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Phosphorylation of Bcl-associated death protein (Bad) by erythropoietin-activated c-Jun N-terminal protein kinase 1 contributes to survival of erythropoietin-dependent cells

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ABSTRACT

The glycoprotein erythropoietin (Epo) is a hematopoietic cytokine necessary for the survival of erythrocytes from immature erythroid cells. The mitogen-activated c-Jun N-terminal kinase 1 (JNK1) plays an important role in the proliferation and survival of erythroid cells in response to Epo. However, the precise mechanism of JNK1 activation promoting erythroid cell survival is incompletely understood. Here, we reported that JNK1 is required for Epo-mediated cell survival through phosphorylation and inactivation of the pro-apoptotic, Bcl-2 homology domain 3 (BH3)-only Bcl-associated death protein (Bad). Upon Epo withdrawal, HCD57 cells, a murine Epo-dependent cell line, displayed increased apoptotic cell death that was associated with decreased JNK1 activity. Epo withdrawal-induced apoptosis was promoted by inhibition of JNK1 activity but suppressed by expression of a constitutively active JNK1. Furthermore, Epo-activated JNK1 phosphorylated Bad at threonine 201, thereby inhibiting the association of Bad with the anti-apoptotic molecule B-cell lymphoma-extra large (Bcl-X_L). Replacement of threonine 201 by alanine in Bad promoted Epo withdrawal-induced apoptosis. Thus, our results provide a molecular mechanism by which JNK1 contributes to the survival of erythroid cells.

1. Introduction

The glycoprotein erythropoietin (Epo) is a hematopoietic cytokine synthesized in the kidney in response to hypoxia, and it is necessary for the survival and production of mature erythroid progenitors. Although it has been demonstrated to effectively promote proliferation, differentiation, and survival of erythroid progenitors, the role of Epo to prevent apoptosis of late erythroid progenitors is now considered the primary effect of Epo action (Noguchi et al., 2008; Zhande and Karsan, 2007). The anti-apoptotic function of Epo has been the subject of many studies performed in the past few years (Abutin et al., 2009; Ghezzi and Brines, 2004). Epo exerts its effects on erythroid progenitors by binding to its cognate receptor, EpoR, and activates pathways that include phosphatidylinositol 3-kinase (PI3K), c-Jun N-terminal protein kinase (JNK), the extracellular signal-regulated kinase (ERK), and signal transducer and activator of transcription (STAT) pathways (Maiese et al., 2005; Richmond et al., 2005). Recently, it was suggested that Epo could play a role in non-erythroid tissues as numerous other cell types (endothelial- and cancer cells) express EpoR (Acs et al., 2001; Maiese et al., 2005). However, the underlying molecular mechanism of Epo–EpoR signaling on cell survival remains unclear.

The c-Jun N-terminal protein kinase (JNK) has been implicated in the regulation of erythroid proliferation and survival (Jacobs-Helber and Sawyer, 2004; Lee et al., 2010). JNK is a member of the MAPK superfamily and the JNK MAPK subfamily has two ubiquitously expressed isoforms, JNK1 and JNK2, and a tissue-specific isoform, JNK3 (Lin, 2006; Wagner and Nebreda, 2009). Between JNK1 and JNK2, JNK1 is the main c-Jun kinase, whereas JNK2 may interfere with JNK1 activation (Lin, 2006; Liu et al., 2004). JNK is known to be activated by a number of extracellular stimuli including growth factors, cytokines, oncogenes, and environmental stresses (Liu and Lin, 2007). Deregulation of JNK activity has been implicated in many human diseases and certain types of cancer (Liu and Lin, 2007; Wagner and Nebreda, 2009). The activation of JNK was initially reported to be induced by Epo (Nagata et al., 1997), and recent reports have suggested that JNK activation is necessary for the initiation of erythroid proliferation and differentiation

Abbreviations: Epo, erythropoietin; Bad, Bcl-associated death protein; JNK, c-Jun N-terminal protein kinase; Bcl-X_L, B-cell lymphoma-extra large; KA, kinase assays; IB, immunoblotting; IP, immunoprecipitation; CBB, coomassie brilliant blue staining; GST, glutathione S transferase.

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(Jacobs-Helber and Sawyer, 2004; Lee et al., 2010). However, the mechanism of JNK1 activation in the Epo survival signals has not yet been elucidated.

Bcl-associated death protein (Bad) is a BH3-only pro-apoptotic Bcl-2 family protein and plays a critical role in the crosstalk between the growth/survival factor signaling pathway and the intrinsic death machinery (Chipuk et al., 2010). The pro-apoptotic activity of Bad is regulated by extracellular stimuli via post-translational modifications including phosphorylation (Deng et al., 2008; Yu et al., 2004; Zhang et al., 2005). In response to the stimulation of growth/survival factors such as interleukin-3 (IL-3) and insulin-like growth factor-I (IGF-I), Bad is phosphorylated at several serine and/or threonine residues (Ser112, Ser136, Ser155, or Thr201) by a group of protein kinases such as PKA, Akt, and JNK (Cieslak and Lazou, 2007; Yu et al., 2004). Phosphorylation of Bad makes it inactive as it is sequestrated in the cytoplasm via interaction with 14-3-3, a group of cytoplasmic anchorage proteins that interact with proteins through specific phospho-serine/threonine motifs (Sunayama et al., 2005), and thereby preventing Bad interaction with Bcl-2/Bcl-X_L on the mitochondria membrane (Yu et al., 2004). JNK1 suppresses IL-3 withdrawal-induced apoptosis in hematopoietic FL-5.12 cells via phosphorylation of Bad (Deng et al., 2008; Yu et al., 2004). In the present study, we demonstrated that JNK1 activation is also required for the survival of murine erythroleukemia cells through phosphorylation and inactivation of pro-apoptotic molecule Bad, thus our finding indicates an important role of JNK1 signaling in Epo-mediated cell survival.

2. Materials and methods

2.1. Reagents

Antibodies against Bad (C-20), Bcl-X_L (H5), HA (F7), were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against phospho-Bad Ser136 was from Cell Signaling Technology (Danvers, MA, USA). Antibodies against JNK (G151-333 for immunoprecipitation and G151-666 for immunoblotting) were from BD PharMingen (San Diego, CA, USA). The specific JNK inhibitor SP600125 was synthesized and purified (Tang et al., 2002). [γ-^{32}P]ATP and [^{35}S]methionine were from Dupont NEN (Boston, MA, USA). Caspase pan inhibitor Z-VAD-FMK was from Promega (Madison, WI, USA). Anti-phospho-BadT201 antibody was generated by injecting KLH-conjugated phosphopeptide (CRNLGKGGSpTPSQ) into rabbits (Cocalico Biologicals Inc.) and purified (Yu et al., 2004). Propidium iodide (PI) and anti-M2 antibody were from Sigma (St. Louis, MO, USA).

2.2. Plasmids

pCDNA3.1/Hygro (+)-M2-Bad wt or T201A mutant was described previously (Deng et al., 2008). Glutathione S-transferase (GST)–JNK1 and GST–JNK1 (APF), pSRα-hemagglutinin (HA)-MKK7–JNK1, and pSRα-HA-MKK7 (KM)–JNK1 was described previously (Yu et al., 2004; Zheng et al., 1999). Murine Bcl-X_L was subcloned into pCDNA3.1-HisC vector between BamHI and XhoI, HA-MKK7-JNK1 and HA-MKK7 (K149M)-JNK1 were subcloned into pCDNA3.1/Hygro (+) between HindIII and BamHI. All constructs were confirmed by DNA sequencing.

2.3. Cell culture and transfection

Epo-dependent erythroleukemia cell line HCD57 was cultured in Iscove's modified Dulbecco medium (IMDM, Invitrogen, Carlsbad, CA, USA) supplemented with 25% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 10 μg/ml gentamicin (Invitrogen, CA, USA), and 1 U/ml Epo (Amgen, Thousand Oaks, CA, USA) at 37 °C in 5% CO_2. For Epo deprivation studies, HCD57 cells were washed with IMDM three times and re-cultured in IMDM media supplemented with 25% FCS and 10 μg/ml gentamicin in the absence of Epo for 18 h. For Epo stimulation, cells were deprived of Epo and stimulated with 10 U/ml Epo for the indicated times. pCDNA3.1/Hygro (+)-M2-Bad wt or T201A, pCDNA3.1/Hygro (+)-HA-MKK7-JNK1 or pCDNA3.1/Hygro (+)-HA-MKK7 (KM)-JNK1 was transfected into HCD57 cells using Gene Electroporator (Bio-Rad, Hercules, USA) with 250 V, 950 μF, and 50 Ω resistance in a 0.4-cm cuvette. Stable clones were selected against hygromycin B (1 mg/ml).

2.4. Protein kinase assays and immunoblotting

Immune complex kinase assays and GST–c-Jun pull down kinase assays were performed and quantitated as described previously (Deng et al., 2008). Briefly, kinase assays were carried out for 60 min at 30 °C in 20 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 1 mM DTT, 10 μM nonradioactive ATP, and 10 μCi [γ-^{32}P]ATP. Wild type (wt) or mutant GST–Bad or GST–c-Jun (2–4 μg) was used as substrate as indicated. Reactions were terminated by the addition of 4× SDS sample buffer and heating at 95 °C for 5 min. The proteins were separated by SDS-PAGE and visualized by coomassie brilliant blue staining. Phosphorylated proteins were detected and quantitated by PhosphorImager (Molecular Dynamics, PA, USA). Active JNK1 was immunoprecipitated from the lysates prepared from cells stimulated by Epo (10 U/ml), using anti-JNK1 antibody (antibody G151-333).

Immunoblotting was performed as described previously (Deng et al., 2008).

2.5. In vitro translation

The pCDNA3.1 based Bcl-X_L was translated *in vitro* using the Rabbit Reticulocyte Lysate TNT Coupled Transcription/Translation System (Promega, Madison, WI, USA), following the manufacturer's instructions.

2.6. Apoptosis assays

Apoptosis was determined by propidium iodide (PI) staining using flow cytometry analysis. Following cell treatment, cells were fixed in 70% ethanol overnight at 4 °C. Cells were then washed twice in phosphate-buffered saline and stained for 3 h in 3 mM sodium citrate, 2 mM PI, and 50 mg/ml RNase A at 4 °C in the dark. Cells were analyzed by flow cytometry (FACS Caliber, BD, USA). Sub-G_0/G_1, indicative of apoptosis, was gated and a percentage of the total number of cells was determined.

2.7. Statistical analysis

All statistical analyses were performed by using the Student *t*-test. A probability level of 0.05 or less was considered to be significant. Data are presented as mean values ± standard error.

3. Results

3.1. JNK1 is an Epo-activated protein kinase

To determine if JNK1 is involved in the Epo dependent cell survival, we used HCD57 cells to examine the role of JNK1 in apoptosis. HCD57 cells have successfully been used as a model to study the molecular mechanism underlying Epo withdrawal-induced apoptosis (Jacobs-Helber et al., 2000). As expected, HCD57 cells underwent apoptosis upon Epo withdrawal, which was reversed by Epo readdition (Fig. 1A). To further confirm Epo withdrawal-induced apoptosis in HCD57 cells, cells were pretreated with