Granulocyte colony-stimulating factor induces the binding of STAT1 and STAT3 to the IFNγ response region within the promoter of the FcγRI/CD64 gene in human neutrophils

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Abstract Granulocyte colony-stimulating factor (G-CSF) has been recently shown to induce the high-affinity Fc receptor for IgG (FcγRI/CD64) in human polymorphonuclear neutrophils (PMN). To elucidate the molecular mechanisms whereby G-CSF exerts this effect, we examined whether the cytokine induces the binding of transcription factors to the IFNγ response region (GRR), a well characterized regulatory element in the FcγRI promoter that is responsible for the transcriptional induction of this gene. Using electrophoretic mobility shift assays, we show that in human PMN, G-CSF activates a GRR-binding complex which contains members of the signal transducer and activator of transcription (STAT) family of proteins, namely STAT1 and STAT3. In keeping with this result, treatment of neutrophils with G-CSF led to tyrosine phosphorylation of STAT3, as determined by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies. This is the first demonstration that in human neutrophils, the induction by G-CSF of FcγRI gene expression may be mediated by the binding of STAT1 and STAT3 to the GRR sequence.

Key words: Neutrophil; G-CSF; FcγRI/CD64; STAT1; STAT3

1. Introduction

In recent years, many studies have established that under appropriate stimulatory conditions, human polymorphonuclear neutrophils (PMN) can synthesize cytokines, as well as several other proteins that are involved in their effector functions [1,2]. One such molecule is the high-affinity receptor for the Fc portion of IgG, FcγRI/CD64, which mediates phagocytosis and antibody-dependent cellular cytotoxicity in activated granulocytes [3,4]. Whereas this receptor is rarely detectable on mature PMN, its surface expression can be greatly upregulated by interferon-γ (IFNγ), both in vitro and in vivo [5-8]. This action of IFNγ has been shown to involve the binding of the activated transcription factor, STAT1, to the IFNγ response region (GRR) of the FcγRI gene promoter [9], resulting in an increased transcription of the FcγRI gene [8]. Although the transcriptional activation of the FcγRI/CD64 gene has long been considered an IFNγ-specific effect in myelomonocytic cells, it has been recently reported that granulocyte colony-stimulating factor (G-CSF) also has the ability to induce FcγRI mRNA accumulation and surface expression in mature neutrophils [10]. The present work was therefore undertaken to define which transcription factors are involved in the G-CSF-mediated induction of FcγRI/CD64 gene expression in human neutrophils.

2. Materials and methods

2.1. Cells and reagents

Neutrophils were isolated from buffy coats of healthy donors under endotoxin-free conditions, as previously described [11]. Highly purified PMN (>99.5% pure) were resuspended in RPMI 1640 medium containing 10% low-endotoxin fetal calf serum (FCS, Hyclone Laboratories Inc., Logan, UT, USA), and stimulated for the indicated times with 1000 U/ml recombinant human G-CSF (Granulokine, Hoffman-La Roche, Basel, Switzerland), or with 100 U/ml recombinant human IFNγ (kindly provided by Dr. G. Garotta, Hoffman-La Roche, Basel, Switzerland). An anti-STAT1 rabbit polyclonal IgG (raised against amino acids 688–710), and two anti-STAT3 rabbit polyclonal IgG (C-20 and K-15, raised against amino acids 750–769 and 626–640, respectively) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Northern blot analyses

Northern blots were performed as described [11]; FcγRI, gp91-phox (the heavy chain subunit of the cytochrome b component of the NADPH oxidase) and actin mRNAs were detected by sequential hybridizations of the nylon filters with purified, denatured, and $[^{32}P]dCTP$-labeled cDNA fragments.

2.3. Cell fractionation and electrophoretic mobility shift assays (EMSA)

After stimulation with 1000 U/ml G-CSF or its diluent (20 min, 37°C), neutrophils (10^8/condition) were diluted in ice-cold phosphate-buffered saline (PBS) and centrifuged ($500 \times g$, 10 min, 4°C). Cells were resuspended in 1.3 ml of relaxation buffer [12] supplemented with an antiprotease and antiphosphatase cocktail (3 mM diisopropyl-fluorophosphate (DFP), 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.25 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 33 μg/ml aprotinin, 1 mM Na_3VO_4 and 50 mM NaF), and disrupted in a nitrogen bomb (Parr Instruments, Mobile, IL, USA) as described [12]. The cavitates were centrifuged ($12000 \times g$, 30 min, 4°C) to pellet unbroken cells, nuclei, and granules. The resulting supernatants (referred to as cytoplasmic preparations) were either processed for immunoprecipitation, or (when used in EMSA experiments) further concentrated using Microcon 10 columns (Amicon, Beverly, MA, USA). EMSAs were performed as described previously [13], using either the 39-bp GRR element of the FcγRI/CD64 gene promoter [14], or the high-affinity c-*sis*-inducible element (hSIE), m67 [15], as oligonucleotide probes. The murine leukemia virus upstream conserved region (UCR) [16] was used as a non-specific competitor.

2.4. Immunoprecipitations and immunoblots

Cytoplasmic preparations (2 mg/condition) were incubated with 20 μl of a 50% slurry of protein A agarose (Boehringer, Mannheim, Germany) in the presence of a 1:300 dilution of anti-STAT3 antibody (C-20), for 4 h at 4°C on a rotating wheel. Immunoprecipitates were washed six times with 1 ml of washing buffer (150 mM KCl, 10 mM Tris pH 7.4, 10% glycerol, 0.05% Triton X-100, 0.1% BSA, 1 mM Na_3VO_4 and 50 mM NaF) prior to electrophoretic separation on 7.5% SDS-PAGE and subsequent transfer to nitrocellulose by electroblotting. Nitrocellulose membranes were blocked in 5% BSA, 20 mM Tris pH 7.6, 137 mM NaCl, 0.05% Tween-20 for 4 h at room tem-

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perature and further incubated (overnight at 4°C) with a 1:1000 dilution of anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY, USA), or with a 1:2000 dilution of anti-STAT3 antibody (K-15). Antibody binding was visualized by peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:15000) and revealed using the enhanced chemiluminescence system (Amersham, Little Chalfont, UK) according to the manufacturer's instructions.

3. Results

Fig. 1 shows a representative Northern blot performed on total RNA purified from human neutrophils cultured for up to 18 h in the presence or absence of G-CSF, or (for comparison) with IFNγ. In agreement with a recent report [10], G-CSF induced the selective accumulation of FcγRI mRNA in human PMN. This effect of the cytokine was already detectable after 1 h, and increased thereafter, peaking at about 5 h. In contrast, G-CSF failed to influence the steady-state levels of mRNA encoding the NADPH oxidase component, gp91-phox. By comparison, IFNγ induced the accumulation of mRNA transcripts encoding both FcγRI and gp91-phox, as previously described [8,11].

To elucidate the molecular mechanisms whereby G-CSF induces FcγRI/CD64 mRNA in PMN, we examined whether the cytokine could induce the binding of protein complexes to the GRR. This regulatory element, located within the promoter of the FcRI gene, contains a typical 9-bp IFNγ-activated sequence (GAS) consensus core, and is necessary and sufficient for IFNγ-induced activation of FcγRI/CD64 [14,17-20]. Cytoplasmic preparations from untreated and G-CSF-treated neutrophils were therefore incubated with a ^{32}P-labeled GRR probe and analyzed in EMSA. As shown in Fig. 2 (left panel), a GRR-binding complex was induced in G-CSF-treated cells, compared to untreated cells. This DNA-binding complex was specific, since it was competed by an excess of unlabeled GRR, but not by an excess of unrelated oligonucleotide (UCR) [16].

Recent studies have shown that stimulation of monocytes with IL-10 results in FcγRI induction [21], as well as in the binding to the GRR element of protein complexes containing

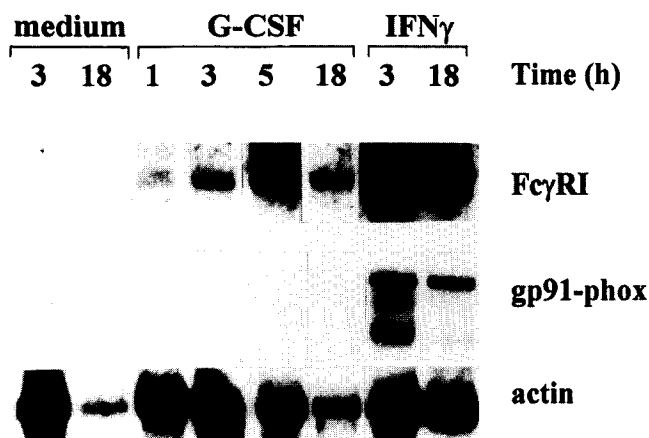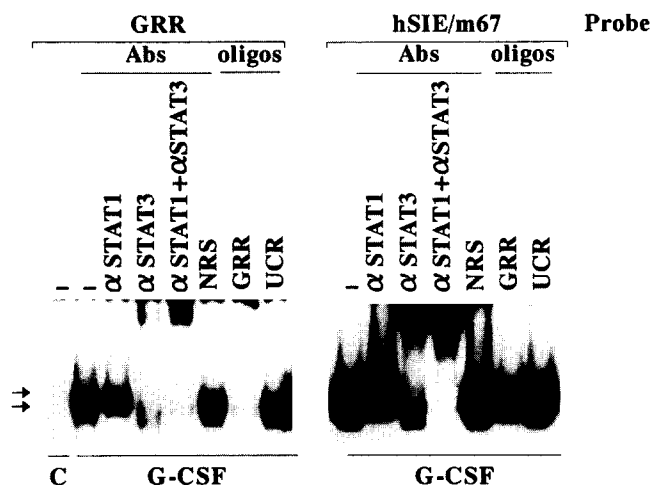Fig. 2. Characterization of DNA-binding protein complexes induced by G-CSF in human PMN. Neutrophils were incubated for 20 min at 37°C in the presence or absence (C) of 1000 U/ml G-CSF, prior to disruption by nitrogen cavitation. The resulting cytoplasmic preparations were incubated with ^{32}P-labeled oligonucleotides (using 60 μg/lane for GRR probe and 40 μg/lane for hSIE/m67 probe), and analyzed in EMSA. For competition and supershifting studies, cytoplasmic preparations were first incubated (30 min, 4°C) with unlabeled competitor (at a 100-fold molar excess), or with anti-STAT1, anti-STAT3, or normal rabbit serum (NRS), prior to incubation with the labeled GRR or hSIE/m67 probes. The experiment depicted in this figure is representative of three.

STAT1 and STAT3 [20,22,23]. We therefore investigated whether the same proteins can bind the GRR sequence following G-CSF treatment of human PMN. To this end, antibodies to STAT1 and STAT3 were added to PMN cytoplasmic preparations prior to incubation with the ^{32}P-labeled GRR probe and subsequent EMSA analysis. As shown in Fig. 2 (left panel), anti-STAT1 antibodies eliminated the lower part of the specific GRR-binding complex, whereas the upper part could be supershifted by anti-STAT3 antibodies. Moreover, when both antibodies were used in combination, the whole GRR-binding complex was efficiently supershifted. By comparison, pretreatment of the same cytoplasmic preparations with normal rabbit serum had no detectable effect. These results clearly show that G-CSF treatment of PMN results in the binding of STAT1 and STAT3 to the GRR. This conclusion was further supported by EMSA experiments in which m67, a synthetic derivative of the hSIE, was used as a probe. This element is known to bind with particularly high affinity the STAT proteins activated by a variety of cytokines [15], and has been previously utilized to study G-CSF-induced STAT protein activation in the human myeloid leukemia cell line, AML-193 [24], and more recently, in human neutrophils [25]. Fig. 2 (right panel) shows that cytoplasmic preparations from G-CSF-treated PMN interacted with the hSIE/m67 probe to form a DNA-binding complex identical in composition to that observed using the GRR probe. Indeed, supershift experiments identified both STAT1 and STAT3 as constituents of this complex. Noteworthy is that an excess of unlabeled GRR oligonucleotide probe did not completely compete out the m67-binding complex, confirming that hSIE/m67 has a higher affinity than GRR for STAT proteins (Fig. 2, right panel).

Since the binding of STAT3 to GAS elements is known to

Fig. 1. Effect of G-CSF on the steady-state levels of FcγRI/CD64 mRNA in human PMN. Neutrophils (10^6 cells/300 μl) were cultured with 1000 U/ml G-CSF, 100 U/ml IFNγ, or their diluent (RPMI 1640), for the indicated times. Total RNA was isolated and analyzed by Northern blot, using FcγRI/CD64, gp91-phox and actin cDNA probes. The experiment depicted in this figure is representative of two.
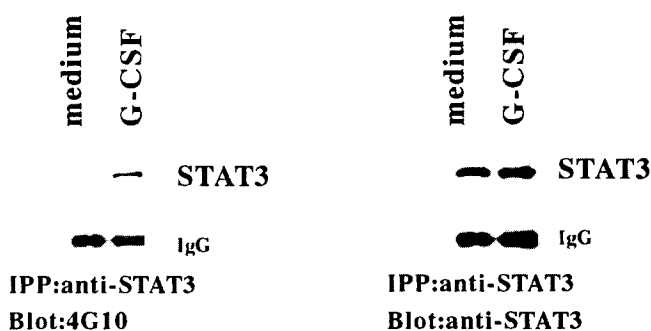
Fig. 3. Effect of G-CSF on the tyrosine phosphorylation of STAT3 in human PMN. Neutrophils were incubated in the presence or absence of G-CSF and disrupted as described in the legend to Fig. 2. The resulting cytoplasmic preparations were immunoprecipitated with an anti-STAT3 antibody and processed for immunoblot using anti-phosphotyrosine antibodies. The nitrocellulose membrane was then stripped and reprobed using an anti-STAT3 antibody.

depend upon its phosphorylation on tyrosine residues [24,26], we examined whether G-CSF treatment of PMN might induce the tyrosine phosphorylation of STAT3. For this purpose, cytoplasmic preparations from PMN treated for 20 min with G-CSF (or its diluent) were immunoprecipitated with anti-STAT3 antibodies, and processed for immunoblot using an anti-phosphotyrosine antibody (Fig. 3). To ascertain that equal amounts of immunoprecipitated proteins were loaded on the gels, the blots were stripped and reprobed with anti-STAT3 antibodies. The results of these experiments make it clear that in human PMN, STAT3 becomes tyrosine phosphorylated in response to G-CSF.

4. Discussion

In the present study, we show that in G-CSF-treated human PMN, the inducible accumulation FcγRI/CD64 mRNA is preceded by the binding of STAT1 and STAT3 to the GRR, a GAS-related enhancer sequence located in the FcγRI gene promoter. These findings, in conjunction with a recent study [10], strongly suggest that the binding of STAT1 and STAT3 to the GRR plays a major role in the induction by G-CSF of FcγRI/CD64 gene expression in human PMN. That both IL-10 and IFNγ induce FcγRI/CD64 gene expression in monocytes, presumably through the binding of DNA-binding complexes containing STAT1 and STAT3 to the GRR [20-23], further supports this conclusion.

In addition to showing that human PMN express substantial amounts of STAT3 protein, our immunoprecipitation studies revealed that STAT3 becomes tyrosine phosphorylated following G-CSF treatment, in agreement with observations made in other cell types [24,27]. Although the G-CSF receptor lacks intrinsic tyrosine kinase activity, G-CSF has been shown to promote the activation of JAK2 in the AML-193 human myeloid leukemia cell line [24], in the immature hematopoietic cell line BAF/BO3 [27], and more importantly, in human neutrophils [28]. In view of the ability of JAK2 to phosphorylate STAT proteins on tyrosine residues [9], these observations raise the possibility that in neutrophils stimulated by G-CSF, there might be a direct link between the onset of JAK2 kinase activity and the activation of DNA-binding complexes containing STAT1 and STAT3.

While this article was in preparation, Tweardy and collea-gues [25] reported that G-CSF treatment of human neutrophils induces a DNA-binding activity that interacts with a hSIE/m67 oligonucleotide probe. Because this hSIE/m67-specific DNA-binding activity was recognized neither by an anti-STAT3 antibody (the same that was used in this study), nor by antibodies directed against other members of the STAT family, the authors concluded that the hSIE/m67-binding complex contains a novel STAT-like protein present in mature neutrophils [25]. While the results of the above study contrast with those reported herein in many respects, these apparent discrepancies are likely to reflect fundamental differences in the respective procedures used to disrupt neutrophils. In this regard, it is noteworthy that our preliminary studies, in which whole-cell neutrophil extracts were prepared by freeze-and-thaw cycles, which results in the partial solubilization of the granules and concomitant release of proteolytic enzymes, yielded identical results to those reported by Tweardy et al. [25]. However, by preparing neutrophil lysates by nitrogen cavitation, which preserves neutrophil granule integrity [12], we were able to detect STAT3 in supershift and immunoblot experiments, as demonstrated herein. Thus, we propose that in neutrophils disrupted by freeze-and-thaw cycles or detergent lysis, STAT3 is subjected to proteolytic cleavage and thus becomes not recognizable by anti-STAT3 antibodies.

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