The International Journal of Biochemistry & Cell Biology 43 (2011) 1134–1146

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Hepatocyte growth factor-induced c-Src-phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathway inhibits dendritic cell activation by blocking IκB kinase activity

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ARTICLE INFO

Article history:
Received 18 January 2011
Received in revised form 23 March 2011
Accepted 12 April 2011
Available online 19 April 2011

Keywords:
c-MET
Dendritic cell
Hepatocyte growth factor
NF-κB
Signaling

ABSTRACT

Hepatocyte growth factor modulates activation and antigen-presenting cell function of dendritic cells. However, the molecular basis for immunoregulation of dendritic cells by hepatocyte growth factor is undefined. In the current study, we demonstrate that hepatocyte growth factor exhibits inhibitory effect on dendritic cell activation by blocking IκB kinase activity and subsequent nuclear factor-κB activation. Inhibition of IκB kinase is mediated by hepatocyte growth factor-induced activation of c-Src. Proximal signaling events induced in dendritic cells by hepatocyte growth factor include a physical association of c-Src with the hepatocyte growth factor receptor c-MET and concomitant activation of c-Src. Activation of c-Src in turn establishes a complex consisting of phosphatidylinositol 3-kinase and c-MET, and promotes downstream activation of the phosphatidylinositol 3-kinase/AKT pathway and mammalian target of rapamycin. Blocking activation of c-Src, phosphatidylinositol 3-kinase and mammalian target of rapamycin prevents hepatocyte growth factor-induced inhibition of IκB kinase, nuclear factor-κB and dendritic cell activation. Notably, hepatocyte growth factor-stimulated c-Src activation results in induction of phosphatidylinositol 3-kinase complexes p85α/p110α and p85α/p110δ, which is required for activation of mammalian target of rapamycin, and consequent inhibition of IκB kinase and nuclear factor-κB activation. Our findings, for the first time, have identified the c-Src-phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathway that plays a pivotal role in mediating the inhibitory effects of hepatocyte growth factor on dendritic cell activation by blocking nuclear factor-κB signaling.

1. Introduction

Among antigen-presenting cells (APCs), only dendritic cells (DCs) are capable of activating naïve T cells to initiate a primary immune response (Hilkens et al., 1997). However, the outcome of a T cell response is largely dependent on the activation and maturation status of DCs. Immature DCs, which express low levels of major histocompatibility complex (MHC) and costimulatory molecules, exhibit a reduced T cell stimulatory capacity (Jonuleit et al., 2000). Upon maturation, DCs upregulate expression of MHC and costimulatory molecules, and gain competency to promote expansion and effector T cell differentiation (Jonuleit et al., 2000). The maturation status of DCs is generally influenced by local cytokine milieu. Importantly, hepatocyte growth factor (HGF), which is a pleiotropic cytokine and expressed in immunosuppressive conditions such as tumors, is known to promote differentiation of monocytes to tolerogenic DCs (Rutella et al., 2006a,b). HGF treatment also results in suppression of DC function (Okunishi et al., 2005). Furthermore, a recent study demonstrates a role for HGF in induction of DC tolerance (Benkhoucha et al., 2010). However, the molecular mechanism of HGF-induced inhibition of DC function has not been defined.

Although HGF was originally discovered as a potent mitogen for hepatocytes (Nakamura et al., 1984), it exhibits multiple effects on many other cell types. For example, HGF promotes mitogenesis, motogenesis and morphogenesis of a wide variety of cells; stimulates antigen-specific B cell differentiation, and migration of DCs and T cells; enhances neuron survival; and regulates leukocyte homing and lymph node organization (van der Voor et al., 1997; Zarnegar and Michalopoulos, 1995; Kurz et al., 2002; Beilmann et al., 2000). Various studies further indicate that HGF exhibits immunoregulatory properties. For example, HGF is shown to counteract a potent immunosuppressive cytokine transforming growth factor β (TGFβ) (Ueki et al., 1999), which suggests that HGF might be an immunopotentiator. In contrast, the immunosuppressive effect of HGF is indicated by a report demonstrating that HGF prevents acute and chronic rejection of cardiac allograft by inducing TGFβ1 and IL-10 expression (Yamaura et al., 2004). HGF can mediate these different cellular functions because of its ability to activate

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doi:10.1016/j.biocel.2011.04.006

multiple cytoplasmic signaling mediators. In fact, HGF triggers recruitment of a number of SH2-containing signal transducers such as phosphatidylinositol (PI) 3-kinase, Grb2-SOS complex, phospholipase C (PLC)-γ and c-Src to a multifunctional docking site YVHVNATYVNV located within HGF receptor cMET (Ponzetto et al., 1993, 1994). Accordingly, it is important to define which signaling proteins are essential for inhibition of DCs by HGF.

Expression of several genes critical for activation, maturation and APC function of DCs is primarily regulated by a transcription factor nuclear factor-kappa B (NF-κB) (Rescigno et al., 1998; Weaver et al., 2001). NF-κB is a dimer composed of the Rel family of proteins and is sequestered in the cytoplasm of resting cells by inhibitory IκB proteins (Ghosh and Karin, 2002). Many external stimuli induce phosphorylation of IκBs by IκB kinase (IKK) complex, followed by proteolytic degradation of IκB proteins (Ghosh and Karin, 2002). However, the role of the NF-κB pathway in HGF-mediated immunoregulation of DCs remains unknown.

The signaling pathway transduced by c-MET in DCs upon HGF treatment is not yet defined. Therefore, the current study was initiated to determine the mechanism by which HGF regulates DC activation. We demonstrate that HGF-induced DC suppression requires activation of c-Src, which blocks the NF-κB pathway and subsequent DC activation via a signaling cascade involving sequential activation of the PI3K/AKT pathway and mammalian target of rapamycin (mTOR).

2. Materials and methods

2.1. Mice

BALB/c and C57/BL6 (B6) mice were maintained and bred under specific pathogen-free conditions. The use of mice was approved by the Institutional Animal Ethics Committee of the Institute of Microbial Technology, India.

2.2. DC preparation

Bone marrow-derived DCs (BMDCs) and splenic DCs (sDCs) were prepared from male or female BALB/c and B6 mice between 8 and 12 weeks of age as described (Bhattacharyya et al., 2004; Weaver et al., 2001). Flow cytometric analyses indicated ~90% purity of BMDCs and sDCs based on CD11c expression.

2.3. DC pretreatment with HGF, αc-MET Ab or inhibitors of signaling molecules

DCs (5×10^6 cells/well) were pretreated with specified concentrations of recombinant human HGF (Sigma–Aldrich, USA) for indicated times in 6-well low-cluster plates (Corning, USA) in RPMI 1640 complete medium (10% FBS, penicillin/streptomycin, L-glutamine, sodium pyruvate, non-essential amino acids, 2-mercaptoethanol). Subsequently, DCs were washed twice, resuspended in complete medium, and stimulated with lipopolysaccharide (LPS) (500 ng/ml) (Sigma–Aldrich, USA) or tumor necrosis factor α (TNFα) (20 ng/ml) (R&D Systems, USA) for specified times. In some experiments, DCs were treated for 1 h with (1) c-Src inhibitor E804 (10 nM; Calbiochem, USA), (2) PI3K inhibitors wortmannin (Wort) (200 nM; Cell Signaling Technology, USA) or Ly294002 (LY) (50 μM; Cell Signaling Technology, USA), (3) mTOR inhibitor rapamycin (100 nM; Calbiochem, USA), or (4) 10 μg/ml αc-MET antibody (Ab) or isotype control Ab (R&D Systems, USA); or for 3 h with AKT inhibitor IV (AI-IV; 20 μM; Calbiochem, USA) before HGF treatment.

2.4. Electrophoretic mobility shift assay (EMSA) and immunoblotting

Nuclear and cytoplasmic extracts were prepared from DCs as described (Beg et al., 1993). EMSA was performed using ^{32}P-labeled DNA probe containing NF-κB binding sites derived from MHC-I H2K promoter: 5′-CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC-3′ (Haldar et al., 2010). A double-stranded OCT-1 DNA probe, 5′-TGTCGAATGCAAATCACTAGAA-3′ was used as control (Haldar et al., 2010). Supershift EMSAs were carried out as described (Haldar et al., 2010) using Abs: αp50, αRelB (Active Motif, USA); αp65 (Abcam plc., USA); αp52, αcRel and rabbit IgG (Santa Cruz Biotechnology, USA). Bands were visualized using a phosphorimager (Bio-Rad Molecular Imager FX, USA).

Western and dot blotting were performed as described (Bhattacharyya et al., 2004; Toulouse et al., 2005). Blots were probed with Abs specific for: IκBα, IκBβ, IκBε, IKK1, PI3K/p110β and PI3K/p110δ (Santa Cruz Biotechnology, USA); phospho-IκBα, phospho-IKK1/phospho-IKK2 (Ser180/Ser181), IKK2, phospho-AKT (Ser473), AKT, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho-p38MAPK (Thr180/Tyr182), p38MAPK, phospho-ERK1/phospho-ERK2 (Thr202/Tyr204), ERK1/ERK2, phospho-c-Src (Tyr416), c-Src, phospho-p70S6 kinase (phospho-p70S6K) (Thr389), p70S6K, phospho-eukaryotic initiation factor 4E binding protein 1 (phospho-4E-BP1) (Thr37/46), 4E-BP1, phospho-mTOR (Ser2448), mTOR, phospho-glycogen synthase kinase 3β (phospho-GSK3β) (Ser9), GSK3β, influenza hemagglutinin (HA), PI3K/p110α and PI3K/p85α (Cell Signaling Technology, USA); c-Met and phospho-Tyrosine (phospho-Tyr) (Millipore, USA); and β-actin (Sigma–Aldrich, USA). Binding of secondary HRP-labeled goat-αrabbit (Santa Cruz Biotechnology, USA) or goat-αmouse (Sigma–Aldrich, USA) Abs was analyzed using SuperSignal® West Pico or West Dura Chemiluminescent Substrate (Pierce, USA).

2.5. siRNA and DNA transfection of BMDCs

BMDCs were transfected with either a scrambled control set of oligos or siRNAs (60 nM) specific for c-Src, mTOR or PI3K subunits p110α and/or p110δ (Santa Cruz Biotechnology, USA) using Lipofectamine LTX (Invitrogen, USA) as per the manufacturer's instructions. Transfection efficiency was determined by transfecting DCs with fluorescein isothiocyanate (FITC)-conjugated control siRNA, and measuring the frequency of FITC-positive DCs via flow cytometry. Approximately, >80% of BMDCs were transfected under the conditions used.

The expression vector pcDNA3 encoding HA-tagged constitutively active GSK3β, in which regulatory serine 9 (Ser9) was changed to alanine (HA-GSK3β-S9A), and HA-tagged empty pcDNA3 vector (HA-EV) were provided by Dr. Rujun Gong (Brown University, USA) and Dr. Eminy H.Y. Lee (Academia Sinica, Taiwan), respectively (Gong et al., 2008; Chao et al., 2007). BMDC transfection with expression plasmid or empty vector was performed using TransIT-2020 transfection reagent (Mirus, USA) following the manufacturer's instructions. BMDCs were then subjected to different treatments as specified. Approximately, 70% BMDCs were transfected under the conditions used, which was determined by transfecting BMDCs with pmaxGFP (Lonza Cologne AG, Germany) and measuring the frequency of GFP$^+$BMDCs via flow cytometry.

2.6. Immunoprecipitation of c-MET-associated protein complexes

For "pull-down" experiments, DCs (10^7) were treated with HGF (60 ng/ml) for varying times, chilled on ice, resuspended in 1 ml of the cell-permeable protein cross-linker dimethyl 3,3-dithiopropionimidate dihydrochloride (Sigma–Aldrich, USA) in PBS (2 mg/ml) and incubated at room temperature for 20 min. In some