attenuate SFK phosphorylation in untreated and RA-stimulated cells. NB-4 cells were left untreated or were treated with RA alone, PP1 alone, PP2 alone, RA plus PP1, or RA plus PP2, for 2 h at 37°C. Following treatment, cells were harvested, and whole cell lysates (40 μg per lane) were electrophoresed on a 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane. Filters were probed with anti–phosphoSrc Y418 polyclonal antibody. The membrane was stripped and reprobed with anti–β-actin monoclonal antibody to show equal protein loading. The experiment was done thrice, with similar results each time. B, enhancement of NB-4 differentiation by SFK inhibitors. NB-4 cells were left untreated or were treated with RA alone (0.01 μmol/L), PP1 alone (10 μmol/L), PP2 alone (10 μmol/L), RA plus PP1, or RA plus PP2, for 72 h. Cells were then harvested, stained with anti-CD11b antibody, and analyzed by flow cytometry. A total of 10,000 cells per sample were analyzed for expression of CD11b. Open histograms, staining with isotype control antibody; shaded histograms, cell surface staining for CD11b. Numbers, percentage of CD11b-positive cells. The experiment was done thrice, with similar results each time. C, granulocytic differentiation of NB-4 cells was assessed by morphologic analysis of Hema 3–stained cytospin preparations. The data represent the mean ± SD of three different fields, with 100 cells counted per field. Morphologic evaluations were done in two independent experiments, with similar results each time. D, treated NB-4 cells were evaluated for granulocytic differentiation on the basis of NBT reduction. The plotted data depict the mean ± SD of three different fields, with 100 cells counted per field. Analysis of NBT reduction was done in two independent experiments, with similar results obtained.
concentrations of RA for 72 h (Supplementary Fig. S1). Following treatment, cells were harvested, and graulocytic differentiation was assessed by flow-cytometric analysis of CD11b expression, morphologic analysis, and NBT staining. A dose-dependent response was observed, and good agreement was seen using the three different assays of differentiation (Supplementary Fig. S1A–C). Based on these data, we selected the suboptimal dose of 0.01 μmol/L RA for subsequent combination studies in NB4 cells.

To determine the impact of SFK inhibition on RA-induced differentiation, NB4 cells were treated with RA in the absence or presence of the SFK inhibitors PP1 or PP2. The PP1 and PP2 inhibitors are selective inhibitors of SFKs and exhibit a broad spectrum activity against multiple members of the SFK family (26, 27). Initial experiments were done to determine appropriate concentrations of PP1 and PP2 for inhibiting SFK activation in the cells. Figure 1A shows that unstimulated NB4 cells contained readily detectable levels of phosphorylated/activated SFKs, as assessed by immunoblotting with an antibody (α-phosphoSrc Y418) that recognizes a conserved phosphotyrosine residue present in different SFK members. Phosphorylation of this residue corresponds closely with enzymatic activation (28).

Figure 2. The ability of PP2 to enhance RA-induced differentiation closely correlates with its ability to inhibit SFK phosphorylation/activation. A, NB-4 cells were treated with RA alone (0.01 μmol/L) or RA plus various concentrations of PP2 (0.01–40 μmol/L) for 2 h. Following treatment, cells were harvested, and whole cell lysates (40 μg per lane) were electrophoresed on a 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane. Filters were probed with anti-phosphoSrc Y418 polyclonal antibody. The membrane was stripped and reprobed with anti-β-actin monoclonal antibody to show equal protein loading. The blots are representative of two independent experiments. B, to generate a dose-response curve for PP2 inhibition of SFK phosphorylation, densitometry of a representative phosphoSrc band (* in A) was carried out. The densitometric value obtained at 40 μmol/L PP2 was set at 100% reduction, and the value obtained with RA alone was set at 0% reduction. C, NB-4 cells were treated with RA alone (0.01 μmol/L) or RA plus various concentrations of PP2 (0.01–40 μmol/L). After 72 h of treatment, cells were harvested and assessed for CD11b expression by flow cytometry. The maximal enhancement of RA-induced differentiation obtained with 40 μmol/L PP2 was set at 100% enhancement, and the value obtained with RA alone was set at 0% enhancement. The percent enhancement obtained with intermediate doses of PP2 were calculated relative to that obtained at 40 μmol/L PP2. The results were obtained in two independent experiments.

3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Figure 1B–D shows that inhibition of SFKs dramatically enhanced RA-induced differentiation of NB4 cells. Treatment of NB4 cells for 72 h with the dose of 0.01 μmol/L RA resulted in very little induction of differentiation, as assessed by CD11b expression (7.0% CD11b-positive cells; Fig. 1B). Similarly, treatment for 72 h with 10 μmol/L PP1 alone or 10 μmol/L PP2 alone resulted in only 5.4% or 5.0% CD11b-positive cells, respectively. By contrast, when cells were treated simultaneously with RA and PP1, 49.8% of cells were scored CD11b positive, whereas cotreatment with RA and PP2 resulted in 67.9% CD11b-positive cells. Quantitatively and qualitatively similar results were obtained when differentiation was assessed by either morphologic analysis (Fig. 1C) or NBT staining (Fig. 1D). Additionally, flow-cytometric analysis of Annexin V– and propidium iodide-stained cells revealed that treatment for 72 h with PP1 or PP2, alone or in combination with RA, did not result in induction of apoptosis (Supplementary Fig. S2).3 Similarly, inclusion of PP1 or PP2 did not alter the cell cycle profile of the cells (Supplementary Fig. S2).3 Thus, the abilities of PP1 and PP2 to enhance RA-induced differentiation were not due to effects on apoptosis or cell cycle. Taken together, these results strongly implicated SFKs in the negative regulation of RA-induced granulocytic differentiation of NB4 cells.

We next sought to verify that the abilities of the SFK inhibitors to enhance RA-induced differentiation closely correlated with their potencies for inhibiting the phosphorylation/activation of SFKs in intact cells. In Fig. 2A, NB-4 cells were treated with RA in the presence of varying doses of PP2, followed by immunoblotting of whole cell lysates to detect phosphorylated/activated SFKs. Densitometry of a representative phospho-SFK band (indicated by asterisk) was then used to generate a dose-response curve (Fig. 2B). In separate aliquots of the treated cells, enhancement of RA-induced differentiation was determined by flow-cytometric analysis of CD11b staining, and a dose-response curve was generated (Fig. 2C). As can be seen, the dose-response curve for inhibition of SFK phosphorylation was highly similar to the dose-response curve for the enhancement of RA-induced differentiation, with IC50 values for PP2 being ~5 μmol/L in both cases. Similar results were obtained with PP1 (data not shown). These experiments supported the contention that the enhancement of RA-induced differentiation by PP1 and PP2 was due to the inhibition of intracellular SFK enzymes.

To determine whether SFKs might be acting to inhibit early events in RA-induced differentiation, experiments were done wherein SFK inhibitors were added at varying times before or after RA stimulus. As shown in Fig. 3, simultaneous addition of RA and PP2 inhibitor again resulted in a dramatic enhancement of RA-induced differentiation, with 62.0% of cells scoring as CD11b positive (versus 5.8% and 1.6% in cells treated with RA alone or PP2 alone, respectively). Pretreatment of the cells with PP2 for 1 h before the addition of RA did not markedly affect this level of enhancement. Interestingly, when PP2 was added 2 or 6 h after stimulation with RA, the level of differentiation enhancement also remained roughly the same (66.9% and 63.5% CD11b-positive cells, respectively). However, when PP2 was added either 24 or 48 h after RA stimulation, there was a significant reduction in the level of CD11b-positive cells (37.9% and 25.0% CD11b-positive cells, respectively). Taken together, these findings strongly implicated SFKs in the negative regulation of RA-induced granulocytic differentiation of NB4 cells.
SFK Inhibitors Enhance RA-Induced Differentiation in HL-60, U937, and Primary AML Cells

We next sought to determine whether SFK inhibition would enhance RA-induced differentiation in other myeloid cell lines and in primary AML cells. HL-60 is an AML cell line, and U937 is a myelomonocytic cell line; both represent non-APL forms of AML. In response to RA treatment, HL-60 cells undergo granulocytic differentiation, whereas U937 cells undergo monocytic differentiation (29, 30). In Fig. 4A and B, HL-60 cells were treated with a suboptimal dose of RA in the absence or presence of PP1 or PP2. In the absence of SFK inhibitor, RA treatment resulted in only 8.2% CD11b-positive cells, whereas cotreatment with RA plus PP1 or RA plus PP2 led to 43.9% and 54.1% CD11b-positive cells, respectively (Fig. 4A). These results were confirmed in assays that scored differentiation on the basis of morphology or NBT staining (Fig. 4B). Similarly, inhibition of SFKs was found to enhance RA-induced monocytic differentiation of U937 cells (Fig. 4C). Thus, the negative regulation of RA-induced differentiation by SFKs seems to be a general phenomenon in RA-responsive myeloid cell lines.

To determine the impact of SFK inhibition on the differentiation of primary AML cells, CD33+ blasts were purified from the peripheral blood of a patient with FAB M5 AML. In Fig. 4D, the CD33+ primary blasts were treated with RA in the absence or presence of PP1 or PP2, and 5 days later, expression of CD11b was analyzed. Although a high level of CD11b-positive cells was observed in the DMSO-treated control cells, the results indicate that both PP1 and PP2 promoted a modest enhancement of RA-induced differentiation in the primary AML cells.

SFKs Negatively Regulate RA-Induced Gene Expression

To determine a possible mechanism for the negative influence of SFKs on RA-induced differentiation, we investigated the impact of SFKs on RAR-mediated gene transcription. We first examined the effect of SFK inhibition on the expression of four proteins (C/EBPα, PU.1, ICAM-1, cathepsin D; refs. 31–34) derived from RAR target genes (Fig. 5). In the absence of any treatment, NB4 cells indicated that the negative impact of SFKs on RA-induced differentiation was not due to a negative regulation of events occurring within the first few hours after RA stimulation.

SFK Inhibitors Enhance RA-Induced Differentiation in HL-60, U937, and Primary AML Cells

Figure 4. SFK inhibitors enhance RA-induced myeloid differentiation of HL-60, U937, and primary AML cells. HL-60 cells were treated for 72 h with 2 μmol/L RA alone or in combination with PP1 or PP2 (10 μmol/L), and granulocytic differentiation was assessed by A, CD11b staining (open histograms: isotype control; shaded histograms: anti-CD11b; results representative of three independent experiments) and B, morphologic analysis of Hema 3–stained cytospin preparations or NBT reduction (two independent experiments) as described in Fig. 1. In C, U937 cells were treated for 5 d with 0.1 μmol/L RA alone or in combination with PP1 or PP2 (10 μmol/L) and analyzed for monocytic differentiation by flow-cytometric determination of CD11b expression (results representative of three independent experiments). D, CD33+ primary blasts were isolated from the peripheral blood mononuclear cells of an AML patient (FAB M5) and treated with RA alone (1 μmol/L) or RA plus SFK inhibitors (10 μmol/L). Cells were harvested on day 5 and analyzed for CD11b expression by flow cytometry.

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expressed only very low levels of these proteins (lane 1). Surprisingly, treatment with PP1 or PP2 alone caused a modest induction of C/EBPε, PU.1, ICAM-1, and cathepsin D (lanes 2 and 3), indicating that endogenous SFK activity suppressed the basal expression levels of these proteins. As expected, treatment with RA alone (lane 4) induced the expression of all four proteins. However, this induction by RA was further enhanced when cells were cotreated with RA in combination with PP1 or PP2 (lanes 5 and 6). This suggested that SFKs may be acting to negatively regulate RAR-mediated gene transcription.

To directly assess whether SFKs can negatively regulate RAR-mediated gene transcription, we transiently transfected HeLa cells with pRAR-Luc. The pRAR-Luc reporter construct contains five RA response elements (RARE) upstream from the firefly luciferase gene. Cells were also transfected with phRL-TK, which constitutively expresses Renilla luciferase, and Renilla luciferase readings were used to normalize for transfection efficiencies. In addition to the luciferase constructs, some of the cells were also transfected with increasing amounts of a construct (CA-Src) encoding an epitope-tagged (HA epitope), constitutively active form of c-Src (HA-CA-Src). The HA-CA-Src protein contains a Y529F mutation rendering the enzyme constitutively active (35). As shown in Fig. 6A, RA treatment resulted in a 5.5-fold induction of the RARE luciferase activity, relative to untreated cells. However, transfection of the cells with increasing amounts of the CA-Src construct led to a dose-dependent decrease in RARE-luciferase activity. Confirmation of HA-CA-Src expression in the transfected cells is shown in Fig. 6B. To verify that the negative regulation of RAR-mediated gene transcription was due to the enzymatic activity of HA-CA-Src, the experiment was repeated by transfecting cells with a construct encoding a kinase-dead mutant of c-Src (KD-Src; containing the K296R inactivating mutation; ref. 36). In the experiment shown in Fig. 6C, RA stimulation resulted in a 4.7-fold induction of RARE luciferase activity, which was markedly attenuated in cells expressing HA-CA-Src. However, the ability of HA-CA-Src to abrogate RA-induced RARE luciferase activity was potently inhibited by PP2. Moreover, in contrast to HA-CA-Src, expression of kinase-dead Src (KD-Src) had little impact on RA induction of RARE-luciferase activity. These results support the hypothesis that SFKs can negatively regulate RAR-mediated gene transcription.

**Discussion**

The use of RA results in an high cure rate in APL patients and underscores the potential promise of differentiation therapies (12–14). Treatment with RA overcomes the blockade to differentiation in APL leukemic blasts, serving to clear the bone marrow and peripheral blood of high levels of the blasts. Although RA differentiation therapy has proven highly successful in APL, other subtypes of AML are insensitive to RA. The molecular bases for RA insensitivity in AML are poorly understood, and our understanding of the cellular factors that regulate RA-induced gene transcription and myeloid differentiation is incomplete. In this study, we found that inhibition of SFKs resulted in a dramatic enhancement of RA-induced myeloid differentiation. This effect was seen in three different AML cell lines, as well as a primary AML of M5 subtype, and was not due to effects of the SFK inhibitors on cell cycle status or apoptosis. These findings show that one or more members of the SFK family act to inhibit RA-induced myeloid differentiation. Moreover, they suggest that pharmacologic inhibition of SFKs may have a therapeutic value as part of a differentiation-inducing regimen. The combination of RA with an SFK inhibitor might result in more efficient and rapid differentiation induction in APL patients. It also remains possible that SFK inhibition may promote sensitivity to RA in certain forms of AML that are typically RA insensitive. Further testing of these hypotheses is clearly warranted.

Recent studies using gene knock-out mice have indicated that SFKs, most notably Lyn, Fgr, and Hck, play a role in regulating myelopoiesis in vivo. Lyn-deficient mice exhibit increased levels of myeloid progenitor cells in the bone marrow (21), whereas progenitors derived from Hck-deficient mice exhibit increased proliferation in response to G-CSF (20). Similarly, triple knock-out mice that are deficient in Hck, Lyn, and Fgr manifest increased levels of myeloid progenitors, and these cells show a hyperproliferative response to G-CSF (20). Thus, SFKs have been shown to act as negative regulators of progenitor pool expansion and G-CSF–stimulated granulopoiesis (15). Interestingly, however, defects in myeloid differentiation have not been reported in SFK knock-out mice. These defects may be difficult to observe in vivo if SFK deletion simply accelerates the pace of normal differentiation or if compensation by another SFK member occurs. Alternatively, SFKs may exert little, if any, effect on cytokine-induced myeloid differentiation, whereas exerting a significant inhibitory effect on myeloid differentiation resulting from...
RA treatment, as seen in our study. Additional studies are needed to determine which members of the SFK family are acting to negatively regulate differentiation in myeloid lineage cells and the precise roles they play in myelopoiesis. The application of small interfering RNA strategies or pharmacologic inhibitors with specificity for different SFK members should facilitate these studies.

We have also consistently observed a modest enhancement of SFK phosphorylation, representing SFK activation, in AML cells treated with RA for 2 or 6 h (Fig. 1A and data

Figure 6. Constitutively active Src kinase inhibits RAR-mediated gene transcription. A, HeLa cells were transiently transfected with a RARE luciferase (firefly) construct (pRAR-Luc) along with varying amounts of constitutively active Src construct (CA-Src; 100–400 ng). To control for transfection efficiency, cells were also transfected with a construct (phRL-TK) that constitutively expresses Renilla luciferase. Twenty-four hours after transfection, cells were treated with RA (1 μmol/L) for 16 h. Cells were then harvested, and luciferase activity was determined in cell lysates. The data are expressed as the fold increase in firefly luciferase activity over untreated controls, following normalization for transfection efficiency using Renilla luciferase activity. Results represent the mean ± SD from three identical wells. *, P < 0.05, using one-way ANOVA test when comparing empty vector versus CA-Src (400 ng) in the presence of RA. Similar results were obtained in two independent experiments.

B, equal quantities of whole cell lysates (20 μL) used in the luciferase assay in A were separated on a 10% SDS/PAGE gel, transferred to a nitrocellulose membrane, and probed with anti-HA antibody for detection of the epitope-tagged HA-CA-Src protein. The blot was stripped and reprobed with anti-β-actin antibody. Similar results were obtained in two independent experiments.

C, HeLa cells were transfected with RARE-luciferase plasmid (pRAR-Luc), along with CA-Src construct or a construct encoding a kinase-dead c-Src (KD-Src). Twenty-four hours after transfection, cells were treated for 60 min in the absence or presence of PP2 (20 μmol/L). RA (1 μmol/L) was then added, and incubation was continued for 16 h. The data are expressed as the fold increase in firefly luciferase activity over untreated controls and are normalized for Renilla luciferase activity as in A. The results represent the mean ± SD from three identical wells. *, P < 0.05, using one-way ANOVA when comparing CA-Src versus KD-Src – transfected cells in the presence of RA. **, P < 0.01 (one-way ANOVA,) for CA-Src versus CA-Src + PP2 in the presence of RA. Similar results were obtained in two independent experiments.
not shown). Consistent with these results, Katagiri et al. (37) have reported increased expression and activation of Lyn and Fgr during RA-induced granulocytic differentiation of HL-60 cells. Together, these findings suggest that RA treatment promotes the up-regulation of a negative feedback loop involving SFK enzymes. However, because the basal level of SFK activation in AML cells seems to be quite high, the significance of further up-regulation of SFK activity by RA is unclear. It is intriguing, however, that enhancement of RA-induced differentiation by SFK inhibitors can be achieved even when the inhibitors are added several hours after RA stimulation (Fig. 3). This may mean that SFKs act to negatively regulate secondary events that are significantly downstream from RAR-mediated gene transcription. Alternatively, RA-induced myeloid differentiation may require RAR-mediated transcription for extended periods of time, and SFKs may simply be impacting that transcription at any point throughout this required interval. Attempts to determine the length of time myeloid cells need to be exposed to RA (and hence, undergoing RAR-mediated transcription) to commit to differentiation have proven difficult because complete depletion of RA from RA-treated cells is complicated by the presence of cytoplasmic RA-binding proteins (e.g., cellular retinol-binding protein and cellular retinoic acid-binding protein). Nonetheless, our data favor the hypothesis that SFKs act, at least in part, by negatively regulating RAR-mediated transcription.

The impact of SFKs on RA-induced gene expression was shown by two different experiments. First, we showed that inhibitors of SFKs enhanced RA-induced up-regulation of key RAR target genes, including C/EBPα, PU.1, ICAM-1, and cathepsin D (Fig. 5; refs. 31–34). Second, we showed that constitutively active c-Src inhibited RA-induced expression from a RARE-luciferase reporter construct (Fig. 6A). By contrast, a kinase-dead c-Src had little impact on RA-induced expression from the reporter construct (Fig. 6C). These findings provide the first demonstration that SFKs can regulate transcription mediated by a nuclear hormone receptor, however, is not unprecedented, as others have reported SFK inhibition of estrogen receptor–mediated transcription (38, 39).

Our results raise the possibility that SFKs may be impacting the phosphorylation status and, thereby, the activity and/or stability of RAR. Although SFKs may directly phosphorylate RAR, it is also possible that another kinase may act as an intermediary between activated SFKs and RAR. In this regard, although SFKs have not been shown to directly phosphorylate RAR, several other intracellular kinases do have the ability to directly phosphorylate the receptor. Both PKC and Akt directly phosphorylate RAR and inhibit RAR-mediated transcription, whereas c-jun-NH2-kinase directly phosphorylates and induces proteasomal degradation of the receptor (40–42). In addition, PKA, p38, and CDK7 have been shown to directly phosphorylate RAR (43–45). It is also possible that SFKs may inhibit RAR-mediated gene transcription by promoting the phosphorylation of its dimerization partner, retinoid X receptor, or by promoting phosphorylation of coactivators or co-repressors that are important in the regulation of RAR-mediated transcription. A careful and detailed analysis is needed to identify the kinases that phosphorylate each of these proteins and to determine which of these key proteins may be targets of SFK-mediated signaling in myeloid cells.

By inhibiting the expression of RAR target genes, SFKs inhibit the expression of proteins that play key roles in driving myeloid differentiation. This includes the transcription factors C/EBPα and PU.1 (Fig. 5). C/EBPα is induced by both RA and G-CSF, and mice that are deficient in C/EBPα exhibit functional and maturational defects in granulocytes and macrophages (46, 47). Forced expression of C/EBPα results in granulocytic differentiation of murine 32Dcl3 cells. Mice that are deficient in PU.1 show aberrant development of monocytes and granulocytes, as well as T and B lymphocytes (48). In myeloid cells, the relative expression level of PU.1 is important, with high levels promoting monocytic differentiation, lower levels promoting granulocytic differentiation, and absence resulting in blockade of differentiation (49). Recent studies from Mueller et al. (50) have shown that expression of PML/RAR, the primary fusion oncoprotein in APL, inhibits PU.1 expression, an effect that is overcome by treatment with RA. Moreover, expression of PU.1 in the absence of RA treatment was sufficient to induce differentiation in APL cells. The ability of SFK inhibitors to enhance PU.1 expression in either the absence or presence of RA (Fig. 5) suggests that this effect may be key to the ability of these inhibitors to enhance RA-induced differentiation. It also raises the question of whether enhanced PU.1 expression, resulting from SFK inhibition, may be sufficient to promote RA-induced differentiation in non-APL subtypes of AML.

Our preliminary results suggest that this may be the case for some non-APL AMLs (Fig. 4D) and support the need for further investigation.

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References


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Inhibition of Src family kinases enhances retinoic acid–induced gene expression and myeloid differentiation

Michelle B. Miranda, Robert L. Redner and Daniel E. Johnson


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