Ligand-engaged urokinase-type plasminogen activator receptor and activation of the CD11b/CD18 integrin inhibit late events of HIV expression in monocyctic cells

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Urokinase-type plasminogen activator (uPA) signaling via its receptor uPAR inhibits late events in HIV-1 replication in acutely infected primary monocyte-derived macrophages (MDMs) and promonocytic U937 cells. Here we show that U937-derived, chronically infected U1 cells stimulated with phorbol 12-myristate 13-acetate (PMA) express integrins, uPA, and soluble uPAR at levels similar to those of MDMs. uPA inhibited HIV expression in U1 cells incubated with either PMA or tumor necrosis factor-α (TNF-α), but not with other HIV-inductive cytokines or lipopolysaccharide. Of interest, only PMA and TNF-α, but not other HIV-inductive stimuli, induced surface expression of the α5 chain CD11b in U1 cells constitutively expressing CD18, the β2 chain of the Mac-1 integrin. Like uPA, fibrinogen, a Mac-1 (CD11b/CD18) ligand, and M25, a peptide homologous to a portion of the β-propeller region of CD11b preventing its association with uPAR, inhibited HIV virion release in PMA-stimulated U1 cells. Both uPAR small-interference RNA (siRNA) and soluble anti-β1/β2 monoclonal antibodies abolished the anti-HIV effects of uPA, whereas CD11b siRNA reversed the anti-HIV effect of M25, but not that induced by uPA. Thus, either uPA/uPAR interaction, Mac-1 activation, or prevention of its association with uPAR triggers a signaling pathway leading to the inefficient release of HIV from monocyctic cells. (Blood. 2009;113:1699-1709)

Introduction

Urokinase-type plasminogen activator (uPA), a serine protease that activates plasminogen to plasmin,1 is synthesized as an inactive precursor (pro-uPA) that undergoes a rapid proteolytic activation. uPA binds to a specific glycosyl-phosphatidyl-inositol (GPI)–anchored receptor, uPAR, localized at the cell surface.2 Both uPA and uPAR are expressed by inflammatory cells, including neutrophils, monocytes, macrophages, and activated T lymphocytes,2 in which they play important roles in cell activation, adhesion, and migration.3,4 In addition to localizing the enzymatic activity of uPA on the leading edge of migrating cells, uPAR mediates signaling by uPA.5 The binding of uPA to uPAR induces migration, adhesion, and proliferation of different cell types, independent of the catalytic activity of uPA.6,7 As a GPI receptor lacking an intracellular domain, uPAR requires the interaction with transduction-competent receptors, such as the G-protein–coupled receptor formyl peptide receptor-like–1 (FPRL1),8 the gp130 signal–transducing chain of the interleukin-6 (IL-6) receptor family,8 or integrins such as αvβ3 in epidermal cancer cells and CD11b/CD18 (Mac-1) in monocyte-macrophages.5,6,9,10

High serum and cerebrospinal spinal fluid levels of soluble uPAR (suPAR) have been correlated with the severity of HIV-1 disease independent of CD4+ T-cell counts or viremia levels.11-14 Furthermore, uPA expression has been observed in the brains of HIV+ individuals whose brains stained negatively for both HIV-1 p24 Gag antigen and uPAR,15 suggesting a potential role of uPA as a negative regulator of HIV-1 expression. In vitro, uPA inhibits HIV-1 replication in lymphoid histocultures, primary monocyte-derived macrophages (MDM), promonocytic U937 cells acutely infected with HIV, and chronically infected promonocytic U1 cells stimulated with the differentiating agent phorbol 12-myristate 13-acetate (PMA) or tumor necrosis factor-α (TNF-α).16,17 In particular, uPA was shown to promote the sequestration of HIV particles in cytoplasmic vacuoles, likely belonging to multivesicular bodies,18-20 an effect that was fully accounted for by the signaling-competent amino-terminal fragment (ATF) of uPA.5 More recently, we showed that vitronectin (VN)–dependent cell adhesion is crucial for uPA-mediated inhibition of virus replication in MDM and in PMA-stimulated U1 cells.17 A better definition of this signaling pathway and of its determinants may be relevant for understanding the dynamics of tissue seeding by infected leukocytes that may affect their ability or efficiency to establish HIV reservoirs in sanctuary sites21 and cause organ/tissue-specific pathology, such as HIV-associated dementia, interstitial lung disease, nephropathy, enteropathy, and wasting syndrome.22,23

In the present study we investigated which among the known uPAR-associated signaling-competent receptors mediate its inhibitory signal on HIV-1 expression in monocyctic cells. Our findings indicate that such an inhibitory effect is mediated by β1 and/or β2 integrins, but does not require the expression of CD11b. In addition, we demonstrated that stimulation of the Mac-1 integrin by fibrinogen (FNG) or prevention of the association between CD11b and uPAR fully mimicked uPA/uPAR-dependent inhibition of late events in virus expression. These findings reinforce the hypothesis of a common pathway controlling the late phase of HIV assembly and release from infected monocyctic cells.
Methods

Reagents

Lipopolysaccharide (LPS)-free (<2 × 10^{-5} EU/μL, corresponding to <10^{-10} EU/ng) human pro-uPA (52 kDa) was provided by Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL). The ATF peptide was purchased from American Diagnostica (Stamford, CT). Pro-uPA and ATF were used at 10 nM. FNG, phosphatidylinositol-specific phospholipase C (PIPLC) from Bacillus cereus of Bacillus cereus were purchased from Sigma-Aldrich (St Louis, MO). Interleukin-6 (IL-6) and interferon-γ (IFN-γ) were purchased from R&D Systems (Minneapolis, MN). MDMs were resuspended as recommended by the manufacturers and used at final concentrations of 6, 10, and 50 ng/mL, respectively, based on previous studies. PIPLC was resuspended at 20 U/mL in culture medium and used at the final concentration of 10 U/mL. M25 (PRYQHGVLAM-FRQNTG) and its scrambled peptide (scM25, HQIPGAYRGVQFR-TFML) were purchased from PRIMM (Milan, Italy) and dissolved at 20 mM in dimethylsulfoxide (DMSO) and used at the indicated concentrations. A list of antibodies used and their sources is provided in Document S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article). All experiments were performed in 96-well microtiter plates (Falcon; BD Biosciences, Bedford, MA) unless otherwise specified.

Quantification of uPA and suPAR secretion

Both molecules were measured in culture supernatants of U1 cells and MDM. uPA concentrations were measured by a commercial kit (IMUBIND uPA ELISA kit no. 894; American Diagnostica, Greenwich, CT) with a lower detection limit of 10 pg/mL. Concentrations of suPAR and the uPA ELISA kit no. 894; American Diagnostica, Greenwich, CT) with a lower detection limit of 10 pg/mL. Concentrations of suPAR and the uPA-suPAR complex were determined using an in-house sandwich ELISA.

Chromically HIV-infected U1 cell line

The promonocytic U1 cell line contains 2 copies of integrated X4 HIV-1LAMIN provirus per cell, and it is characterized by a constitutive state of relative viral latency.23 High levels of virus expression are rapidly induced by stimulation of U1 cells with either PMA or cytokines.23,24 U1 cells were stimulated at the concentration of 2 × 10^5 cells/mL in RPMI 1640 containing 10% of heat-inactivated fetal bovine serum (FBS), and were first incubated with either uPA or peptides 30 minutes before the addition of PMA or HIV-inductive stimuli.

U1 cell adhesion assay

U1 cells were resuspended in culture medium containing 10% FBS and left either unstimulated or stimulated and seeded in triplicates into 96-well tissue-culture plates. At the indicated time points, cells were washed with warm culture medium, fixed for 15 minutes with 3% paraformaldehyde in phosphate-buffered saline (PBS), stained for 10 minutes with 0.5% crystal violet (in 20% methanol/80% water), washed 3 times and lysed with 100 μL 1% sodium dodecyl sulfate (SDS) in water. The results were obtained by measuring the absorbance at 570 nm.

Acute CCR5-dependent infection of primary human monocyte-derived macrophages

Circulating monocytes were isolated from Ficoll-Hypaque purified peripheral blood mononuclear cells (PBMC) of several independent, healthy, HIV-1–seronegative donors by iso-osmotic Percoll gradient.27 Their purity was approximately 90% based on morphology and CD14 staining (data not shown). Monocytes were allowed to differentiate for 7 days in culture in Dulbecco modified eagle medium (DMEM) enriched with 10% FBS and 10% human AB serum before infection.17 Thus, human MDMs were treated for 30 minutes with uPA or ATF and then infected with the CCR5-dependent (R5) laboratory-adapted HIV-1LAMIN strain at the multiplicity of infection (MOI) of 0.1.17 MDM cultures were carried on for 3 to 4 weeks after infection and supernatants were collected every 3 to 4 days and stored at −20°C for determination of their reverse transcriptase (RT) activity content. Collected culture supernatants were replenished with fresh culture medium, with uPA or ATF for treated cultures.

Acute X4 HIV-1 infection of the human promonocytic U937 cell line

U937 cells were resuspended in RPMI 1640 plus 10% FBS and infected with the laboratory-adapted X4 HIV-1LAMIN strain at the MOI of 1 after 30 minutes’ preincubation with FNG. Cell cultures were carried on for 3 to 4 weeks after infection and supernatants were collected every 2 to 3 days and stored at −20°C for determination of their RT activity content. Collected culture supernatants were replenished with fresh culture medium, with FNG for treated cultures.

HIV-1 quantification by RT activity assay

HIV-1 expression was monitored by determination of Mg^{++}-dependent RT activity in culture supernatants, reflecting the production of new progeny virions.28 Release of cell-associated virions by 5 consecutive cycles of cell freezing and thawing was performed as previously described.16

Cytolfluorimetric analysis

U1 cells were washed twice in 2% FBS/PBS and stained by using the indicated Ab at 1 μg/10^6 cells unless otherwise specified. After 30 minutes of incubation on ice, U1 cells were centrifuged twice in 2% FBS/PBS at 390g for 5 minutes at +4°C; the cells were then incubated with 1 μg of the secondary anti–mouse-FITC or anti–rabbit-FITC Ab. The cells were spun again after 30 minutes of incubation on ice and resuspended in 2% formaldehyde/PBS. Fifteen thousand cells were acquired using a FACSscan (Becton Dickinson, Franklin Lakes, NJ) flow cytometry apparatus and analyzed by CellQuest software (Becton Dickinson).

Cell proliferation

Cell proliferation was assessed by the uptake of [3H]-thymidine. [3H]-thymidine (3.7 × 10^7 Bq) was added to 4 × 10^4 cells in 200 μL medium and incubated 16 hours at 37°C, 5% CO2. Cells were harvested and the β emission was counted in a 1450 β-counter (1450 Microbeta Plus; PerkinElmer Life and Analytical Sciences, Waltham, MA).

siRNA transient transfection

Three pairs of validated stealth small-interference RNA (siRNA; 25 nt) for each target antigen were purchased from Invitrogen (Paisley, United Kingdom) and resuspended at the final concentration of 20 μM in diethyl pyrocarbonate (DEPC)–treated water, as detailed in Document S1. Typically, 2 × 10^6 cells were transfected with 1 μM siRNA, then resuspended at 10^6 cells/mL and finally diluted 4 hours later at 2 × 10^5 cells/mL before incubation with uPA and stimuli. Cells transiently transfected with irrelevant stealth siRNA duplex containing 45% to 55% GC (Mock siRNA; Invitrogen) were used as negative controls.

Statistical analysis

Results are reported as mean values plus or minus standard deviation (SD), and the analysis of variance was performed by a 2-tailed Student t test to calculate P values for paired observations (unless otherwise specified). The observed differences were considered significant if the P value obtained was below .05.
Figure 1. UPA inhibits HIV expression in chronically infected U1 cells via binding of its ATF domain to uPAR. (A) U1 cells were preincubated with different concentrations of either uPA or peptides representing the low molecular weight (LMW), ATF, growth factor domain (GFD), or omega loop components of uPA. Cells were then stimulated with PMA and followed for virus expression in culture supernatants. All peptides significantly inhibited (P < .001) virus expression in the same range of concentrations, except for LMW, which was ineffective at all tested concentrations. (B) U1 cells were analyzed for uPAR expression 2 and 24 hours after incubation with PiPLC (10 U/mL). Down-modulation of uPAR expression was clearly detectable at both time points. Geometric mean fluorescence intensities (MFI) are shown: isotype, MFI: 4; Nil, unstimulated cells, MFI: 31; PiPLC, MFI: 14. (C) U1 cells were incubated with PiPLC for 2 hours and then stimulated with PMA in the presence or absence of uPA (10 nM). The RT activity levels were measured in the culture supernatants at the peak of virus expression (day 4 after stimulation). PiPLC abolished the inhibitory effect of uPA on virus expression without affecting the inductive effect of PMA (P < .001). (D) U1 cells were stimulated with PMA in the presence or absence of suPAR and uPA, and RT activity was determined in the culture supernatants after 3 days of culture (P < .001); a similar pattern of virus expression was observed even at days 2 and 4 of culture (data not shown). The error bars indicate the SD of duplicate samples. All the described experiments were performed in duplicate wells and repeated 3 times, and provided identical results. cpm indicates counts per minute.

Results

The anti-HIV activity of uPA depends on its ATF–receptor-binding domain and the cell-surface expression of uPAR

Exogenously added pro-uPA is immediately cleaved in vitro into its active form, uPA, by the plasmin present in the cell-culture serum-enriched medium.30 The anti-HIV effect of uPA on stimulated U1 cells was already shown to be dependent on its receptor-interacting component, ATF, and not on its catalytic activity.16,17 This finding was confirmed here, in that only uPA peptides maintaining an ATF growth factor-like domain,30 including the so-called Omega loop,30 but not the enzymatically competent low molecular weight fragment, inhibited HIV expression in chronically infected U1 cells stimulated with either PMA (Figure 1A) or TNF-α (data not shown).

To demonstrate the essential role of the GPI-anchored protein uPAR31 in uPA-mediated HIV inhibitory signals, U1 cells were incubated with PiPLC from B cereus that specifically removes GPI anchors.32 PiPLC indeed reduced the expression of uPA on U1 cell surfaces 2 to 24 hours after incubation, as measured by cytofluorimetric analysis (Figure 1B). Concomitantly, PiPLC promoted the release of suPAR in the culture supernatant, at concentrations from 33 pM (Table 1) to 220 pM (not shown), while it did not modulate the cell-surface expression of non–GPI-anchored receptors including integrins, as measured by FACS analysis (data not shown). PiPLC did not

Table 1. Concentrations of uPA, suPAR, and the uPA-suPAR complex in culture supernatants of U1 cells and of primary MDMs acutely infected or not infected with HIV-1

<table>
<thead>
<tr>
<th>Cell type/ Stimulus/infection</th>
<th>RT activity, cpm/µL</th>
<th>suPAR, pM</th>
<th>uPA, pM</th>
<th>suPAR/uPA ratio</th>
<th>suPAR-uPA complex, pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>&lt; 100</td>
<td>33 ± 0</td>
<td>2.9 ± 0.6</td>
<td>11.38</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>PMA</td>
<td>1657 ± 221†</td>
<td>49 ± 12</td>
<td>2.5 ± 1</td>
<td>19.66</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2021 ± 310†</td>
<td>34 ± 1</td>
<td>4.6 ± 1</td>
<td>7.4</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>2421 ± 212†</td>
<td>33 ± 1</td>
<td>2.7 ± 0.6</td>
<td>12.2</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>MDM Control†</td>
<td>NA</td>
<td>217 ± 102</td>
<td>8 ± 2</td>
<td>27.2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>HIV</td>
<td>6967 ± 882†</td>
<td>182 ± 93</td>
<td>8 ± 2</td>
<td>22.8</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

The levels of expression of the analytes were measured 48 hours after stimulation of U1 cells and at the peak of viral replication in MDMs. Because MDMs were seeded at twice the concentration of U1 cells, the results shown have been normalized by dividing by a factor of 2. Results are expressed as mean plus or minus SD of the concentrations determined from 3 independent experiments with U1 cells and from MDM cultures from 5 independent donors (Figure 2).

Significantly higher levels of HIV production were observed in PMA or cytokine-stimulated U1 cells versus unstimulated cells (Nil; †P = .001), as well as in infected versus uninfected MDMs (†P = .001). Furthermore, increased levels of suPAR were observed after U1 cell stimulation with PMA, but not with cytokines (|P = .001 vs Nil). There were also increases in the suPAR/uPA ratio in PMA and TNF-α–stimulated U1 cells (|P = .0045 and |P = .01, respectively) and the suPAR-uPA concentrations in cells stimulated with TNF-α (|P = .01).

NA indicates not applicable.

*Control: uninfected MDM.
interfere with HIV expression in either unstimulated or PMA-stimulated U1 cells (Figure 1C), suggesting that neither uPAR per se nor endogenous uPA play a significant role in HIV-1 expression in U1 cells. In contrast, PIPLC abrogated the anti-HIV activity of exogenous uPA (Figure 1C) without any cytotoxic or cytostatic effects, as tested by [3H]-thymidine uptake (data not shown).

We next determined the concentrations of uPA, suPAR, and of their complex in culture supernatants of both unstimulated and stimulated U1 cells (Table 1). Most of the uPA released from stimulated U1 cells was indeed complexed with suPAR (Table 1), as previously reported in vivo. Addition of suPAR did not alter HIV expression per se from either unstimulated or PMA-stimulated U1 cells (Figure 1D). Furthermore, suPAR did not interfere with HIV-1 replication in acutely infected U937 cells (data not shown, n = 3) reinforcing the concept that its role in virus production is strictly related to mediating uPA-dependent cell signaling. In contrast, a 20:1 molar excess of suPAR to uPA abrogated the anti-HIV activity of uPA (Figure 1D), whereas, vice versa, a 5:1 excess of uPA to suPAR overcame the inhibitory effect of suPAR (data not shown).

**ATF inhibits HIV replication in primary human MDMs**

We have previously reported that uPA inhibits virus replication in primary MDM and we here report that exogenously added ATF exerts an anti-HIV activity in vitro–infected MDMs established from several donors (Figure 2A). The anti-HIV activity of uPA and ATF were tested on primary MDMs infected with an R5 HIV-1. The compounds were added to the cell cultures at the time of infection and every 3 days after infection. The results of a single experiment, representative of 5 independently performed experiments with cells from different donors, are shown. The error bars indicate the SD of triplicate samples, whereas asterisks at the indicated time points indicate statistical significance (P < .001) among Nil and uPA or ATF treated cells; no significant differences were observed in the inhibition levels induced by uPA or ATF.

(B) Culture supernatants of uninfected MDMs and in vitro–infected MDMs (C) were analyzed for the levels of uPA and suPAR at the time corresponding to the peak of viral replication (RT activity) in 5 independent experiments performed with cells from different donors. Differences in the levels of suPAR, uPA, the uPA-suPAR complex, and the suPAR/uPA ratio between uninfected and infected cells tested were not significant. R² was calculated by linear regression and P values by Spearman rank test. cpm indicates counts per minute.

Unstimulated U1 cells showed constitutive cell-surface expression of uPAR and CD18, but negligible presence of CD11b (Table 2; Figure 3A). PMA stimulation up-regulated the levels of cell-surface uPAR (reaching a plateau approximately 20 hours after stimulation, as previously reported in both U937 and MCF-7 cells) and induced the expression of CD11b, thus allowing the formation of Mac-1 (Figure 3A top panels). Superimposable results, although inducing lower levels of CD11b expression, were observed by TNF-α stimulation (Figure 3A middle panels). In contrast, IL-6 strongly up-regulated both HIV-1 and uPAR expression, but failed to induce detectable levels of CD11b (Figure 3A bottom panels). Of interest, uPA inhibited HIV expression in U1 cells stimulated with both PMA and TNF-α, but not with IL-6 (Figure 3B). U1 cells were also stimulated with IFN-γ or granulocyte-macrophage colony-stimulating factor (GM-CSF) plus LPS, which up-regulated virus expression to comparable levels, in the presence and absence of uPA. However, both IFN-γ and GM-CSF plus PLS did not induce expression of CD11b (data not shown); concomitantly, uPA failed to inhibit HIV production induced by these stimuli.

Among other uPA coreceptors and integrin chains expressed by U1 cells, neither FPRL1, nor α3β1, α6, and α5 integrin chains were involved in uPA-mediated inhibition of HIV expression (see Document S1 and Figures S1, S2).
Table 2. Expression of integrin chains and cell-surface receptors in U1 cells

<table>
<thead>
<tr>
<th>Integrin chain</th>
<th>Nil</th>
<th>PMA</th>
<th>IL-6</th>
</tr>
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<tbody>
<tr>
<td>α6 (CD41)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α6 (CD11a)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α6 (CD103)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α6 (CD11b)</td>
<td>−</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>α6 (CD11c)</td>
<td>−</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>α6 (CD51)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α7β3 (VN-R)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α9β3 (FN-R, VN-R)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>αβ (FN-R)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α1 (VLA1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α2 (CD49b)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α2 (CD49c)</td>
<td>−</td>
<td>−</td>
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<tr>
<td>α2 (CD49d)</td>
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<td>α2 (CD49e)</td>
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<tr>
<td>α9 (CD49f)</td>
<td>−</td>
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<td>*</td>
</tr>
<tr>
<td>β1 (CD29)</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>β2 (CD18)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β2 (CD61)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>β2 (CD104)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

Cell-surface expression of the indicated molecules was evaluated by cytofluorometric analysis after 24 hours of stimulation (n = 4 for all antigens except uPAR and CD11b, which were tested in 9 independent experiments). An identical pattern of expression was observed up to 72 hours after stimulation. Up-regulation of CD11b and CD11c in U937 cells stimulated with PMA was previously reported.43

The Mac-1 ligand FNG inhibits late events of HIV expression in U1 cells and in acutely infected U937 cells

We next evaluated whether known Mac-1 ligands could also affect HIV expression in either PMA or TNF-α-stimulated U1 cells in the absence of uPA. U1 cells were incubated with FNG, known to bind to both Mac-138 and the VN receptor α6β3 (which, however, was not expressed by U1 cells, as shown in Table 2)17; the cells were then stimulated by either PMA or HIV-inductive cytokines. Incubation of U1 cells with FNG in the absence of other stimuli did not activate HIV expression (data not shown); however, FNG inhibited HIV expression in U1 cells stimulated with PMA (Figure 4A), but not with IL-6 (data not shown). As with uPA inhibition,16 FNG inhibition of HIV expression in PMA-stimulated U1 cells was reversed by cell disruption (Figure 4B). Furthermore, FNG inhibited the acute replication of X4 HIV-1 in U937 cells (Figure 4C). As observed with U1 cells, U937 cell disruption at time points before and after the peak of HIV replication restored the levels of RT activity to those of control cells (Figure 4D).

Preventing association of CD11b/CD18 from uPAR in the absence of receptor ligands inhibits HIV-1 expression

To investigate the role of Mac-1 interaction with uPAR in terms of inhibition of HIV expression, we tested the potential effect of M2, a peptide known to prevent or disrupt the integrin association with uPAR.39 Surprisingly, M25 alone, but not its control scrambled peptide, inhibited HIV expression in PMA-stimulated U1 cells in the absence of uPA in a concentration-dependent fashion, reaching more than 90% inhibition at 80 μM (Figure 5A). Furthermore, M25 inhibited the residual levels of RT activity in the presence of uPA (Figure 5A); identical results were obtained in U1 cells stimulated with TNF-α (data not shown). The M25 anti-HIV mechanism resembled that induced by both uPA and FNG in that it was fully reverted by cell disruption (Figure 5B), suggesting that prevention of the association between Mac-1 and uPAR triggers an inhibitory pathway of HIV-1 particle release.

The anti-HIV effect triggered by uPA/uPAR interaction is independent of CD11b

Experiments were next carried out after transfection of U1 cells with siRNA directed to either uPAR or CD11b. As shown earlier, the constitutive levels of expression of uPAR in U1 cells were enhanced by 24-hour stimulation with PMA (Figure 6A top left panel), while CD11b, which was not expressed by unstimulated cells, was clearly induced by PMA (Figure 6A top right panel). Transfection with their respective siRNA partially reduced the expression of uPAR (Figure 6A bottom left panel) while it abrogated PMA-induced CD11b expression (Figure 6A bottom right panel); these suppressive effects were stable for at least 3 days after cell stimulation (data not shown).

Mock siRNA did not alter the inhibitory effects of either uPA or M25 on HIV production, while CD11b siRNA abolished the anti-HIV activity of M25 but did not affect the inhibitory capacity of both uPA and M25 peptide (Figure 6B middle panel). Given the dependence of uPA anti-HIV effect on VN-mediated cell adhesion,40 we next investigated the potential interference of these siRNAs on U1 cell adhesion to the plastic substrate in the presence of either uPA or M25. As expected, M25 did not affect plastic adhesion of both unstimulated (not shown) and PMA-stimulated U1 cells (Figure 6C). Transfection with CD11b siRNA did not alter the ability of uPA to induce U1 cell adhesion, whereas this effect was lost in U1 cells transfected with uPAR siRNA (Figure 6C).

Cell adhesion–dependent uPA inhibition of HIV expression involves integrin β chains

We then investigated the potential contribution of β1 and β2 integrin chains. Different anti-integrin blocking Abs were used to coat the plastic of tissue-culture microwells, thereby inducing integrin-dependent cell adhesion.41 In addition, we tested the same Abs in suspension to prevent cell clustering by homotypic interactions.41

Plates coated with Abs recognizing antigens not expressed by unstimulated U1 cells, such as CD3 and CD11b (Table 2), failed to induce cell adhesion (Figure 7 top panel). In contrast, Abs recognizing β1, but not β2, integrin chains induced cell adhesion in both unstimulated and uPA-stimulated cells (Figure 7 top panel). PMA stimulation enhanced the levels of cell adhesion in plates coated with anti-CD11a, anti-CD11b, and anti-β1, but not anti-β2, integrin chains (Figure 7 top panel). Consistently, uPA increased the levels of cell adhesion in PMA-stimulated cells in plates coated with Abs directed against CD11a, CD11b, β1, but not β2, integrin chains. As expected, when Abs were added in suspension, either no adhesion or reduced levels of adhesion were observed in comparison to what was observed in the analogous Ab-coated plates (Figure 7 top panel).
None of the Abs tested modified the levels of HIV expression in unstimulated cells or cells incubated with uPA alone (Figure 7 bottom panel). When U1 cells were stimulated with PMA, no effects were observed in the presence of all tested Abs, except in plates coated with anti-CD11b that inhibited virus production, while no effects were observed when this Ab was diluted in cell culture supernatants. UPA maintained its anti-HIV activity in all tested conditions except in the presence of soluble anti-β1 and anti-β2 integrin chains that abolished its antiviral effect, without affecting uPA-mediated enhancement of PMA-induced cell adhesion.

Thus, the cell adhesion–dependent antiviral signal triggered by uPA binding to uPAR requires downstream activation of β integrin chains.

Discussion

In the present study we investigated the role of potential signaling partners mediating uPA/uPAR-dependent inhibition of late events in HIV-1 replication in monocytic cells such as primary MDMs, and promonocytic U1 and U937 cells lines. UPA anti-HIV activity in U1 cells was restricted to PMA and TNF-α stimulation, but was ineffective when these cells were stimulated with other cytokines or LPS. This restricted pattern of the uPA-mediated anti-HIV effect was paralleled by the up-regulation of CD11b on the surface of U1 cells which constitutively expressed the β2 chain CD18 to form Mac-1 (also known as complement receptor-3) known to provide signaling competence to the uPA/uPAR complex in myelomonocytic cells. Independent of uPA/uPAR, FNG binding to Mac-1 inhibited late events in HIV production in both stimulated U1 cells and acutely infected U937 cells. Furthermore, prevention of uPAR association with CD11b by the M25 peptide showed similar HIV-inhibitory effects in PMA-stimulated U1 cells. siRNA targeting uPAR reversed the inhibitory effect of both uPA and M25, whereas siRNA-mediated down-regulation of CD11b abolished M25 but did not affect uPA-dependent antiviral effect. Soluble Ab directed against β1 and/or β2 integrin chains, but not against the α chains of CD11a-b-c, prevented uPA interference with HIV expression. These findings suggest a model whereby a similar, if not identical, anti-HIV
signal, interfering with late events in the virus life cycle, is triggered by uPA binding to uPAR and by uPAR-independent ligation of Mac-1 or prevention of the interaction between CD11b and uPAR.

As observed in cancer^42 the uPA/uPAR system plays a relevant role in HIV disease. Soluble levels of suPAR represent a potent predictor of HIV disease progression, independent of the levels of peripheral CD4^+ T-cell counts and viremia. 11 These observations were extended and confirmed in patients with HIV-associated dementia. 14,15 Cultivation and infection of both primary MDMs and promonocytic cell lines, such as U1 and U937, revealed that both uPA and suPAR levels are expressed in vitro at levels similar to those observed in vivo. 15 Furthermore, in vitro–released uPA was mostly complexed with suPAR (Table 1), as described in vivo. 15 This observation likely accounts for the lack of evidence of an autocrine/paracrine role of endogenous uPA on HIV replication in our model systems. Because free uPA inhibits acute HIV-I

Figure 4. FNG inhibits HIV expression in PMA-stimulated U1 cells and in U937 cells acutely infected with an X4 HIV-1. (A) U1 cells were incubated with the indicated concentrations of FNG and then stimulated with PMA. (B) Cell disruption rescues the RT activity levels of U1 cells stimulated with PMA in the presence of FNG, as detected after 4 days of culture (arrowhead in panel A). (C) U937 cells were incubated with FNG (100 ng/mL) and then infected with the X4 strain HIV-1LAI. FNG was added to the cell cultures every 72 hours, when 50% of the culture supernatant was replaced with culture medium. (D) Cell disruption rescued the RT activity levels of acutely infected U937 cells in the presence of FNG at the indicated days after infection (arrowheads in panel C). *P < .001. The results shown were derived from 1 experiment, representative of 3 (A,B) and 2 (C,D) independently performed experiments. cpm indicates counts per minute.

Figure 5. M25 inhibits HIV expression in PMA-stimulated U1 cells both in the absence and presence of uPA. (A) U1 cells were incubated with different concentrations (5-80 μM) of the M25 peptide or its scrambled form (M25scr) in the presence or absence of uPA (10 nM) and were then stimulated with PMA. A concentration-dependent inhibition of HIV expression was observed in the presence of M25, but not of its control peptide, both in the presence and absence of uPA. (B) Cell disruption rescued HIV expression from the inhibitory effect of M25 (40 μM) or uPA (10 nM) after 5 days of culture (*P < .001; **P < .001). The results shown were derived from one experiment representative of 6 (A) and 3 (B) independently performed experiments. cpm indicates counts per minute.
replication in primary MDMs and U937 cells as well as virus expression in chronically infected U1 cells, its sequestration by suPAR to form a biologically inactive uPA-suPAR complex may indeed prevent the anti-HIV effects of uPA. These findings provide a potential explanation for the CD4 and viremia independent prognostic value of increased suPAR concentrations in HIV disease progression.

Furthermore, HIV-1 infection of primary MDMs did not alter levels of expression of uPA and suPAR (Table 1). This observation may account for the lack of correlation between circulating levels of uPA and suPAR and viremia. In contrast, suPAR levels were found to be strictly correlated with the state of immune activation in HIV-infected individuals, as determined by the serum levels of TNF-α, sTNFR-I, CCL2, CCL4, and CCL5. suPAR levels also correlate with lipid and glucose metabolism in HIV-infected individuals, as was reflected in vitro by a trend of association among the levels of HIV-1 replication in acutely infected MDMs and the levels of suPAR and uPA-suPAR complexes (Figure 2).

uPA-dependent inhibition of virus expression in U1 cells was restricted to PMA and TNF-α stimulation, while other HIV-inductive stimuli (including IL-6, IFN-γ, and GM-CSF plus LPS) were unaffected. Of interest, both PMA and TNF-α up-regulate HIV expression via activation of NF-κB, whereas the other stimuli act by activation of an ERK-1/-2 AP-1–dependent pathway up-regulating virus transcription. Since the inhibitory effect of uPA occurs at a posttranscriptional/posttranslational level resulting in the accumulation of virions in intracellular vacuoles, the selectivity of the anti-HIV effect of uPA was unlikely related to interference with virus transcription, as previously described.

We previously demonstrated that the catalytic component of uPA is dispensable for its anti-HIV effects, unlike its signaling component ATF, as here confirmed in stimulated U1 cells with smaller ATF peptides and primary MDMs. Removal of uPAR, by either cleavage of its GPI anchor or reduction of its expression by means of siRNA, resulted in the abolition of the antiviral effect of uPA. Because uPAR is a GPI-anchored receptor, signal transduction is usually mediated by other receptors and, in monocytic cells, either by FPRL1 or by integrins such as Mac-1. Indeed, both FPRL1 and some integrins, including αvβ5, αvβ1, and other chains, were constitutively expressed by U1 cells and not affected by...
HIV-inductive stimuli. However, no evidence of interference with uPA-dependent anti-HIV effects was obtained by incubating U1 cells with agonists of either FPRL1 or these integrins.

Unstimulated U1 cells express abundant levels of the integrin β chain CD18, but not of the α chains CD11b or CD11c. Only PMA and TNF-α up-regulated the expression of CD11b and CD11c, as reported, suggesting that the uPA anti-HIV effect could be mediated by either Mac-1 (CD11b/CD18) or p150/95 (CD11c/CD18). However, prevention of CD11c expression by means of siRNA did not affect HIV expression in PMA-stimulated cells (data not shown), while different CD11b ligands, including FNG and anti-CD11b mAb (on coated plates) triggered an HIV-inhibitory signal in PMA-stimulated U1 cells. Like uPA, FNG interfered with the late stage of virus production both in U1 and acutely infected U937 cells. Before our observation, Mac-1 was shown to enhance CD4/CCR5-dependent HIV entry in monocytes, macrophages, and dendritic cells, whereas, conversely, several Mac-1 ligands, including FNG, have shown inhibitory effects on the enhancement of HIV expression and cell adhesion. These results suggest that interference with either uPAR or CD11b expression by siRNA resulted in different biologic effects. UPAR targeting siRNA, as well as PIPLC treatment, only partially decreased the levels of uPAR expression in PMA-stimulated U1 cells, yet it abolished uPA and M25 anti-HIV effects. Conversely, almost complete prevention of CD11b expression by siRNA resulted in the expected loss of the M25 anti-HIV effect, but was irrelevant for uPA-dependent effects on HIV expression and cell adhesion. These results suggest that UPAR may functionally replace CD11b in terms of triggering a β chain–dependent anti-HIV signal, as previously suggested. In this regard, uPA may promote an indirect activation of β1 and β2 integrin chains after the recruitment of VN to form a uPA/uPAR/VN supercomplex competent of mediating cell adhesion and
mechanotransduction. In support of this interpretation, soluble anti-β1 and anti-β3 Ab did not prevent uPA induced cell adhesion, as recently reported, while they fully prevented the anti-HIV effect of uPA. Overall these results suggest that an interplay between integrins and uPAR may occur in lipid rafts known to play a fundamental role in both viral entry and in the exit of new progeny virions from infected cells.

In addition to uPA/uPAR and Mac-1 and its ligands, other extracellular stimuli, including IFN-γ and CCL2/MCP-1, have been previously shown to inhibit virion release by favoring intracellular virion accumulation. In this regard, assembly and release of HIV virions in multivesicular bodies belonging to the hexosomal pathway are crucial aspects characterizing the infection of macrophages. Although the relevance of intracellular virion accumulation in macrophages has been recently questioned, different monocytic cells, including U937, HL-60, THP1, and MonoMac cells, primary monocytes, and macrophages derived from CD34+ bone marrow precursors show accumulation and release of HIV virions in intracellular compartments for several weeks, in addition to their generation at the plasma membrane. Even more important, these morphogenetic features of virion assembly in subcellular vacuolar compartments have been documented in vivo in the brain of individuals with HIV encephalitis.

In conclusion, our results suggest a general model whereby interacting, yet distinct, ligands can trigger a signaling pathway resulting in the inhibition of HIV spread by interfering with late-stage virion assembly and release in macrophages. At least 3 converging signals leading to this common final pathway can be postulated based on the present results: (1) ligation of uPAR by its natural ligand uPA via its ATF domain; (2) ligand binding and cross-linking of Mac-1; and (3) prevention of uPAR interaction with CD11b resulting in the assembly of Mac-1 dissociated from uPAR. As for other examples of biologic redundancy, these observations suggest that control of HIV assembly, maturation, and virion release are crucial checkpoints for efficient viral spreading from macrophages to other susceptible cell types.

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Authorship

Contribution: M.A. designed and performed experiments, analyzed and interpreted the results, and contributed to the drafting of the manuscript; S.A.M. and C.E. performed experiments and interpreted the results; R.P. and F.B. designed experiments and contributed to the interpretation of the results and drafting of the manuscript; and G.P. designed experiments, analyzed and interpreted the results, and contributed to the drafting of the manuscript.

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Ligand-engaged urokinase-type plasminogen activator receptor and activation of the CD11b/CD18 integrin inhibit late events of HIV expression in monocytic cells

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