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Normal Neutrophil Maturation is Associated with Selective Loss of MAP Kinase Activation by G-CSF

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Abstract

Although both GM-CSF and G-CSF activate p42/44 MAPK in neutrophil progenitors, the ability of G-CSF to cause MAPK activation is lost in mature neutrophils, while GM-CSF exposure still causes activation. The mechanism of this differential effect related to maturation status has not been explored. We verified that G-CSF and GM-CSF receptors remain functional on purified mature neutrophils by demonstrating that both cytokines caused phosphorylation of STAT3. However, only GM-CSF was capable of activating MAPK as assessed by gel shift and in vitro kinase assay. Both G-CSF and GM-CSF caused activation of p21 ras in neutrophils, demonstrating that early events in the ras-MAPK pathway remain functional after stimulation by either cytokine. Inhibition of tyrosine phosphatase activity by pervanadate restored the ability of G-CSF to activate MAPK in mature neutrophils. Specific inhibition of the SHP-1 phosphatase, known to be activated by G-CSF but not GM-CSF also restored the ability of G-CSF to activate MAPK in neutrophils. These studies suggest that G-CSF activation of SHP-1 may be an important regulatory step for permitting optimal terminal differentiation during neutrophil production and add to our knowledge of the instructional role of G-CSF and GM-CSF for balancing proliferation and differentiation of neutrophil progenitor cells. This information may prove useful for the understanding of conditions in which neutrophil proliferative/differentiative balancing is dysregulated, such as myeloid leukemia and myelodysplastic disorders.

Keywords

Neutrophils; Map Kinase; G-CSF; GM-CSF; Tyrosine Phosphatase; SHP-1

INTRODUCTION

The continuous production of mature neutrophils is dependent upon a balance between progenitor cell proliferation and differentiation. Two cytokines with important roles in this process are GM-CSF and G-CSF, which are known to activate similar signal transduction pathways after binding to their specific cell surface receptors [1,2]. One of these pathways is the ras-MAPK pathway, which is thought to be involved in neutrophil progenitor proliferation, but not differentiation [3]. Although both GM-CSF and G-CSF activate MAPK in neutrophil progenitors, the ability of G-CSF to cause MAPK activation is lost in mature neutrophils [3], while GM-CSF exposure still causes activation. The mechanism of this differential effect related to maturation status has not been explored. We have

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previously demonstrated in a progenitor cell line modeling system that inhibition of MAPK activation during G-CSF stimulation shifted proliferative/differentiative balancing to neutrophil maturation [4]. This suggests that inhibition of MAPK activation by G-CSF may be an important physiologic event permitting terminal differentiation. These studies were undertaken to uncover the mechanism by which the ability of G-CSF to activate MAPK is lost by the time of neutrophil maturity. We found that inhibition of tyrosine phosphatase activity restored the ability of G-CSF to activate MAPK in mature neutrophils, and that the phosphatase responsible for down regulation of MAPK activation by G-CSF is likely SHP-1.

MATERIALS AND METHODS

Reagents

Anti-phospho-STAT3, anti-STAT3, anti-phosphotyrosine (4G10), anti-p42/44 Map Kinase and anti-Ras antibodies were obtained from Upstate Biotechnology Inc., Lake Placid, New York. Sodium orthovanadate, an inhibitor of tyrosine phosphatases, was obtained from Sigma, St. Louis, MO, Sodium stibogluconate, an inhibitor of the SHP-1 hematopoietic tyrosine phosphatase [5], was obtained from the Centers for Disease Control, Atlanta, GA. G-CSF was obtained from Amgen, Thousand Oaks, CA. GM-CSF was obtained from Sandoz, Vienna, Austria. The MPD cell line, used as a model of neutrophil progenitor differentiation in response to G-CSF, was established and characterized by the authors [6].

Isolation of Mature Peripheral Blood Neutrophils

Purification of neutrophils from peripheral blood was performed as previously described [7]. Following informed consent, 50 ml of blood was collected from the antecubital vein of healthy volunteers using sodium citrate as anticoagulant. Blood was mixed with 15 ml of 6% dextran, allowed to settle, and the plasma and buffy coat were removed and centrifuged at 800×g for 5 minutes. The pellet was resuspended in 35 ml of saline and centrifuged again for 15 minutes at 10°C in a Ficoll-Histopaque discontinuous gradient. Neutrophils were recovered and contaminating erythrocytes were lysed by hypotonic shock. Cells were washed and the pellet resuspended in Hank's balanced salt solution. Isolated neutrophils were used within 5 hours of isolation.

Immunoblotting

Neutrophil lysates were prepared by pelleting purified neutrophils at 7000 × g and resuspension in 200 µl lysis buffer (12 mM Tris-HCl, pH 7.2, 14 mM NaCl, 1mM EDTA, 100 µM sodium orthovanadate, 10 µM phenylmethylsulfonyl fluoride, 5 µg/ml each of aprotinin, pepstatin A and leupeptin, and 0.12% Triton X-100) on ice. Total cell lysates were centrifuged at 4°C at 14,000 × g and supernatants were saved for immunoblotting.

For immunoblotting, cell lysate supernatants prepared from 5 × 10⁶ cells were subjected to SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes. Blots were blocked in Tris-buffered saline-Tween 20 (TBS-T) (20mM Tris-base, pH 7.6, 137 mM NaCl, 0.1% Tween 20) supplemented with 2.5% bovine serum albumin, followed by incubation with an optimal concentration of primary antibody. This was followed by a 1:7000 dilution of horseradish peroxidase-conjugated secondary antibody. Signals were obtained by enhanced chemiluminescence and autoradiography. For some experiments, blots were stripped by a 15 minute incubation at 50°C in 62.5 mM Tris-HCl, 2% SDS and reprobed with a second primary antibody to verify equal loading of lanes.

MAPK Enzymatic Activity Measurement

The enzymatic activity of 42MAPK was measured in whole cell lysates essentially as initially described [8] with optimization for mature neutrophils. 1 mM of MBP peptide (APRTPGGRR), used as the phosphoacceptor, was diluted in freshly prepared kinase buffer (13.4 mM HEPES, pH 7.3, 25 mM MgCl₂, 30 μM Na₂VO₃, 5 mM p-nitrophenyl phosphate, 2 mM EGTA, 2 μM cAMP-dependent kinase inhibitor TTYADFIASGRTGRRNAIHD, 0.420 μCi [γ -³²P]ATP (7 nM), and 68 μM unlabeled ATP). To initiate the phosphotransferase reaction, aliquots (20 μl) of kinase buffer were mixed 1:1 (v/v) with cell lysates. The reaction was carried out at room temperature for 10 min and terminated by blotting 20 μl of the reaction mixture onto P81 ion exchange chromatography paper. Filter squares were washed, dried, and counted for radioactivity. Controls were run in parallel with no S6 kinase substrate peptide. Control counts were subtracted from samples.

Ras Activation Assay

Experiments were performed using the Ras Activation Assay Kit (Upstate Biotechnology), following the manufacturer's instructions. Briefly, Glutathione agarose bead-bound GST fusion protein corresponding to the human activated-ras binding domain of raf-1 was used to specifically bind and precipitate activated ras (ras-GTP) from cell lysates. The beads were then boiled in reducing sample buffer, centrifuged, and the supernatant resolved by electrophoresis and immunoblotted with a monoclonal anti-ras antibody.

Phosphatase Inhibition

Freshly isolated neutrophils were treated with increasing concentrations of pervanadate (freshly prepared by addition of hydrogen peroxide to sodium orthovanadate) [9] or sodium stibogluconate for 10 minutes at room temperature before the addition of 20 ng/ml G-CSF for 5 minutes at 37°C. Cells were then lysed and whole cell lysates were electrophoresed and immunoblotted or used for in vitro kinase assay as described above.

RESULTS

Isolated Mature Neutrophils Express Functional Receptors for GM-CSF and G-CSF

To verify that isolation procedures did not cause loss of receptor expression or function, we verified the presence of both GM-CSF and G-CSF receptors on the surface of purified neutrophils by flow cytometric analysis using fluorochrome conjugated monoclonal antibodies specific for each receptor (CD116, GM-CSFR α and CD 114, G-CSFR, PharMingen, San Diego, CA) (data not shown). The functionality of both receptors was shown by immunoblotting of neutrophil lysates to demonstrate phosphorylation of STAT3 in response to stimulation by either 20 ng/ml GM-CSF or 20 ng/ml G-CSF (fig. 1A). Furthermore, the integrity of early events in the ras-MAPK pathway following stimulation by either cytokine was demonstrated by detection of ras activation (fig. 1B).

GM-CSF, but not G-CSF, Causes Activation of MAPK in Mature Neutrophils

Immunoblotting of stimulated neutrophil lysates with anti-MAPK demonstrated gel shifting of MAPK in response to 20 ng/ml GM-CSF or PMA, but not 20 ng/ml G-CSF (fig. 2A). These results were corroborated by an in vitro MAPK enzymatic assay, which also showed absence of activated MAPK in lysates from G-CSF stimulated neutrophils (fig. 2B).

Loss of Activation of MAPK by G-CSF is Related to Differentiation Level

To ascertain whether loss of MAPK activation by G-CSF is related to the maturational state of neutrophil development, we employed the MPD myeloid cell line which differentiates to mature neutrophils following 5 day incubation with G-CSF [6]. MAPK was activated by

both 20 ng/ml G-CSF and 20 ng/ml GM-CSF in untreated MPD cells. After 5 day incubation with G-CSF, short-term re-stimulation with G-CSF could no longer activate MAPK while GM-CSF stimulation still caused activation (fig. 3). These results were not simply related to down-regulation of G-CSF receptor signaling ability due to the 5 day G-CSF pre-incubation because inhibition of phosphatase activity by pervanadate permitted G-CSF to induce tyrosine phosphorylation of multiple species in the differentiated cells (not shown).

Restoration of G-CSF activation of MAPK in Neutrophils by Pervanadate Inhibition of Tyrosine Phosphatases

Initial experiments were performed to determine the maximum concentration of sodium pervanadate that would not cause a significant increase in background tyrosine phosphorylation of proteins in unstimulated neutrophils. A 1:500 dilution was found to give optimal results (not shown). For subsequent experiments, neutrophils were incubated at room temperature in pervanadate for 10 minutes before stimulation with 20 ng/ml G-CSF, preparation of cell lysates and immunoblotting. Preincubation with pervanadate resulted in detection of phosphorylated MAPK in G-CSF-stimulated neutrophil lysates (fig. 4A). In vitro kinase assay confirmed that pervanadate-treated lysates contained significantly high levels of activated MAPK enzymatic activity following G-CSF stimulation (fig. 4B).

Evidence Suggesting that SHP-1 is the Tyrosine Phosphatase that Downregulates G-CSF Activation of MAPK in Mature Neutrophils

Since GM-CSF causes activation of MAPK in neutrophils and G-CSF does not unless tyrosine phosphatases are inhibited, we reasoned that a phosphatase that is activated by G-CSF, but not GM-CSF might be responsible. A candidate was identified in the SHP-1 hematopoietic tyrosine phosphatase that is known to be activated by G-CSF, but not by GM-CSF [10]. Sodium stibogluconate has been shown to be a specific inhibitor of SHP-1 at a concentration of 10 µg/ml [5]. Preincubation of neutrophils in sodium stibogluconate for 10 minutes allowed detection of phosphorylated MAPK in G-CSF-stimulated neutrophil lysates (fig. 5).

DISCUSSION

These studies demonstrate that the selective loss of G-CSF activation of MAPK in mature neutrophils can be abrogated by inhibition of tyrosine phosphatases and that the SHP-1 phosphatase is likely involved in this process. We believe that this observation is important for understanding proliferative/differentiative balancing during neutrophil production. Although both GM-CSF and G-CSF are known to promote neutrophil progenitor proliferation and differentiation, studies in knockout mice suggest that GM-CSF is not necessary for physiologic neutrophil production while G-CSF is absolutely required [11]. Taken with the observation that MAPK activation is associated with proliferative, but not differentiative signaling in neutrophil progenitors [2], G-CSF activation of SHP-1 may be an important regulatory step for permitting optimal terminal differentiation during neutrophil production. This idea is supported by previous studies showing upregulation of SHP-1 during G-CSF-induced granulocytic differentiation of the 32D.c13 myeloid cell line [10] and dephosphorylation of the ERK-2 MAP kinase by activated SHP-1 [9]. In addition, SHP-1 knockout mice produce morphologically and phenotypically immature granulocytes [12]. Conversely, GM-CSF may serve a role as a “stress” cytokine, serving to augment the number of neutrophil progenitors during times of increased need. The continued ability of GM-CSF to activate MAPK throughout the maturation process would be expected to shift proliferative/differentiative balancing toward proliferation.

Our results and those of others [9] contradict studies published by Dong et al [13], in which SHP-1 was found to suppress Stat3 activation, but to have no effect on p42/44 MAPK activation. Although we are uncertain of the reason for the discrepancy, we will note that the investigators performed these studies on a non-hematopoietic chicken cell line that had been engineered to lack SHP-1 expression and to express the G-CSF receptor.

These studies add to our knowledge of the instructional role of G-CSF and GM-CSF for balancing proliferation and differentiation of neutrophil progenitor cells. This information may prove useful for the understanding of conditions in which neutrophil proliferative/differentiative balancing is dysregulated, such as myeloid leukemia and myelodysplastic disorders.

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ABBREVIATIONS

G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
MAPK	mitogen-activated protein kinase
STAT3	signal transduction activator of transcription-3
SHP-1	src-homology phosphatase-1

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Figure 1.

A. Assessment of functional integrity of G-CSF and GM-CSF receptors on purified neutrophils by activation of STAT3. Lysates from cytokine stimulated neutrophils were probed with an antibody specifically recognizing phosphorylated (activated) STAT3. The blot was stripped and reprobed with an antibody recognizing total STAT 3 to demonstrate equivalent loading of lanes. B. Activation of p21 Ras by both G-CSF and GM-CSF in purified neutrophils. Cells were incubated with the appropriate cytokine for the indicated time interval before preparation of lysates. Activated Ras was specifically precipitated using a bead-bound construct representing the activated Ras binding domain of Raf-1. The precipitates were then immunoblotted with a p21 Ras antibody.

Figure 2.

GM-CSF, but not G-CSF, causes activation of p42/44 MAPK in purified neutrophils. A. MAPK Immunoblot. G: C-CSF. GM: GM-CSF. C: control. PMA: phorbol 12-myristate 13-acetate, used as a positive control for activation. Cells were stimulated for the indicated periods of time and lysates were immunoblotted with a p42/44 MAPK antibody. Retardation of migration is seen with GM-CSF and PMA stimulated cells reflecting increased molecular mass resultant from phosphorylation. B. In vitro MAP kinase assay demonstrating activation of MAPK by GM-CSF and PMA, but not G-CSF at 3 to 5 minutes. Activation is assessed by incorporation of [$\gamma^{32}\text{P}$]ATP into the MBP substrate and scintillation counting. dpm: disintegrations per minute.

Figure 3.

Loss of ability of G-CSF to activate MAPK in MPD cells following differentiation. A. MPD cells treated for 5 days with G-CSF to differentiate to neutrophils. B. Undifferentiated MPD cells. Cells were stimulated with cytokine for the indicated time and lysates were immunoblotted with a p42/44 MAPK antibody. Activation is detected by retardation of migration due to phosphorylation.

Figure 4.

Preincubation of neutrophils with pervanadate restores the ability of G-CSF to phosphorylate MAPK. A. Analysis by immunoblotting. Neutrophils were incubated in the indicated concentration of pervanadate for 10 minutes before addition of G-CSF for 3–5 minutes. Lysates were then immunoblotted with 4G10 antibody. The 42–44 kD region is shown. The blot was then stripped and reprobed with a p42/44 MAPK antibody (bottom) to demonstrate equivalent loading of lanes. B. In vitro MAP Kinase assay demonstrating activation of MAP kinase by G-CSF in neutrophils preincubated for 10 minutes in the indicated concentration of pervanadate. dpm: disintegrations per minute.

Figure 5.

Specific inhibition of SHP-1 by sodium stibogluconate restores activation of MAPK by G-CSF in neutrophils. Cells were incubated in the indicated concentration of stibogluconate (SS) for 10 minutes before stimulation for 5 minutes with G-CSF. Lysates were then immunoblotted with 4G10 antibody to detect tyrosine phosphorylation. The blot was then stripped and reprobed with a p42/44 MAPK antibody to demonstrate equivalent loading of lanes. The two lanes on the far right are controls to demonstrate that GM-CSF activated MAPK in the cells in the absence of preincubation with SS.