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HIV-1 adaptation to low levels of CCR5 results in V3 and V2 loop changes that increase envelope pathogenicity, CCR5 affinity and decrease susceptibility to Maraviroc

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ABSTRACT

Variability in CCR5 levels in the human population is suggested to affect virus evolution, fitness and the course of HIV disease. We previously demonstrated that cell surface CCR5 levels directly affect HIV Envelope mediated bystander apoptosis. In this study, we attempted to understand HIV evolution in the presence of low levels of CCR5, mimicking the limiting CCR5 levels inherent to the host. HIV-1 adaptation in a T cell line expressing low levels of CCR5 resulted in two specific mutations; N302Y and E172K. The N302Y mutation led to accelerated virus replication, increase in Maraviroc IC50 and an increase in Envelope mediated bystander apoptosis in low CCR5 expressing cells. Analysis of subtype B sequences showed that N302Y is over-represented in CXCR4 tropic viruses in comparison to CCR5 tropic isolates. Considering the variability in CCR5 levels between individuals, our findings have implications for virus evolution, MVC susceptibility as well as HIV pathogenesis.

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1. Introduction

HIV infection in permissive cells requires the presence of the receptor CD4 and a co-receptor CXCR4/CCR5 (Gallo et al., 2003; Wyatt and Sodroski, 1998). Interestingly, the role of CCR5 in HIV infection is not limited to the level of virus entry but also disease progression (Edo-Matas et al., 2011; Ometto et al., 2001). This is in part related to the CCR5 expression levels in the host which are determined via CCR5 gene and promoter polymorphisms (Chalmet et al., 2008; Ometto et al., 2001; Samson et al., 1996) as well as epigenetic factors (Gornalusse et al., 2015). Interestingly, CCR5 levels also vary with the state of T cell activation, a phenomenon that correlates well with HIV infections (Ostrowski et al., 1998). The CCR5 levels in turn affect the process of envelope glycoprotein (Env) evolution thereby dictating virus fitness and disease progression (Jakobsen et al., 2013; Jekle et al., 2003). While it is clear that CCR5 levels play a complex role in HIV infection and disease progression, it remains unknown how HIV evolves in the presence of varying CCR5 levels. This becomes imperative to study with the increasing use of CCR5 antagonists like Maraviroc (MVC) and development of gene therapy approaches targeting CCR5 (Bennett and Akkina, 2013; Didigu and Doms, 2014; Didigu and Doms, 2012; Didigu et al., 2014; Hutter and Ganepola, 2011; Hutter et al., 2009; Tilton and Doms, 2010; Younan et al., 2013, 2014).

The role of CCR5 in HIV disease is multi factorial. Firstly, marked variation in CCR5 cell surface expression in the human population due to CCR5 gene and promoter polymorphisms (Chalmet et al., 2008; Ometto et al., 2001; Samson et al., 1996) have been associated with disease progression rate (Jakobsen et al., 2013; Jekle et al., 2003). As these polymorphisms have been shown to alter the cell surface levels of CCR5; a direct correlation between CCR5 levels and HIV pathogenesis has been suggested (de Roda Husman et al., 1997). In this regard, we have previously demonstrated that CCR5 levels on the cell surface directly affect HIV Env glycoprotein mediated apoptosis in bystander cells (Joshi et al., 2011). Furthermore, the CCR5Δ32 deletion mutation is well characterized for HIV resistance and is frequently associated with long-term non-progressors (Edo-Matas et al., 2011). While CCR5Δ32 homozygous individuals are resistant to HIV infection due to lack of CCR5 expression (Liu et al., 1996); CCR5Δ32

Abbreviations: CCR5, chemokine receptor; Env, Envelope; MVC, Maraviroc; DMEM, Dulbecco's Modified Eagles medium; RT, Reverse Transcriptase; HIV, Human Immunodeficiency Virus; AIDS, Acquired Immune Deficiency Syndrome; FPR, False Positive Ratio

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heterozygous individuals progress either relatively slowly to AIDS or remain long term non-progressors (Liu et al., 1996) possibly due to lower levels of CCR5 on the cell surface. Similarly, polymorphisms in the CCR5 promoter regions have also been associated with disease progression (Catano et al., 2011; Gornalusse et al., 2015; Huik et al., 2014; Ometto et al., 2001) once again as a consequence of lower CCR5 expression (Shieh et al., 2000).

Secondly, CCR5 levels may also play a crucial role in regulating virus tropism. HIV-1 Env glycoprotein is highly variable (Stalmeijer et al., 2004) and accelerated disease progression during late stages of HIV infection is often associated with co-receptor switching to CXCR4 (X4) usage compared to early stage CCR5 (R5) tropic viruses (Jekle et al., 2003; Spijkerman et al., 1998). While co-receptor switch from R5 to X4 tropic precedes a rapid decline in CD4 cells and progression to AIDS (Schuitemaker et al., 1992), the conditions that are required for co-receptor switching remain unclear. One leading hypotheses is that a reduction in the availability of CCR5 facilitates co-receptor switching and may be a driving force in governing the evolution of the HIV repertoire (Cheng-Mayer et al., 2009). Other explanations like occurrence of pre-existing CXCR4 variants, altered levels of ligands for CXCR4/CCR5 and suppression of CXCR4 tropic viruses by the immune system early during the infection have also been suggested (Cheng-Mayer et al., 2009).

In the current study, we investigated the adaptation and evolution of an R5 tropic HIV isolate under limiting CCR5 levels. We found that HIV-YU2 adapted to replicate in cells expressing low CCR5 levels by acquiring changes in the V2 (E172K) and V3 (N302Y) loop. Interestingly, these changes were associated with increased resistance to anti-CCR5 drug MVC as well as enhanced replication and heightened pathogenicity in low CCR5 expressing cells. While molecular modeling suggests that these changes are located in regions associated with CCR5 binding, the virus did not switch co-receptor usage to CXCR4 tropic. Interestingly, the N302Y mutation has been predominantly associated with CXCR4 tropic viruses suggesting likely progression of the virus towards the CXCR4 utilization pathway. These data suggest that in HIV infected people with low CCR5 levels, the virus may evolve by acquiring changes that lead to better replication, enhanced fitness and eventually increased pathogenicity prior to AIDS development. Hence, the time course of HIV infection to AIDS may in part be dictated by CCR5 levels and Env evolution.

2. Materials and methods

2.1. Cell lines and transfections

SupT1 cells expressing low (L23), medium (M10) or high (H6) CCR5 have been described previously (Joshi et al., 2011) and were maintained in RPMI medium supplemented with 10% FBS, penicillin streptomycin (5000 U/ml) and Blasticidin (3 μg/ml). 293T, HeLa and TZM-bl cells (NIH AIDS research and reference reagent program) were maintained in Dulbecco's Modified Eagles medium supplemented with 10% FBS and penicillin streptomycin (5000 U/ml). TZM-bl are HeLa derived cells that express the HIV receptor CD4 and the co-receptors CXCR4 and CCR5 along with the luciferase and beta galactosidase genes under the control of HIV LTR (Platt et al., 2009). These cells readily support HIV infection/replication. U87 cells expressing CD4 and either CXCR4 or CCR5 were obtained from the NIH AIDS reagent program and were maintained in DMEM-10 supplemented with 1 μg/ml Puromycin and 300 μg/ml G418. All transfections were conducted using the Turbofect transfection reagent (Fisher Scientific) following the manufacturer's instructions.

2.2. Generation of virus stocks and infectivity assays

293T cells were transfected with the pNLLuc-R-/E- (He et al., 1995) HIV backbone along with different Env constructs. Virus stocks were harvested 48 h post transfection and Reverse Transcriptase (RT) activity determined. Virus stocks were titrated in TZM-bl cells using 2-fold serial dilutions of the stocks. Infectivity assays were conducted in different cell lines in the presence of 20 μg/ml DEAE dextran (Sigma) or 10 μg/ml Polybrene (Sigma) using equal RT cpm of virus stocks. Luciferase activity was determined 48 h post infection using the BriteLite Plus Luciferase assay substrate (PerkinElmer). Infectivity for each Env was calculated as percent of YU-2 Env control. For some experiments, the CXCR4 antagonist AMD-3100 (2 μM) or the CCR5 antagonist MVC (1 μM) was added at the time of infection.

2.3. Adaptation of virus to low levels of CCR5 in SupT-R5-L23 cells

Low CCR5 expressing cells (L23) were infected with approximately 10^6 RT cpm of R5 tropic HIV-1 isolate YU-2 prepared by transfecting 293T cells with the molecular clone pNLYU-2. Cultures were monitored for virus induced cytopathic effects and supernatants harvested every 2–3 days for estimation of RT activity as a measure of virus replication. During the peak of virus replication, cell pellets from infected cells were harvested and DNA isolated using the DNA Blood Mini kit (Qiagen).

2.4. Construction of full length replication competent infectious molecular clones

For construction of full length molecular clones the Env, Rev and Tat region was PCR amplified from the revertant isolates using primers bearing EcoR1 (forward primer) and Xho1 site (reverse primer). The amplified region was cloned into the pCDNA3.1+ vector using the pCDNA3.1 directional TOPO® expression kit (Invitrogen). Thereafter, the EcoR1/Xho1 fragment from the above clones was excised using restriction digest and cloned into the EcoR1/Xho1 sites of pNL-YU-2. The final molecular clones were authenticated by RE digest and sequencing.

2.5. Env cloning and mutagenesis

The full length Env region (containing the open reading frames for the Env and Rev genes) was amplified with subtype B specific primers and a nested PCR reaction using the Phusion High Fidelity PCR kit (New England Biolabs). The amplified Env region was cloned into the pCDNA3.1+ vector using the pCDNA3.1 directional TOPO® expression kit (Invitrogen) followed by full length sequencing analysis to verify the authenticity of the inserts. Contigs were made using the DNA star software (DNASTAR Inc., Madison, WI) and Env open reading frames for all constructs established. Functionality of each Env was determined by generating pseudotyped HIV particles using Env constructs and pNLLucR-/E- backbone as described above. Point mutations were introduced into the YU-2 Env constructs using specific mutagenic primers and the Quick Change site directed mutagenesis kit (Stratagene) strictly following the manufacturer's instructions. The introduced mutations were confirmed by sequencing.

2.6. MVC sensitivity

pNLLuc-R-/E- virus stocks pseudotyped with different Envs were prepared as described above. Virus stocks were added to TZM-bl cells in the presence of different concentrations of MVC. Infection was determined 48 h post infection by measuring luciferase activity in the cultures. Infectivity curves of each Env were

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