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Reactive oxygen species generated by hematopoietic cytokines play roles in activation of receptor-mediated signaling and in cell cycle progression

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Abstract

Hematopoietic cytokines, including interleukin (IL)-3 and erythropoietin (Epo), regulate hematopoiesis by stimulating their receptors coupled with the Jak2 tyrosine kinase to induce receptor tyrosine phosphorylation and activate mainly the STAT5, PI3K/Akt, and Ras/MEK/ERK signaling pathways. Here we demonstrate that IL-3 or Epo induces a rapid and transient (peaking at 30 min) as well as late progressive increase in reactive oxygen species (ROS) in a hematopoietic progenitor model cell line, 32Dcl3, and its subclone expressing the Epo receptor (EpoR), 32D/EpoR-Wt. The cytokine-induced ROS generation was not affected in 32Dcl3 cells depleted of mitochondrial DNA. The antioxidant *N*-acetyl-L-cysteine (NAC) inhibited IL-3-induced tyrosine phosphorylation of Jak2, IL-3 receptor βc subunit (IL-3Rβc), and STAT5 as well as activation-specific phosphorylation of Akt, MEK, and ERK, while treatment of cells with H_2O_2 activated these signaling events. NAC also inhibited the EpoR-induced transphosphorylation of IL-3Rβc. Moreover, NAC treatment reduced the expression levels of c-Myc, Cyclin D2, and Cyclin E, and induced expression of p27, thus inhibiting the G1 to S phase transition of cells cultured with IL-3. Further studies have shown that the degradation of c-Myc was facilitated or inhibited by treatment of cells with NAC or H_2O_2, respectively. These data indicate that the rapid generation of ROS by cytokine stimulation, which is at least partly independent of mitochondria, may play a role in activation of Jak2 and the STAT5, PI3K/Akt, and Ras/MEK/ERK signaling pathways as well as in transactivation of cytokine receptors. The cytokine-induced ROS generation was also implicated in G1 to S progression, possibly through stabilization of c-Myc and induction of G1 phase Cyclin expression leading to suppression of p27.

Keywords: ROS; IL-3; Erythropoietin; Jak2; STAT5; Akt; c-Myc

1. Introduction

Proliferation, apoptosis, and differentiation of hematopoietic progenitor cells are regulated by several cytokines, such as interleukin (IL)-3 and erythropoietin (Epo), which act through the type I cytokine receptors [1,2]. IL-3 stimulates the proliferation and differentiation of various hematopoietic cell lineages and activates the functions of mature macrophages, eosinophils, and mast cells, while Epo regulates the growth and differentiation of the erythroid progenitor cells. The receptor for IL-3 (IL-3R) consists of the α and β subunits, both of which belong to the cytokine receptor superfamily [1]. The mouse has two highly homologous β subunits, βc and β_{IL3}, while the human has only one type of subunit, βc. Both mouse and human βc are shared with the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5. Although lacking the tyrosine kinase domain, the hematopoietic cytokine receptors mainly couple with Jak2, a member of the JAK family of tyrosine kinases, to transduce a growth signal in hematopoietic cells [2]. Through activation of Jak2 and other tyrosine kinases, the cytokine receptors become tyrosine-phosphorylated and thereby recruit various Src homology domain 2 (SH2)-containing signaling molecules to activate common downstream signaling pathways. The latent transcription factor STAT5 is recruited to the receptors for Epo and IL-3 through its SH2 domain to become phosphorylated and activated by Jak2. One of the other main signaling

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pathways is the Ras/ERK activation cascade involving the small GTP-binding protein Ras and the downstream serine/threonine kinases Raf-1, MEK1/2, and ERK1/2 [2–4]. The activated form of Ras also binds the p110 catalytic subunit of phosphatidylinositol 3′-kinase (PI3K) and activates its catalytic activity. PI3K is also recruited to the tyrosine phosphorylated cytokine receptors via the SH2 domain in the regulatory p85 subunit and activated through this interaction. A serine/threonine kinase, Akt, is activated downstream of PI3K and phosphorylates various signaling molecules, including Bad and FKHR, to promote cell survival.

Recent studies have shown that stimulation of a variety of cell surface receptors, including those for platelet-derived growth factor, insulin, and angiotensin I, induces generation of reactive oxygen species (ROS) such as H_2O_2 and superoxide [5–7]. Furthermore, accumulating evidence has also indicated that ROS function as requisite second messengers that are necessary for ligand-mediated regulation of protein kinase activation, gene expression, and/or proliferative responses. While mitochondria, NAD(P)H oxidases, and lipid metabolism are common sources of ROS, NADPH oxidase(s) has been identified as a possible source(s) of ROS generation in many systems of receptor-stimulated ROS production [5–7]. Although the molecular targets that translate the effects of ROS to signaling cascades are still unclear, an interesting potential target is the family of protein tyrosine phosphatases (PTP), which are susceptible to oxidative inactivation [5].

Sattler et al. [8] previously reported that hematopoietic growth factors, including IL-3 and GM-CSF, induce an increase in ROS in hematopoietic cells. Moreover, treatment with antioxidants, which inhibited the increase in ROS, reduced tyrosine phosphorylation and proliferation induced by GM-CSF. It was, thus, suggested that generation of ROS in response to hematopoietic growth factors may contribute to downstream signaling events, especially those involving tyrosine phosphorylation [8]. However, the source and time course of ROS generation by hematopoietic cytokines as well as the mechanisms by which ROS are involved in cytokine receptor signaling to regulate proliferation and apoptosis of hematopoietic cells have remained to be investigated.

In the present study, we demonstrate that hematopoietic cytokines IL-3 and Epo induce both rapid transient and slow progressive increases in ROS, which were not affected by depletion of mitochondrial DNA. Studies with antioxidant treatment of cytokine stimulated cells and with H_2O_2 stimulation indicate that ROS may play roles in cytokine activation of Jak2 as well as the downstream signaling pathways involving STAT5, Akt, and MEK/ERK. The present study also implicates ROS in hematopoietic cytokine-induced cell cycle progression from G1 to S phase through inducing expression of c-Myc, Cyclin D2, and Cyclin E as well as reducing expression of p27.

2. Materials and methods

2.1. Cells and reagents

The IL-3-dependent murine myeloid cell line 32Dcl3 was maintained in RPMI 1640 with 10% fatal calf serum (FCS) and 10% WEHI conditioned medium (as a source of murine IL-3). 32D/EpoR-Wt [9], a clone of IL-3 dependent 32D cells expressing the transfected murine EpoR, and the Epo-dependent human leukemic cell line UT-7 [10] were maintained in RPMI 1640 with 10% FCS and 1 U/ml Epo.

N-acetyl-L-cysteine (NAC), 2′7′-dichlorofluorescin diacetate (DCF-DA), and cyclohexamide were purchased from Sigma-Aldrich. Antibodies were purchased from the following companies: Santa Cruz (Jak2, cyclin D2, cyclin E, α-tubulin, IL-3Rβc, c-Myc), Cell Signaling (phospho-ERK (Thr202/Tyr204), phospho-STAT5 (Tyr694), phospho-Akt (Ser473), phospho-MEK (Ser217/221), phospho-pRb (Ser807/811)), Upstate Biotechnology (Jak2, phosphotyrosine, ERK), and Transduction Laboratories (p27). A rabbit antiserum against the EpoR cytoplasmic domain was previously described [16]. Recombinant murine IL-3 was purchased from PeproTech. Recombinant human Epo was kindly provided by Chugai Pharmaceutical Co Ltd (Tokyo Japan).

2.2. Analysis of intracellular ROS levels

Intracellular ROS levels were measured using DCF-DA, which becomes fluorescent when oxidized by either H_2O_2 or superoxide, essentially as described by Sattler et al. [8]. In brief, cells were labeled with 10 μM DCF-DA for the last 5-min period of cytokine stimulation and were washed once with cold Dulbecco's phosphate-buffered saline (PBS). The fluorescence of oxidized DCF in cells was measured with an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a FACSscan flow cytometer (Becton Dickinson).

2.3. Generation of 32Dcl3 Rho0 cells depleted of mitochondrial DNA

32Dcl3 Rho0 cells depleted of mitochondrial DNA were generated essentially as described previously [11]. In brief, 32Dcl3 were cultured and passage in culture medium supplemented with ethidium bromide (250 ng/ml), uridine (50 μg/ml), and sodium pyruvate (100 ng/ml) for 10 days. The depletion of mitochondrial DNA was verified by examination of the expression of cytochrome oxidase II (Cox II) mRNA, coded by mitochondrial DNA, by the RT-PCR analysis using specific upstream (5′-TGCATGTGGC-TGTGGATGTCATCAA-3′) and downstream (5′-CAC-TAAGACAGACCCGTCATCTCCA-3′) primers. As a control, the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, coded by chromosomal DNA, was also analyzed by RT-PCR using specific