Short Communication

Adhesion Pathways Utilized by HIV-Infected Lymphocytes

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Abstract

We recently reported a novel adhesion pathway in lymphocytes that is mediated by cyclin-dependent kinase (Cdk) 4 activity and mediates lymphocyte interactions with endothelial matrix. We now demonstrate that HIV-infected lymphocytes also use Cdk4 to mediate spontaneous adhesion to fibronectin and endothelial matrix. We further demonstrate that HIV-infected lymphocytes require Rap-1 activity for phorbol-stimulated adhesion. Understanding adhesion pathways used by HIV-infected lymphocytes may lead to interventions to regulate aberrant adhesion and migration.

LYMPHOCYTE EMIGRATION IS A tightly regulated process that is essential for an appropriate inflammatory or immune response. The ability of HIV-infected lymphocytes to adhere and transmigrate through the endothelium contributes to propagation of infection and several disease manifestations, such as lymphocytic interstitial pneumonitis and HIV encephalitis. However, the pathways used by HIV-infected lymphocytes for adhesion are unknown.

We recently reported a new adhesion pathway that is important in posttrafficking, i.e., mediating lymphocyte interactions with and migration through subendothelial extracellular matrix.¹ We showed that leukocytes could adhere to high-density fibronectin or exposed endothelial matrix in the absence of exogenous stimulation. We found several novel features of this pathway that clearly distinguish it from "conventional" phorbol ester-stimulated adhesion: it is dependent on Cdk4 activity, it requires microtubules, it does not require exogenous lymphocyte activation, and it does not require the small GTPase Rap-1 activity.¹ Because this novel pathway allows lymphocyte adhesion to physiologically relevant substrates such as exposed endothelial matrix in the absence of exogenous stimulation, we have termed it "ligand-induced adhesion" (LIA). We demonstrated a role for Cdk4-mediated adhesion in lymphocyte recruitment following lung injury and in thymocyte maturation and adhesion.^{1,2}

Cyclin dependent kinases (Cdk) are serine/threonine kinases that regulate cell cycle progression. Because of the role of Cdks in cell proliferation, much interest has focused on the development of pharmacological Cdk inhibitors as a therapeutic for cancer. Several studies have demonstrated that Cdk

inhibitors can also inhibit replication of several viruses,³ including HIV.⁴⁻⁶ Of additional interest, Cdk9, the catalytic subunit of positive transcription elongation factor b (P-TEFb), is part of the TAK complex (Tat-associated kinase complex), and binds to Tat protein of HIV, suggesting a possible role for Cdk9 in AIDS progression.⁷ Therefore, pharmacologic inhibitors of Cdks have been proposed as therapeutic agents for HIV infection.⁸⁻¹⁰

We questioned whether HIV-infected lymphocytes use the Cdk4-mediated pathway for adhesion, and if so, what the contributions of the different adhesion pathways in lymphocyte adhesion are in HIV-infected cells.

We first compared the adhesion of HIV-infected lymphocytes and mock-infected lymphocytes to high-density fibronectin without phorbol stimulation. Primary blood lymphocytes (PBL) were isolated and infected as previously described.¹¹⁻¹³ As controls, cells were stimulated with PHA alone ("mock-infected"). Infection was monitored by measurement of p24 Ag (Coulter HIV-1 P24 antigen assay kit). Cells were used 7 days after infection. Adhesion assays were performed as previously described.¹ We found HIV-infected lymphocytes had the same adhesion profile to fibronectin as mock-infected cells (Fig. 1A), demonstrating that HIV-infected lymphocytes are similarly capable of ligand-induced adhesion. In addition, HIV-infected Jurkat T cells exhibited similar adhesion to fibronectin as mock-infected Jurkat cells (Fig. 1B). As expected, phorbol ester stimulation of lymphocytes with phorbol dibutyrate (PDBu) increased adhesion to fibronectin (Fig. 1C and D), which was similar in infected versus control lymphocytes. We also examined the spontaneous adhesion of HIV-infected lymphocytes to a more

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FIG. 1. (A, B) HIV-infected lymphocytes participate in ligand-induced adhesion. HIV-infected primary blood lymphocytes (PBL) (A) or Jurkat cells (B) were allowed to adhere for 30 min to indicated concentrations of fibronectin. (C, D) HIV-infected or mock-infected PBL were treated with 100 μ M roscovitrine (C) or 20 μ M purvalanol A (D) for 30 min, with or without stimulation by phorbol dibutyrate (PDBu), and then allowed to adhere to fibronectin (2 μ g/ml) (C) or HUVEC matrix (D) for 30 min. HUVEC matrix was prepared by treating the confluent EC monolayer with 20 mM NH_4OH at 37°C for 5 min. * $p < 0.005$ compared to control. (E) Cdk inhibitors block Rb phosphorylation. Cells were treated with media alone (Ctrl), purvalanol (Pu), or roscovitrine (Ro) for 30 min and then lysed. Equal amounts of protein were separated by SDS-PAGE, then blotted with a phospho-specific Ab to the Cdk4-specific site on Rb (249/252) (top) or Ab to total Rb (bottom) as loading control. (F) Jurkat cells stably transfected with Cdk4 DN or control vector were infected with HIV. At day 7 after infection, Jurkat cells were allowed to adhere to fibronectin (2 μ g/ml). ** $p < 0.01$ compared to control vector. An average of three independent experiments $\pm$ SD is shown. (G) Lysates from Jurkat cells stably transfected with Cdk4 DN or control vector were probed with anti-Cdk4 antibody (top) or phospho-specific Ab to the Cdk4-specific site on Rb (bottom).

physiologic substrate, human umbilical vein endothelial cell (HUVEC)-derived matrix (Fig. 1D). Similar to our results with fibronectin, we found that HIV-infected and control lymphocytes adhered similarly to HUVEC matrix.

To determine whether HIV-infected lymphocytes required Cdk activity for spontaneous adhesion, we tested the effects of the Cdk inhibitors roscovitrine and purvalanol A on adhesion to fibronectin and HUVEC matrix. Treatment of HIV-infected lymphocytes with either Cdk inhibitor significantly decreased adhesion to fibronectin (Fig. 1C) or HUVEC matrix (Fig. 1D). Reduction of inhibition was similar in HIV-infected and mock-infected lymphocytes. In contrast, the Cdk inhibitors had no effect on phorbol ester-stimulated adhesion of HIV-infected or mock-infected lymphocytes (Fig. 1C and D). Of

note, since Cdk inhibitors can target additional pathways,¹⁴ we previously demonstrated that inhibitors of other pathways, including PI3 kinase inhibitor (LY294002), MAPK inhibitor (PD98059), or tyrosine kinase inhibitor (genistein) had no effect on ligand-induced adhesion.¹ We also demonstrated that Cdk4, but not Cdk2, Cdk5, or Cdk6 is involved in ligand-induced adhesion using dominant negative constructs, siRNA knockdown, and thymocytes from Cdk4^{-/-} mice.^{1,2}

The identification of Cdk4 as the relevant substrate was somewhat surprising since roscovitrine has been shown to have limited direct effect against Cdk4.^{15–19} It is possible that Cdk4 inhibition by roscovitrine was indirect, i.e., through the down-regulation of the cyclin partners or other Cdk4s that are required for Cdk4 activity.²⁰ In addition, the absence of

FIG. 2. Phorbol myristate acetate (PMA)-stimulated adhesion, but not ligand-induced adhesion, of HIV-infected lymphocytes is Rap1 dependent. **(A)** HIV-infected lymphocytes and mock-transfected lymphocytes were treated with 10 μ M GGTI, 10 μ M FTI, or 100 μ M roscovitine and then allowed to adhere to HUVEC matrix for 30 min. * $p < 0.001$ compared to control. **(B)** PMA stimulation of cells for 30 min prior to inhibitor treatment. * $p < 0.001$ compared to PMA. Values represent means of three independent experiments performed in triplicate. **(C)** Jurkat cells were treated with the Rho kinase inhibitor (30 μ M Y7632) for 30 min, then allowed to adhere to FN for 30 min alone, or in the presence of phorbol dibutyrate (PDBu). Values of two independent experiments performed in triplicate are shown. **(D)** Jurkat cells were transfected with the Rap1DN construct or control vector and allowed to adhere to FN with or without PDBu.

phosphorylation of reported Cdk4-specific sites on Rb in the presence of roscovitine may be due to regulation by other Cdk4s.^{21,22}

Therefore, to confirm our previous work demonstrating the involvement of Cdk4 in ligand-induced adhesion,¹ we tested the ability of Cdk4 dominant-negative (DN) construct to inhibit ligand-induced adhesion in HIV-infected lymphocytes. Jurkat cells were stably transfected with a Cdk4 DN expression vector that contained a mutation D145N that prevents its activity.²³ We confirmed construct expression and inhibition of phosphorylation of Rb, downstream target of Cdk4 (Fig. 1G). After HIV infection, Cdk4 DN reduced the adhesion in both HIV-infected Jurkat cells and mock-infected Jurkat cells to a similar degree (Fig. 1E).

We previously showed that phorbol ester-stimulated adhesion of Jurkat cells to fibronectin required activation of the small GTPase Rap1.²⁴ To determine whether HIV-infected lymphocytes required Rap1 or Rho1 for spontaneous or phorbol-stimulated adhesion, we treated cells with either GGTI, which inhibits geranylgeranylation of Rap1, or FTI (farnesyltransferase inhibitor), which blocks Rho activity. As expected, roscovitine decreased spontaneous adhesion to fibronectin (Fig. 2A). We found that phorbol myristate acetate (PMA)-stimulated adhesion of HIV-infected lymphocytes and of mock-infected lymphocytes was similarly inhibited by GGTI (Fig. 2B), consistent with a requirement for Rap1 activity in phorbol-stimulated adhesion. In contrast, FTI did not affect PMA-stimulated adhesion in HIV-infected or mock-infected lymphocytes (Fig. 2B). Neither GGTI nor FTI reduced spontaneous adhesion to fibronectin (Fig. 2A), consistent with ligand-induced adhesion being independent of Rap1. As further confirmation that ligand-induced adhesion was independent of Rap1, we demonstrated that transfection of Jurkat cells with a dominant negative construct of Rap1 (N17Rap1)²⁴ did not inhibit ligand-induced adhesion, but did inhibit phorbol ester-stimulated adhesion, confirming our previous work^{1,24} (Fig. 2D). Consistent with a lack of effect of FTI treatment,

the RhoA kinase inhibitor Y7632 did not inhibit either ligand-induced adhesion or phorbol ester-stimulated adhesion. In concert, these data suggest that RhoA kinase and Rap1 activity are not required for ligand-induced adhesion.

In summary, we expand our previous studies to demonstrate that HIV-infected lymphocytes exhibit similar spontaneous adhesion to fibronectin and to HUVEC matrix as mock-infected lymphocytes, demonstrating that HIV-infected lymphocytes participate in ligand-induced adhesion. Using pharmacological inhibitors of Cdk and dominant negative construct of Cdk4, we show that Cdk4 is involved in ligand-induced adhesion of HIV-infected lymphocytes. In addition, we show that HIV-infected lymphocytes use Rap-1 during phorbol-stimulated adhesion. We conclude that HIV infection does not alter utilization of Cdk4-mediated or of Rap1-mediated adhesion pathways. Furthermore, use of Cdk inhibitors as therapeutic for HIV infection should take into account the potential effects on lymphocyte adhesion and transmigration.

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Author Disclosure Statement

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