Role of Phosphoinositide 3-Kinase in Monocyte Recruitment under Flow Conditions*

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Robert E. Gerszten‡‡, Erik B. Friedrich§, Takashi Matsuii, Rebecca R. Hung§, Ling Li‡, Thomas Force‡‡‡, and Anthony Rosenzweig‡‡‡

From the ‡Program in Cardiovascular Gene Therapy, Cardiovascular Research Center and the §Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Charlestown and Harvard Medical School, Boston, Massachusetts and the ¶Molecular Cardiology Research Institute, New England Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02111

Chemokines such as the monocyte chemokine attractant protein-1 (MCP-1) convert monocyte rolling to firm arrest under physiological flow conditions via integrin activation and simultaneously activate phosphoinositide 3-kinase (PI3K). Here we used adenoviral gene transfer and biochemical inhibitors to manipulate PI3K-dependent pathways in human monocytes. In in vitro lipid kinase assays from purified human monocytes, we showed that MCP-1 activates the "classical" PI3Kα pathway and not PI3Kγ, a PI3K isoform thought to be activated only by the βγ complex of heterotrimeric G proteins. The activity of PI3Kα in purified human monocytes was evident within 30 s. MCP-1-induced monocyte arrest was significantly inhibited both by wortmannin (n = 4; p < 0.01) and LY294002 (n = 4; p < 0.01) with restoration of the rolling phenotype (p < 0.05 for both inhibitors, compared with rolling of control monocytes after MCP-1 treatment). To test the hypothesis that activation of PI3K is sufficient to induce monocyte adhesion, we transduced the monocyct TBP-1 cell line with a recombinant adenovirus (Ad) carrying a constitutively active mutant of PI3K (Ad.BD110). We examined the ability of these cells to adhere to human vascular endothelium (HUVEC) transduced with adenoviruses carrying E-selectin, intercellular adhesion molecule-1 (ICAM-1), and VCAM-1. Under flow conditions, ICAM-1- and VCAM-1-dependent firm adhesion of Ad.BD110-transduced TBP-1 cells wasenhanced compared with TBP-1 cells infected with control Ad (n = 4; p < 0.01 for both). Adhesion augmented by constitutive PI3K activation was entirely abrogated by pretreatment with wortmannin (n = 3; p < 0.01). In contrast, a constitutively active Akt construct had no effect on TBP-1 adhesion (n = 3; p = NS). We conclude that PI3K activation is necessary and sufficient to enhance monocyte adhesion under physiological flow conditions. BD110-expressing TBP-1 cells should provide a useful tool for identifying the signaling pathways downstream of PI3K that are necessary for monocyte recruitment relevant to a variety of human vascular pathologies.

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† To whom correspondence should be addressed: Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, East 8307 14th St., Charlestown, MA 02129. Tel.: 617-724-8322; Fax: 617-726-5651; E-mail: gerszten@cvc.mgh.harvard.edu.
‡ A Feodor-Lynen Research Fellow of the Alexander von Humboldt Foundation.
‡‡ Established Investigators of the American Heart Association.

In vitro and in vivo models suggest a role for chemokines in a variety of inflammatory pathologies, including asthma, arthritis, and atherosclerosis. As chemokine attractants, chemokines play an important role in the directional migration of leukocytes through tissues. Recent data have also underscored the importance of chemokines in enhancing the avidity of leukocyte integrins for locally expressed adhesion molecules. Under physiological flow conditions such as seen in the bloodstream, chemokines can induce the rapid conversion of lymphocyte (1) or monocyte (2) tethering (rolling) to firm adhesion via integrin activation. These data speak to an important role for chemokines in the initial step of leukocyte infiltration of the endothelium, which is then followed by transmigration into the surrounding tissues. However, the pathways by which chemokines activate leukocytes, markedly enhancing leukocyte firm adhesion over the course of seconds, remain poorly understood.

Recently the PI3Kγ family has been implicated in chemokine receptor signaling. Chemokines activate PI3K isoforms in nanomolar concentrations, and biochemical inhibitors of PI3K have been shown to inhibit chemokine-triggered chemotaxis of lymphocytes, NK cells, and monocytes (3–5). The PI3Kγ isoform in particular is thought to play an important role in the signaling of G protein-coupled receptors, such as chemokine receptors (5). Neutrophils from mice deficient in PI3Kγ have diminished chemotaxis to specific chemokines (6–8). However, in contrast, neutrophil adhesion induced by several chemokines, is unaffected by the loss of PI3Kγ (6). In addition, data from other lines of investigation suggest roles for other signaling pathways such as Rho GTPases in chemokine-triggered arrest of leukocytes (6, 9).

Here we used biochemical inhibitors and adenoviral gene transfer to manipulate PI3K-dependent pathways in human monocytes and studied the functional effects of these manipulations on adhesion under physiological flow conditions. Interestingly, we found that MCP-1 triggered monocyte adhesion and activation of the PI3Kα isoform, but not PI3Kγ in purified human monocytes. PI3K activation correlated temporally with our functional studies. Furthermore, PI3K was necessary for the chemokine-triggered conversion of monocyte rolling to firm adhesion and sufficient to enhance monocyte arrest under physiological flow conditions.

The abbreviations used are: PI3K, phosphoinositide 3-kinase; NS, not significant; HUVEC, human vascular endothelial cells; MOI, multiplicity of infection; pfu, plaque-forming units; EGFP, enhanced green fluorescent protein; mAb, monoclonal antibody; PI(3,4,5)-trisphosphate; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemo-attractant protein-1.
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EXPERIMENTAL PROCEDURES

Materials—RPMI 1640, Dulbecco’s modified Eagle’s medium, and Dulbecco’s phosphate-buffered saline with or without Ca²⁺ and Mg²⁺ were purchased from BioWhittaker, Inc. Human serum albumin was obtained from Baxter Healthcare Corp (Glendale, CA). Fetal bovine serum was obtained from Hyclone Inc. Recombinant MCP-1 was purchased from R&D Systems. Wortmannin was purchased from Sigma. LY294002 was obtained from Biomol (Plymouth Meeting, PA).

Cell Culture—HEK 293 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in M199 with 20% fetal bovine serum, endothelial cell growth factor (25 μg/ml; Biomedical Technologies, Stoughton, MA), porcine intestinal heparin (50 μg/ml; Sigma), and antibiotics. After infection with adenoviral vectors, HUVEC were cultured as above, but the serum concentration was reduced to 10%. For experimental use in the flow plate apparatus, HUVEC (passage 1–2) were plated at confluence in 0.5-cm² chambers on fibronectin-coated plastic tissue culture slides. On the day of the flow adhesion assay, a fluorescence immunofluorescence assay was performed on HUVEC infected in parallel to document transgene expression and to rule out nonspecific activation of the endothelial monolayer, as previously described (10). THP-1 cells were obtained from ATCC. 1 × 10⁷ cells were infected at an MOI of 25 in 0.5 ml of serum-free RPMI 1640 in a twelve-well dish for 1 h, and the volume was subsequently increased to 3.0 ml and serum added to reach 10%. Experiments were performed and transgene expression was evaluated 24–48 h after infection.

Recombinant Adenoviruses—AdE-sel (2), AdEGFP (12), AdBD110 (12), and AdVE-akt (12) have been described previously. AdICAM was generated using the system recently described by Wang et al. (11). Briefly, AdICAM carries the cDNA for human ICAM-1 (kindly provided by Dr. T. A. Springer) and was initially subcloned into the pLEP shuttle vector. This construct was then linearized and ligated to the pREP vector containing adenoviral (Ad2) backbone. An adenoviral cosmid was then employed generating β phase/Enterobacteria cell extract. Recombinant adenoviruses were propagated in 293 cells by lipofectin-mediated transfection with positive cosmid clones.

Cell Monolayers in 15-cm tissue culture dishes at an MOI of 200 were transduced with adenoviral vectors, HUVEC were cultured as above, but the serum concentration was reduced to 10%. For experimental use in the flow plate apparatus, HUVEC (passage 1–2) were plated at confluence in 0.5-cm² chambers on fibronectin-coated plastic tissue culture slides. On the day of the flow adhesion assay, a fluorescence immunofluorescence assay was performed on HUVEC infected in parallel to document transgene expression and to rule out nonspecific activation of the endothelial monolayer, as previously described (10). THP-1 cells were obtained from ATCC. 1 × 10⁷ cells were infected at an MOI of 25 in 0.5 ml of serum-free RPMI 1640 in a twelve-well dish for 1 h, and the volume was subsequently increased to 3.0 ml and serum added to reach 10%. Experiments were performed and transgene expression was evaluated 24–48 h after infection.

RESULTS

PI3K Activity—Recruitment of monocytes under flow conditions was characterized as either rolling or firm arrest at 2.0 dynes/cm². As shown previously, purified human monocytes predominantly rolled on endothelial cell monolayers transduced with AdE-sel (2). The addition of recombinant MCP-1 (25 nM) to the flow of incoming monocytes, at physiologically relevant concentrations, markedly augmented monocyte adhesion (Fig 1), at least in part, though activation of β₁ and β₂ integrins (2), (data not shown). Pretreatment of monocytes with the PI3K inhibitors, wortmannin (10 nM) or LY294002 (50 μM) (“< 0.01 for both compared with firm adhesion control monocytes after MCP-1 treatment). Representative data from one of four experiments are shown.

We first examined the effects of biochemical inhibition of PI3K activity on monocyte adhesion in a parallel-plate laminar flow model. We qualitatively and quantitatively assessed monocyte interactions with HUVEC cells transduced with adenoviruses carrying cDNAs for the human adhesion molecules, E-selectin (AdE-sel) or ICAM-1. The phenotype of persistent monocyte-endothelial interactions was characterized as either rolling or firm arrest at 2.0 dynes/cm². As shown previously, purified human monocytes predominantly rolled on endothelial cell monolayers transduced with AdE-sel (2). The addition of recombinant MCP-1 (25 nM) to the flow of incoming monocytes, at physiologically relevant concentrations, markedly augmented monocyte adhesion (Fig 1), at least in part, though activation of β₁ and β₂ integrins (2), (data not shown). Pretreatment of monocytes with the PI3K inhibitors, wortmannin (10 nM) or LY294002 (50 μM) (“< 0.01 for both compared with firm adhesion control monocytes after MCP-1 treatment). Representative data from one of four experiments are shown.

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nonspecific effects on alternative pathways (13). Of note, chemokine pretreatment of the endothelial monolayer has no effect on monocyte adhesion in this model (data not shown) (2).

Chemokines activate several PI3K isoforms in leukocytes, including the classical PI3Kα (p85/p110, IA) isoform as well as PI3Kg (p101/p110, IB), which is thought to be activated exclusively by G protein-coupled receptors (3–5). We assessed the kinetics and relative activation of PI3Kα and PI3Kg in purified human monocytes. As seen in Fig. 2A, MCP-1 activated PI3Kα, and pretreatment of monocytes with low-dose wortmannin (or LY294002, data not shown) markedly attenuated this activity. Though baseline PI3K activity varied, an increase in lipid kinase activity of PI3Kα was evident within 30 s of MCP-1 stimulation (Fig. 2B). These data, suggesting rapid activation of the classical PI3K pathway, paralleled our observation that monocyte rolling is converted to firm adhesion in less than 1 min. Interestingly, activity of PI3Kg, previously implicated in chemokine signaling through G protein-coupled receptors (5), was unchanged by MCP-1 at the 30 s and 3 min time points, though background levels were also inhibited by wortmannin. Importantly, the concentration of wortmannin (10 nM) and LY294002 (50 μM) necessary for biochemical inhibition in monocytic cells, as seen in Fig. 2C, was comparable with the concentration necessary for functional inhibition.

As biochemical inhibitors suggested that PI3K is necessary for MCP-1-triggered monocyte adhesion, we next used a constitutively active PI3K construct (AdBD110), which replaces the p85 binding domain fused in-frame with the p110 catalytic subunit of PI3Kα, to address whether PI3K activation is sufficient to recapitulate the augmented adhesion seen by chemokine activation of monocytes under flow. To manipulate signaling pathways using somatic gene transfer, we employed the monocytic THP-1 cell line, which mimics much of the phenotype of human monocytes (14). We first assessed lipid kinase activity in vitro of the monocytic THP-1 cell line transduced with the constitutively active PI3K construct. Cell lysates from the transduced THP-1 cells were concentrated using a combination of mAbs, directed at c-Myc and p110 subunits. PI3K activity was then measured directly as described above.

![Fig. 2. Biochemical analysis of PI3K activation.](http://www.jbc.org/)

A, freshly isolated human monocytes (10⁶) were treated with wortmannin (10 nM, 30 min, 16 °C) or vehicle control (similarly diluted Me₂SO). Monocytes were stimulated with MCP-1 (25 nM) for 3 min and the reaction stopped with lysis buffer. Monocyte lysates were then immunoprecipitated with mAbs directed at either the p85 or p110γ subunits. PI3K activity was measured directly using phosphatidylinositol and [³²P]ATP. Phospholipids were then separated by TLC and [³²P]-labeled PIP was visualized by autoradiography. The upper spot co-migrated with the P(4)P standard (data not shown). B, representative early time points using similar protocol (n = 3). MCP-1 (25 nM) induced an increase in p85- but not p110γ-associated PI3K activity. PI3K activity was inhibited in both cases by wortmannin (10 nM). C and D, PI3K dose-response studies to the inhibitors wortmannin (C) and LY294002 (D). Monocytic THP-1 cells (10⁶) were treated with the indicated inhibitor, stimulated with MCP-1 (25 nM), and PI3K activity was assessed as above. Spots were cut out from the TLC plate and [³²P]-labeled PIP quantitated in a liquid scintillation analyzer (n = 2 for each inhibitor).

![Fig. 3. Analysis of PI3K and Akt activation.](http://www.jbc.org/)

An in vitro kinase assay is shown. 2 x 10⁶ THP-1 cells were transduced with AdEGFP, AdBD110, AdmyrAkt, (MOI = 25 for each) or left uninfected. After 24 h in culture, cells were lysed with lysis buffer. THP-1 lysates were then immunoprecipitated with mAbs directed at c-Myc and p110 subunits. PI3K activity was then measured directly as described above.
with a control adenoviral construct or an adenovirus carrying the cDNA for a constitutively active Akt construct. In contrast, we saw a marked increase in PI3K activity in the AdBD110-transduced THP-1 cells.

We performed flow cytometry experiments on transduced monocytic cells in parallel with functional assays. We saw no effect of adenoviral gene transfer or PI3K inhibitors on surface expression of integrins critical for monocyte-endothelial interactions or on general markers of leukocyte activation (Fig. 4).

Having confirmed that AdBD110 confers increased PI3K activity, we then investigated the functional consequences of this activity in monocytic cells under dynamic conditions. As seen in Fig. 5, uninfected and AdEGFP-transduced THP-1 cells show moderate baseline adhesion to HUVEC monolayers co-expressing E-selectin and ICAM-1. Firm adhesion of both uninfected and AdEGFP-transduced THP-1 cells was similarly enhanced by MCP-1 (25 nM). In contrast, we saw approximately a 3-fold increase in baseline ICAM-1-dependent firm adhesion of the BD110-transduced THP-1 cells, to a level comparable with that seen after THP-1 cells are stimulated with MCP-1. In studies employing VCAM-1-transduced monolayers, we also saw augmented adhesion conferred by transduction of THP-1 cells with the BD110 construct (data not shown). Increased adhesion rendered by constitutive PI3K activation and MCP-1 stimulation was inhibited by pretreatment with wortmannin (10 nM). Interestingly, we saw a further increase in overall adhesion after the addition of MCP-1 to the BD110-transduced THP-1 cells.

The serine-threonine kinase Akt is an early critical branching point downstream of PI3K and has been implicated in chemokine-triggered signaling (15, 16). Its role in protecting cells from apoptosis is well characterized (17). Akt is also activated by chemokines in a variety of leukocyte subsets in a PI3K-dependent manner and in a time course that could be consistent with a role in the adhesive process (18). We tested whether Akt, like PI3K, is sufficient to augment adhesion. We employed Ad.myr-Akt an adenovirus containing the cDNA for human Akt rendered constitutively active by the addition of the Src myristoylation signal (12). As seen in Fig. 6A, MCP-1 rapidly activates Akt in monocytic cells. Transduction of THP-1 cells with BD110 and Ad.myr-akt confers comparable levels of Akt activation as assessed by a mAb specific to Akt phosphorylation at the Ser-473 site. As seen in Fig. 6A, MCP-1 rapidly activates Akt in monocytes. Transduction of THP-1 cells with BD110 and Ad.myr-akt conveys comparable levels of Akt activation as assessed by a mAb specific to Akt phosphorylation at the Ser-473 site. In contrast to PI3K, however, activated Akt did not augment adhesion under flow conditions (Fig. 6B).

**DISCUSSION**

Chemokines can markedly enhance leukocyte interactions with surface-expressed adhesion molecules via the rapid activation of integrins, though the relevant signaling pathways remain poorly characterized. Recent *in vitro* and *in vivo* data have suggested an important role for PI3K in chemokine signaling, as inhibition of PI3K by biochemical inhibitors and genetic manipulation alter chemotaxis and leukocyte accumulation in several animal models. However, the role of PI3K in leukocyte adhesion under dynamic conditions has not been characterized. The data presented here extend the role of PI3K, as it is necessary for the chemokine-triggered conversion of monocyte rolling to firm arrest, an early step in monocyte...
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Studies in knockout mice suggest an important role for PI3Kγ in several in vitro and in vivo inflammatory models, though it should be noted that loss of PI3Kγ had a spectrum of effects on various leukocyte subsets (6–8). Other lines of investigation show that PI3Kγ is activated by chemokines in human NK cells, and mAbs directed against this particular isoform, as opposed to mAbs directed at PI3Kα, inhibit NK cell chemotaxis (5). In this context, it is interesting to note that we did not detect MCP-1-induced activation of PI3Kγ in freshly isolated peripheral human monocytes. Whether the generation of PIP_3 by PI3K isoforms is a general signal for leukocyte activation and migration, or specific PI3K isoforms confer specific downstream signals in individual leukocyte subsets, remains the subject of future investigations.

Data from several lines of investigation, including work in PI3Kγ knockout mice noted previously, now suggest that the signaling pathways necessary for leukocyte chemotaxis differ from those responsible for other functions such as adhesion (2, 22). The CC chemokine MCP-4, for example, is a potent activator of human monocytes in chemotaxis and calcium flux studies, yet has a negligible effect in modulating leukocyte adhesion under flow. The CXC chemokine, interleukin-8, in contrast, has modest effects in calcium flux and chemotaxis, but is a surprisingly potent agonist for monocyte arrest. Somatic gene transfer, in addition to biochemical inhibitors and transgenic methods, may prove a useful tool to dissect pathways downstream of PI3K in chemokine-activated leukocytes responsible for these potentially distinct signaling mechanisms.

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Fig. 5. PI3K is sufficient to augment adhesion. HUVEC were transduced with AdEGFP or AdBD110 in parallel with the biochemical analysis described above. Leukocyte-endothelial interactions were studied at 2.0 dynes/cm². When indicated, interactions were quantified 1 min prior to and 1 min after the addition of MCP-1 (25 nM). Uninfected and AdEGFP-transduced THP-1 cell adhesion is comparably enhanced by MCP-1 (n = 6; **p < 0.01 compared with baseline, p = NS compared with each other.) Adhesion of AdBD110-transduced THP-1 cells is increased significantly (n = 4; ***p < 0.01) and is further enhanced by the addition of MCP-1 (25 nM, n = 3; ****p < 0.05), but entirely abrogated by pretreatment with wortmannin (10 nM; n = 3; ***p < 0.01). Cumulative data from three experiments are shown with the number of coverslips for each condition noted.

Fig. 6. Role of Akt activation in THP-1 cells. THP-1 cells were transduced with AdEGFP, AdBD110, or AdmyrAkt for 48 h or left uninfected. A, for immunoblotting, uninfected cells were treated with MCP-1 (25 nM, lane 4) or vehicle control (lane 5) for 1 min at 37 °C. Western blot analysis was performed on whole cell lysates using mAbs to phospho- (upper panel) and total Akt (lower panel). B, adhesion studies were performed comparing uninfected, EGFP, BD110-, or myr-Akt-transduced cells in the absence of MCP-1 treatment. As noted previously, AdBD110 augmented arrest of THP-1 cells (n = 3; p < 0.01 compared with uninfected or EGFP-infected cells). Ad.myr-Akt-transduction had no effect on MCP-1 adhesion under flow (n = 4; p = NS compared with uninfected or EGFP-infected cells).
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