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Role of different protein tyrosine kinases in fMLP-induced neutrophil transmigration

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Abstract

Protein tyrosine phosphorylation is among the early signaling events in polymorphonuclear leukocyte (PMN) responses to chemoattractant stimulation. We previously showed that tyrosine phosphorylation might serve as the downstream signaling for the modulation of PMN transmigration by CD47. Here, we further investigated the role of various tyrosine kinases in PMN transmigration and identified the potential tyrosine kinases serving as CD47-mediated signaling downstream. We observed that PMN transmigration was significantly enhanced by Src family kinase inhibitors PP1 and PP2 as well as Syk tyrosine kinase inhibitor piceatannol, suggesting that these kinases have negative regulatory roles in PMN chemotaxis. In contrast, PMN chemotaxis was reduced by LFM-A13, an inhibitor of the Tec family tyrosine kinase Btk (Bruton's tyrosine kinase). LFM-A13 also dose-dependently inhibited *N*-formyl-Met-Leu-Phe (fMLP)-induced PMN intracellular [Ca^{2+}] increase. Since LFM-A13 significantly enhanced PMN chemokinesis while other inhibitors had no effect, the inhibition of PMN chemotaxis by LFM-A13 might be due to the promotion of random cell migration. Among the other inhibitors we tested, AG126 significantly inhibited PMN transmigration while the MAP kinase inhibitors SB20358 and PD98059 showed an enhancing effect. No effect of herbimycin A, erbstatin analog, lavendustin A or AG490 on PMN transmigration was observed. Treatment with PP1, PP2 or piceatannol all partially reversed the delay of PMN transmigration caused by inhibitory anti-CD47 antibody. In summary, our results demonstrate distinct roles of different tyrosine kinases in regulating PMN chemotaxis and suggest Src and/or Syk kinases are likely involved in CD47-mediated downstream signaling.

Keywords: Tyrosine phosphorylation; Neutrophil; Transmigration; CD47

Introduction

Neutrophil (PMN) chemotactic transmigration across the vascular endothelium, extracellular interstitium, and mucosal epithelium is a hallmark of acute inflammation and is associated with many pathophysiological conditions (Downey, 1994; Parkos, 1997a, b; Liu et al., 2004). To initiate transmigration, PMN respond to chemotactic factors, including chemokines and chemoattractants, which activate G-protein (Gi family) mediated cascades

Abbreviations: ABTS, 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Btk, Bruton's tyrosine kinase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS-, Hank's balanced salt solution devoid of Ca^{2+} and Mg^{2+}; MAP kinase, mitogen-activated protein kinase; PMN, polymorphonuclear leukocyte

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of intracellular signaling pathways that modulate PMN adhesion, detachment and directional migration. It has been demonstrated that activation of G-proteins by chemoattractants such as *N*-formylated peptides results in an exchange of GTP binding to G-protein α subunit followed by dissociation of the α subunit from the β and γ subunits. The free α subunit then activates downstream effectors including phosphatidylinositol specific phospholipase C (PI-PLC), which produces inositoltrisphosphate (IP3) and DAG. IP_3 mediates an increase of intracellular Ca^{2+} (Siddiqui et al., 2000) and DAG activates protein kinase C (PKC). Activation of PKC along this line further induces many pathways of cell signaling that regulate PMN adhesion and migration, while transient increase of intracellular Ca^{2+} is involved in cytoskeletal reorganization and actin filament uncapping/polymerization, which pushes cell crawling (Downey, 1994). On the other hand, the free G-protein $\beta\gamma$ subunit has been reported to interact with PI3-kinase and isoforms of PLC-β, leading to activation of these proteins and their subsequent attachment to the special domains in plasma membrane (Clapham and Neer, 1993; Zeilhofer et al., 2000). PI3-kinase has been demonstrated to play an important role in PMN directional polarization and migration during chemotaxis (Wang et al., 2002; Weiner et al., 2002), while PLC-β regulates superoxide production and other kinase activities (Li et al., 2000; Zeilhofer et al., 2000; Cicchetti et al., 2002).

In addition to the above regulatory pathways, many other signaling molecules, including protein tyrosine kinases, protein phosphatases, phospholipase A_2, MAP kinases, STAT3, STAT5, etc., are also found to be involved in chemoattractant-induced PMN functions. Among these molecules, activation/inactivation of protein tyrosine kinases and thus changing the intracellular protein tyrosine phosphorylations have been regarded among the earliest signaling events in PMN after chemoattractant stimulation. Both receptor tyrosine kinases and non-receptor tyrosine kinases, including PDGF-receptor, Src family tyrosine kinases, Src related tyrosine kinases and non-Src tyrosine kinases (such as Jaks), have been found in PMN. Studies have demonstrated that Src family kinases (Hck, Fgr, Lyn, etc.) and a non-Src kinase, Syk, play essential roles in integrin-mediated PMN adhesion, PMN spreading, respiratory burst and degranulation (Lowell et al., 1996; Mocsai et al., 1999, 2002; Takami et al., 2001; Nijhuis et al., 2002; Zhang et al., 2005). A report by Nijhuis et al. (2002) also demonstrated that treatment of PMN with PP1, a Src family tyrosine kinase inhibitor, and LY294002, a PI3K inhibitor, resulted in strong inhibition of fMLP-induced superoxide production and cytokine-mediated cell survival but not fMLP-induced migration.

In our previous studies, we kinetically analyzed PMN transmigration using a time-course transmigration setup and demonstrated that genistein, a broad tyrosine kinase inhibitor (Akiyama et al., 1987; Linassier et al., 1990), drastically enhances the onset (within 30 min period) of PMN transmigration towards chemoattractants including fMLP and IL-8 (Liu et al., 2001; Zen et al., 2006). However, this potent enhancing effect of genistein in PMN transmigration revealed by the time-course kinetic assays had been generally missed previously by the traditional one-time point (normally 90–120 min) transmigration assays. Not only being a significant migration enhancer, our study has also demonstrated that genistein, but not its inactive analog daidzein, dramatically reversed anti-CD47 mAb mediated inhibition in PMN transmigration (Liu et al., 2001), suggesting that certain protein tyrosine kinase(s) and its mediated phosphorylation(s) serves as the critical intracellular signaling event(s) downstream of CD47 (Liu et al., 2001). Given these compelling results revealed by the time-course transmigration assays and the importance of tyrosine kinases in PMN activation and chemotaxis, in the present study, we employed the same kinetic assay setups and further analyzed the effects of a panel of other tyrosine kinase inhibitors with narrow inhibitory specificities. In addition to their effects on PMN chemotaxis, we also performed experiments to analyze these tyrosine inhibitors in chemoattractant-induced PMN locomotion, $[Ca^{2+}]$ mobilization and their effects on CD47-mediated transmigration.

Materials and methods

Reagents and materials

The tyrosine kinase inhibitors used in this study, including genistein, AG126, the Src family tyrosine kinase inhibitors PP1 and PP2, herbimycin A, the Bruton's tyrosine kinase (Btk) inhibitor LFM-A13, the JAK-2 kinase inhibitor AG-490, the MAP kinase (p38) inhibitor SB203580, and the MAP kinase (p42/44 MEK) inhibitor PD98059, were purchased from Biomol (Plymouth Meeting, PA). The Syk kinase inhibitor piceatannol, the receptor tyrosine kinase inhibitors lavendustin A and erbstatin analog were purchased from Calbiochem (San Diego, CA). The inhibitory anti-CD47 mAb C5D5 was used as previously described (Parkos et al., 1996; Liu et al., 2001; Zen et al., 2002).

PMN isolation

Human PMN were isolated from whole blood of normal human volunteers following a protocol described previously (Liu et al., 2002; Zen et al., 2006). Briefly, PMN were separated from other blood cells by dextran sedimentation (2% dextran in 0.9% NaCl) and