Extracellular HIV-1 Tat Protein Up-Regulates the Expression of Surface CXC-Chemokine Receptor 4 in Resting CD4+ T Cells

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Extracellular HIV-1 Tat Protein Up-Regulates the Expression of Surface CXC-Chemokine Receptor 4 in Resting CD4+ T Cells

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Here we report that synthetic HIV-1 Tat protein, immobilized on a solid substrate, up-regulates the surface expression of the CXC-chemokine receptor 4 (CXCR4), but not of the CC-chemokine receptor 5 in purified populations of primary resting CD4+ T cells. The Tat-mediated increase of CXCR4 occurred in a well-defined range of concentrations (1–10 nM of immobilized Tat) and time period (4–8 h postincubation). Moreover, the increase of CXCR4 was accompanied by an increased entry of the HXB2 T cell line-tropic (X4-tropic), but not of the BaL macrophage-tropic strain of HIV-1. The ability of Tat to up-regulate CXCR4 expression was abrogated by the protein synthesis inhibitor cycloheximide, clearly indicating the requirement of de novo synthesis. As Tat protein is actively released by HIV-1 infected cells, our data indicate a potentially important role for extracellular Tat in rendering bystander CD4+ T cells more susceptible to infection with X4-tropic HIV-1 isolates. The Journal of Immunology, 1999, 162: 2427–2431.

Materials and Methods

Cells

PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation (Pharmacia, Uppsala, Sweden) of heparinized leukocyte units obtained from healthy adult donors, who gave their informed consent to this research according to the Helsinki declaration of 1975. Resting CD4+ T cells were isolated by stringent immunomagnetic negative selection with Dynabeads M450 (Dynal, Oslo, Norway). For this purpose, we used a mixture of mAbs against CD19 and CD20 present on B cells; CD14, CD56, and CD57 on NK cells; CD14 on monocytes; and CD8 (all mAb were from Coulter-Innontech, Miami, FL). The final cultures of CD4+ T cells thus obtained were always >85% pure, as determined by two-color flow cytometry analysis using a phycoerythrin (PE)-conjugated anti-CD4 mAb (Becton Dickinson, San Jose, CA) in combination with a PC5-conjugated anti-CD3 mAb (Coulter-Innontech). After purification, cells were resuspended in AIM-V serum-free medium (Life Technologies, Grand Island, NY) at 1.8 × 10^6 cells/ml and seeded in 48-well flat-bottom plates (0.6 ml/well).

Adherence of viral proteins to microtiter plates

Full-length synthetic Tat was from Technogen (Caserta, Italy), and recombinant p24 protein and anti-Tat rabbit polyclonal IgG were from Intracell (Cambridge, MA). Viral proteins were resuspended in PBS containing 0.1% BSA (Sigma, St. Louis, MO) and stored in aliquots at −70°C before use. Flat-bottom (48-well) polystyrene plates (Costar, Cambridge, MA) were coated overnight at 4°C with either viral proteins or BSA at the concentrations indicated in the text. Plates were then rinsed with AIM-V serum-free medium to remove nonadherent proteins, and medium was immediately added to the plates after the final wash. The amount of Tat coated to each well was estimated by ELISA using anti-Tat polyclonal IgG (Intracell), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako, Copenhagen, Denmark), as previously described (19). Tat-coated plates were examined before and after 24 h of culture with CD4+ T cells.

In some cases, anti-Tat IgG were added to the wells (5 μg/well) after the final wash and left in the culture medium for all the time of the experiment. In other cases, 200 μg/ml of cycloheximide (Sigma) were added to the culture medium.

Flow cytometric analysis of cell surface molecules

Surface expression of CXCR4, CCR5, CD29, CD49, and CD69 was evaluated by direct staining with the PE-conjugated anti-CXCR4, anti-CCR5,
anti-CD29, anti-CD49 (all from Pharmingen, San Diego, CA), and PC5-conjugated anti-CD69 (Couler-Immunotech) mAbs. Briefly, aliquots of 3 x 10⁶ cells were stained with 5 μl of each mAb in 200 μl of PBS containing 2% FCS at 4°C for 30 min. Nonspecific fluorescence was assessed by using isotype-matched controls. After staining procedures, samples were analyzed using a FACS Calibur flow cytometer (Becton Dickin-
on). Data collected from 10,000 cells are presented as either histograms or mean fluorescence intensity (MFI) values.

In three experiments, cell-associated immuno-fluorescence was calculated using the Quantum 27 fluorescence quantitation kit (Flow Cytometry Standards, San Juan, PR) following manufacturer’s instructions. This system allows for the direct quantitation of the fluorescence intensity of a test sample in terms of the number of molecules of equivalent soluble fluoro-

HIV infection assay

At 4 h postseedling, cells were infected with HIV-1 (HXB2 and BaL strains: Advanced Biotechnologies, Columbia, MD; multiplicity of infection of 0.01) for 3 h and then washed three times with PBS. Of note, viral stocks were treated before use with RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) to remove contaminating DNA. Fourteen hours postin-
fection, CD4⁺ T cells were collected and lysed in protease K-lysis buffer and allowed to incubate at 56°C for 60 min, then at 98°C for 20 min. Serial dilutions of cell lysates were subjected to HIV-1 DNA PCR by using the following primers designed based on previously published sequences (23): 5'–primer, 5'TCTCTCTGGTTAGACCAGATCTG; 3'–primer, 5'–ACTGCTAGAGATTCTCCACTG. These primers, which amplify a 180-bp fragment in the long terminal repeat (LTR) R/U5 region, were designed to detect early steps in reverse transcription (23). Samples were subjected to 40 cycles of amplification (95°C for 1 min, 50°C for 1 min, and 72°C for 1 min). Negative controls were performed by samples containing buffer only or uninfected cells. The PCR products were separated on a 2% agarose gel, transferred to a nylon membrane, and hybridized with a 32P-labeled oligonucleotide probe (5'–CTCAATAAAAGCTTGCCGTTGAGTCT TCAAGTGTGGTGTC) against an internal sequence of the HIV PCR product and analyzed after exposure to x-ray film. To normalize for the quantity of DNA in each sample, β-globin PCR was conducted using β-globin primers (Stratagene, La Jolla, CA). β-Globin PCR products were visualized under UV light after staining of agarose gels with ethidium bromide. Each sample was amplified in duplicate or triplicate.

Statistical analysis

The results were expressed as means ± SD of three or more experiments performed in duplicate. Statistical analysis was performed using the twotailed Student’s t test.

Results

**Immobilized extracellular Tat rapidly and selectively up-regulates the surface expression of CXCR4 in resting CD4⁺ T cells**

To investigate the role of Tat protein on the expression of surface CXCR4 and CCR5, we have chosen a previously established model system that allows us to assess the response of resting CD4⁺ T cells to truly extracellular Tat protein by using protein immobilized on plastic (5). The doses of Tat reported in all experiments are those added overnight to the plates to coat the wells and do not represent the amount of protein bound to each well. The HIV-1 protein p24 and/or BSA were used as controls.

The purity of freshly isolated CD4⁺ T cell populations ranged from 85 to 98% in different experiments, reaching 95% in the representative experiment illustrated in Fig. 1. A–D. CD4⁺ T cells constantly expressed detectable, albeit variable, levels of surface CXCR4 that were rapidly enhanced following incubation at 37°C in serum-free medium (Fig. 1B). Culture of CD4⁺ T cells in Tat-coated plates resulted in a clearly detectable increase in CXCR4 expression with respect to the spontaneous CXCR4 induction within 2–8 h, with maximal (p < 0.01) effect being observed after 4 h (Figs. 1, B–C). On the other hand, when T cells were seeded in p24-coated or BSA-coated plates, no effect on the spontaneous induction of CXCR4 expression was observed.

Since similar results were obtained in experiments performed with CD4⁺ T cell populations showing a variable degree of puri-

**Immobilized extracellular Tat up-regulates the surface expression of CXCR4 in a well-defined range of concentrations**

To evaluate the dose-dependence of Tat-mediated CXCR4 up-regulation, plates were next coated with synthetic Tat at a concentra-
tion range comprised between 0.01 and 100 nM (Fig. 3). After 4 h of incubation, immobilized Tat up-regulated the surface expression of CXCR4 in a bell-shaped fashion. Plates coated with 1–10 nM Tat showed a statistically significant (p < 0.01) increase of CXCR4 levels with respect to plates coated with 0.01, 0.1, and 100 nM Tat or with 10 nM p24 or 0.1% BSA. The specificity of the effect was demonstrated by the ability of anti-Tat polyclonal IgG to abrogate (p < 0.01) the Tat-mediated up-regulation of surface CXCR4. Of note, when soluble Tat was used, a greater variability in CXCR4 modulation was noticed with respect to the experiments performed with immobilized Tat. In general, the concentrations of soluble Tat required to observe up-regulation of the surface CXCR4 after 4 h of culture were one log higher (100 nM) than those required to obtain similar effects with immobilized Tat. To further ascertain whether the Tat-mediated up-regulation of CXCR4 was truly due to extracellular Tat, the amount of protein immobilized on plastic was evaluated before and after seeding CD4⁺ T cells on Tat-coated plates for 24 h by ELISA. In three separate experiments, the enzyme immune adsorbance OD values were similar before (0.93 ± 0.23) and after (0.85 ± 0.15) performing the cell cultures. Taken together, these data indicate that internalization of extracellular Tat by CD4⁺ T cells is not required to up-regulate CXCR4.
Tat-mediated CXCR4 up-regulation requires de novo protein synthesis

To determine whether the Tat-induced up-regulation of CXCR4 surface expression was due to de novo protein synthesis or merely relocation of preexisting receptor to the cell surface, we performed the experiments in the presence of cycloheximide, a pharmacological inhibitor of protein synthesis. When primary CD4\(^+\) T cells were seeded in culture for 4 h in the presence of 200 \(\mu\)g/ml of cycloheximide, a significant (\(p < 0.01\)) inhibition of surface CXCR4 was observed (Fig. 4), indicating that neosynthesis of CXCR4 takes place as soon as freshly isolated CD4 cells are seeded in culture. Of note, the Tat-mediated up-regulation of CXCR4 was also almost completely abolished by the addition of cycloheximide (Fig. 4).

Tat-mediated CXCR4 up-regulation correlates with enhanced infectivity of the HXB2 X4-tropic strain

To define the role of CXCR4 up-regulation in CD4\(^+\) T cell infection by HIV-1, we used an X4-tropic strain of HIV-1. Primary CD4\(^+\) T cells cultured for 4 h in BSA- or Tat-coated plates were infected with HXB2 (multiplicity of infection (MOI) = 0.01) for 3 h. After an additional 14 h of culture in fresh medium, samples were analyzed by PCR for the presence and amount of viral DNA as a measurement of viral entry. Semiquantitative PCR of strong-stop DNA (with LTR R/U5 primers), an early product of reverse transcription, revealed a significant higher level of proviral DNA in cells seeded on Tat-coated plates with respect to those seeded on control (BSA-coated) plates (Fig. 5). In contrast, infection with 0.01 MOI of the M-tropic strain BaL was undetectable in both the Tat-treated samples and the control (BSA- or p24-treated) samples.
BSA- and Tat-treated cultures (data not shown). These results indicate that the Tat-induced up-regulation of CXCR4 affects the susceptibility of CD4+ T cells to infection by X4 HIV-1 strains.

Discussion

In this study, we have established that extracellular Tat selectively up-regulates the levels of surface CXCR4 in resting CD4+ T cells. This receptor belongs to a family of G-protein-coupled receptors that are involved in regulation of numerous biological processes and is selective for a single chemokine, the potent lymphocyte chemoattractant stromal cell-derived factor-1 (SDF-1) (24). Moreover, CXCR4 plays a prominent role as a coreceptor for the highly cytopathic T cell line-adapted isolates of HIV-1 (22). On the other hand, we did not observe any Tat-mediated effect on the surface expression of CCR5, the major coreceptor for M-tropic strains of HIV-1, in resting CD4+ T cells.

The specificity of the Tat-mediated CXCR4 up-regulation was underlined by the fact that Tat was unable to up-regulate other surface markers, such as CD69, CD4, CD29, and CD49. In particular, the inability of Tat to up-regulate CD69, which represents an early activation Ag, clearly indicates that the Tat-mediated CXCR4 up-regulation cannot be considered the consequence of a generic CD4+ T cell activation. In this respect, we have previously demonstrated that immobilized Tat was unable to induce the proliferation of resting CD4+ T cells by itself and required the presence of a second stimulus (5).

In agreement with Jourdan et al. (25), we observed that freshly isolated CD4+ T cells expressed low levels of cell surface CXCR4 that were rapidly enhanced following incubation of the cells in medium alone, even in absence of serum. Extracellular Tat induced a further increase in CXCR4 expression, with maximal effect observed after 4 h of incubation. The efficient inhibitory effect of cycloheximide on the surface expression of CXCR4 induced by extracellular Tat suggests that the Tat-mediated CXCR4 up-regulation is not due to merely relocation of preexisting receptor to the cell surface, but requires de novo protein synthesis of CXCR4.

A number of studies have shown that CXCR4 expression can be rapidly up-regulated or down-regulated depending upon the conditions used to stimulate resting T cells (25–28). While down-regulation of surface CXCR4 appears to be the consequence of rapid endocytosis of cell surface receptor molecules (29), the mechanisms responsible for CXCR4 up-regulation are less clear. Nevertheless, the Tat-mediated up-regulation of CXCR4 surface expression was not accompanied by modifications of surface CCR5. Consistent with our data, other authors have previously shown that CXCR4 and CCR5 are differentially regulated on T
lymphocytes (27–28). CXCR4 and CCR5 are the predominant chemokine receptors used as coreceptors in HIV-1 entry, and as such, their expression is important for determining viral tropism. In the course of HIV-1 infection, M-tropic viral strains predominate during the early phase of infection, while dual-tropic and T cell line-tropic viral strains appear late during disease progression to AIDS. Since CXCR4 was already expressed by resting CD4+ T cells, a crucial issue was to evaluate whether the Tat-mediated increase of surface CXCR4 could impact HIV-1 infectivity. We were able to demonstrate that immobilized Tat was associated to an increased entry of X4-tropic but not of an M-tropic strain of HIV-1 to demonstrate that immobilized Tat was associated to an increased entry of X4-tropic but not of an M-tropic strain of HIV-1. We were able to demonstrate that immobilized Tat was associated to an increased entry of X4-tropic but not of an M-tropic strain of HIV-1.

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