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Contribution of STAT3 to the activation of survivin by GM-CSF in CD34$^+$ cell lines

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Objective. **Granulocyte macrophage colony-stimulating factor (GM-CSF) has been shown to specifically stimulate proliferation of CD34$^+$ hematopoietic progenitor cells. Although signal transducers and activators of transcription 3 (STAT3) is believed essential for transduction of GM-CSF–induced cell proliferation, the signaling mediated by STAT3 is not completely understood. Because survivin regulates cell proliferation and survival via its antiapoptotic function, we studied the link between STAT3 signaling and survivin expression in CD34$^+$ cells.**

Methods. **GM-CSF–induced STAT3 and survivin expression in CD34$^+$ cells was examined by Western blot assay. GM-CSF–activated survivin promoter activity was analyzed by gene transfection and reporter assays. The binding of STAT3 to the survivin promoter was evaluated by chromatin immunoprecipitation and electrophoretic mobility shift assay. Western blotting and flow cytometry were utilized to test the effect of Janus family of tyrosine kinases (JAK) inhibitor and STAT3 small interfering RNA (siRNA) on cell apoptosis.**

Results. **We found that GM-CSF stimulates survivin promoter activity in CD34$^+$ KG-1 cells, and STAT3 binds to the core survivin promoter containing a STAT response element TT(N)$_5$AA at sites −264 to −256. Mutation or deletion of this STAT response element completely abolished the effects of GM-CSF on survivin promoter activity. Furthermore, addition of either JAK inhibitor or STAT3 siRNA was able to inhibit GM-CSF–induced survivin promoter activity and survivin expression. Inhibition of survivin by STAT3 siRNA or by withdrawal of GM-CSF in a GM-CSF–dependent, CD34$^+$ line TF-1 decreased cell growth and increased apoptosis.**

Conclusion. **Altogether, our results suggest that survivin is a transcriptional target of STAT3, and that GM-CSF–stimulated CD34$^+$ cell proliferation is regulated by the JAK/STAT3/survivin signaling pathway.** © **2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

Granulocyte-macrophage colony-stimulating factor (GM-CSF) specifically stimulates proliferation and growth of hematopoietic cells, including CD34$^+$ progenitors [1]. In addition, this cytokine has been shown to induce survival signals that suppress apoptosis in both normal and malignant hematopoietic cells [2,3]. The biological functions of GM-CSF are mediated through its binding to the cell surface receptor that is predominantly expressed on hematopoietic progenitor cells, mature granulocytes, and mature macrophages. Because of its roles in stimulating the proliferation, expansion, and mobilization of hematopoietic progenitor cells from bone marrow to peripheral blood, GM-CSF is now the second most commonly used hematopoietic cytokine therapy (after granulocycte colony-stimulating factor [G-CSF] for mobilization of hematopoietic progenitor cells or direct stimulation of bone marrow cells in transplantation [4].

The binding of GM-CSF to its receptor has been reported to evoke signal transduction by activating the receptor-associated Janus family of tyrosine kinases (JAK) [5]. Activated JAK phosphorylates residues in the cytosolic tail of the GM-CSF receptor, allowing subsequent recruitment of downstream signaling proteins to that receptor complex. Members of the signal transducers and activators of transcription (STAT) family are recruited and phosphorylated by JAK. Subsequently, these phosphorylated STATs

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homodimerize or heterodimerize and translocate to the nucleus, where they are able to regulate transcription by binding to specific DNA promoter elements [6]. Although the DNA sequence of the natural palindromic STAT response elements (SRE) vary considerably, they conform to the general structure $TT(N)_5AA$ [7]. At least seven members of the STAT family are able to bind SRE, and most of these are ubiquitously expressed. However, individual STAT proteins can be differentially activated, depending on the cell type or tissue and the specific stimuli applied. For instance, persistent activation of STAT3 and to a lesser extent STAT5, but not activation of STAT1, STAT2, STAT4, or STAT6 have been observed with unexpectedly high frequency in a variety of tumor tissues [8,9]. In hematopoietic cells, STAT5 is strongly activated by GM-CSF and interleukin-3 [10,11], whereas STAT3 is mainly activated by G-CSF [12–14]. STAT3 is also reported to be activated by GM-CSF in both neutrophils and $CD34^+$ cells [5,15].

Numerous studies have evaluated the role of the JAK/STAT3 signaling pathway in malignant as well as normal cells. STAT3 is constitutively activated in many human cancers, where it functions as a critical mediator of oncogenic signaling through its transcriptional activities. It has been found that STAT3 induces activation of genes encoding proteins such as cyclin D1 and c-Myc that regulate cell-cycle progression and cell proliferation [16]. STAT3 participates in cancer cell survival through the upregulation of genes encoding the Bcl-2 family proteins Bcl-XL and Mcl-1 [17,18]. In addition, it has been found that STAT3 induces vascular endothelial growth factor expression, resulting in promotion of tumor angiogenesis [19]. Besides these roles in cancer cells, STAT3 provides a positive function in normal hematopoiesis. STAT3-mediated signaling has been implicated in hematopoietic progenitor cell proliferation, lineage determination, and granulocytic differentiation [20]. It is well established that JAK/STAT3-mediated cell proliferation and differentiation collaborates with hemapotoietic growth factors and cytokines [21]. Experiments have shown that expression of dominant negative STAT3 or hemapotoietic growth factor mutants blocks granulocytic differentiation [13,22], indicating that there is a requirement for STAT3 in hemapotoietic cytokine-regulated hematopoiesis.

Although the downstream signals mediated by constitutively activated STAT3 contributing to cancer cell growth and progression have been elucidated, the molecular mechanisms by which STAT3 regulates hemotopoiesis have not been completely understood. In particular, the downstream signals mediated by GM-CSF or G-CSF–activated STAT3 in cell proliferation and growth have not been defined. It has been previously reported that GM-CSF is able to induce survivin expression in both normal CD34 hematopoietic progenitor cells and myeloid leukemia, and that GM-CSF–induced survivin expression contributes to cell-cycle entry and proliferation [23,24]. Linked with the observation that the JAK/STAT3 signaling pathway is induced by GM-CSF, we chose to investigate whether JAK/STAT3 is implicated in GM-CSF–induced survivin expression in $CD34^+$ cells and evaluate the cellular consequence as well as GM-CSF–induced survivin expression by targeting the JAK/STAT3 pathway.

Materials and methods

Cells

Two $CD34^+$ leukemia cell lines were used in this study. The KG-1 and TF-1 cell lines were obtained from the American Type Culture Collection. The KG-1 human lymphomyeloid progenitor cells were $>95\%$ $CD34^+$ and 100% $CD4^-$ [25]. The TF-1 human erythromyeloid progenitor cells were over 95% $CD34^+$ and 10% $CD4^+$. TF-1 cells are GM-CSF−dependent [26]. Both KG-1 and TF-1 cell lines were grown in standard culture medium (RPMI-1640 containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin) and incubated at 37°C in 5% CO_2. In addition, a final concentration of 2 ng/mL recombinant GM-CSF was added to the TF-1 culture medium.

Bone marrow aspirates were taken from five normal donors after informed consent. These normal mononuclear cells were first separated by centrifugation on Ficoll-Hypaque (Seromed, Hamburg, Germany; 1.077 g/mL). The $CD34^+$ cells were isolated using the Dynal CD34 Progenitor Cells Selection System (Oslo, Norway) according to manufacturer's instructions. Mononuclear cells unreactive with the immunomagnetic beads were designated as $CD34^-$ controls.

Plasmids, transfection, and luciferase activity assay

The core promoter region of survivin has been previously characterized within the proximal −230 nt of the human survivin gene, which regulates the majority of transcriptional activity [27]. For construction of the reporter plasmid pLuc-269 containing the survivin core promoter and pLuc-250 with a deleted SRE, polymerase chain reaction (PCR) primer pairs were determined from the corresponding site sequences on the full-length survivin promoter. The mutant pLuc-269 m construct made using mutated nested primers at the T and A residues within the SRE, strongly interfered with binding to STAT proteins. Constructs including deleted or mutated fragments were then ligated to the pGL3 basic vector. DNA sequencing was performed to confirm that the sequence of the PCR products were correct as compared to the survivin promoter published in the Human Genome database.

Gene transfection was performed to analyze the effect of GM-CSF on survivin promoter activity. KG-1 cells were transfected with the survivin promoter constructs by electroporation at 350 V, 950 μF using a Gene Pulser II System (Bio-Rad, Hercules, CA). The pRL (Renilla luciferase)-CMV vector was cotransfected to provide an internal control. Transfected cells were resuspended in 10 mL RPMI containing 10% FBS, and incubated with GM-CSF (R&D System), and/or JAK inhibitor 1 (Calbiochem, San Diego, CA) for 24 hours. Cell extracts were prepared with 1× lysis buffer, and then 20 μL aliquots of the supernatant were mixed first with 100 μL Luciferase Assay Reagent II (Promega, Madison, WI) to measure the firefly luciferase activity, and then the Renilla luciferase activity was determined by adding Stop & Glo Reagent to the same sample. The luciferase activities were analyzed on