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HIV internalization into oral and genital epithelial cells by endocytosis and macropinocytosis leads to viral sequestration in the vesicles

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ABSTRACT

Recently, we showed that HIV-1 is sequestered, i.e., trapped, in the intracellular vesicles of oral and genital epithelial cells. Here, we investigated the mechanisms of HIV-1 sequestration in vesicles of polarized tonsil, foreskin and cervical epithelial cells. HIV-1 internalization into epithelial cells is initiated by multiple entry pathways, including clathrin-, caveolin/lipid raft-associated endocytosis and macropinocytosis. Inhibition of HIV-1 attachment to galactosylceramide and heparan sulfate proteoglycans, and virus endocytosis and macropinocytosis reduced HIV-1 sequestration by 30–40%. T-cell immunoglobulin and mucin domain 1 (TIM-1) were expressed on the apical surface of polarized tonsil, cervical and foreskin epithelial cells. However, TIM-1-associated HIV-1 macropinocytosis and sequestration were detected mostly in tonsil epithelial cells. Sequestered HIV-1 was resistant to trypsin, pronase, and soluble CD4, indicating that the sequestered virus was intracellular. Inhibition of HIV-1 intraepithelial sequestration and elimination of vesicles containing virus in the mucosal epithelium may help in the prevention of HIV-1 mucosal transmission.

1. Introduction

Mucosal epithelia are the first sites of contact between HIV-1 and the human body during the course of infection, and they play a critical role in determining the success of HIV-1 in establishing systemic infection. It has been shown in primate models that application of HIV-1 to the surfaces of intact oropharyngeal (Joag et al., 1997), anal/rectal (Bosch et al., 1997), cervicovaginal and foreskin/penile (Carias et al., 2013; Dinh et al., 2015; Girard et al., 1998; Joag et al., 1997) epithelia can lead to systemic infection of HIV-susceptible immune cells. Application of simian immunodeficiency virus to undamaged oral and vaginal mucosal epithelia also results in the transmigration of simian immunodeficiency virus across these epithelia (Miller et al., 2005; Milush et al., 2004; Stahl-Hennig et al., 1999). Similarly, application of HIV-1 to human foreskin, vaginal and cervical tissue explants *ex vivo* leads to the transmission of HIV-1 across these epithelia (Carias et al., 2013; Dinh et al., 2015; Ganor et al.; Hladik et al., 2007; Maher et al., 2005; Stoddard et al., 2009; Zhou et al., 2011). Furthermore, interaction of HIV-1 with the mucosal surface of oropharyngeal tissue explants of the fetus or infant leads to infection of CD4+ T lymphocytes, Langerhans/dendritic cells and macrophages, which is critical for HIV-1 mother-to-child transmission (MTCT) (Tugizov et al., 2012). None of these studies showed epithelial infection with HIV-1, indicating that the virus can migrate across intact mucosal epithelia without infecting them.

Recently, we showed that the majority of infectious virions internalized in tonsil, cervical and foreskin epithelial cells do not cross the epithelium but rather are sequestered in their vesicular/endosomal compartments for several days (Yasen et al., 2017). The interaction of activated lymphocytes with epithelial cells containing HIV-1 facilitates the release of virus and its spread from epithelia into lymphocytes. In the present study we investigated the mechanism of HIV-1 sequestration in endosomes of mucosal epithelial cells.

Mucosal epithelial cell surface proteins, including galactosylceramide (GalCer) and heparan sulfate proteoglycans (HSPG), facilitate HIV-1 internalization into epithelial cells (Bobardt et al., 2007; Bomsel and Alfsen, 2003; Fantini et al., 1997; Herrera et al., 2016; Tugizov et al., 2011). HIV-1 internalization into epithelial cells can occur by endocytosis (Bobardt et al., 2007; Daecke et al., 2005; Herrera et al., 2016; Miyauchi et al., 2009; Tugizov et al., 2012; van den Berg et al., 2014; Vidricaire and Tremblay, 2007). HIV-1 internalization in endothelial cells is mediated by macropinocytosis (Liu et al., 2002). Endocytosis could be due to clathrin-, caveolin- and/or lipid raft-associated mechanisms (Mercer et al.). Macropinocytosis is an actin-dependent process induced by membrane ruffling and the formation of large vacuoles, i.e., macropinosomes (Mercer and Helenius, 2009;

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Tugizov et al., 2013b).

Macropinocytosis plays a critical role in the uptake of viruses belonging to various families, including poxvirus, adeno, and picorna (Mercer and Helenius, 2008, 2009; Mercer et al., 2010; Schelhaas, 2010). Binding of viral envelope-associated phosphatidylserine (PS) to its receptor T-cell immunoglobulin and mucin domain 1 (TIM-1) triggers macropinocytosis (Mercer and Helenius, 2008; Mercer et al., 2010; Shiratsuchi et al., 2000). The outer leaflet of the HIV-1 envelope contains PS (Aloia et al., 1988, 1993b; Callahan et al., 2003; Gekonge et al., 2006), and HIV-1-associated PS facilitates the viral infection of macrophages (Callahan et al., 2003). TIM-1 also promotes HIV-1 entry into CD4+ T lymphocytes (Li et al., 2014). However, the interaction of TIM-1 with HIV-PS during the release of progeny virions inhibits virus release, retaining viral particles to the cell surface (Li et al., 2014). Expression of TIM-1 has been shown in epithelia of oral, lung, cornea, conjunctiva, and kidney (Freeman et al., 2010; Ichimura et al., 2008, 1998; Kondratowicz et al., 2011; Setty and Betal, 2008).

Virus internalized by endocytosis and macropinocytosis may follow intracellular trafficking pathways via early and late endosomes (Mercer and Helenius, 2009; Mercer et al.). Macropinosomes may also fuse with each other and form large vacuoles, which may exist independently from the early and late endosomes (Araki et al., 2006; Falcone et al., 2006; Hamasaki et al., 2004; Hewlett et al., 1994; Racoosin and Swanson, 1993). Early endosomes may serve as sorting compartments, regulating the delivery of internalized virus to various destinations by transcytosis and/or recycling (Jovic et al., 2010; Tuma and Hubbard, 2003). Early endosomal compartments have tubular and vacuolar domains, and the vacuolar domains mature into late endosomes (Huotari and Helenius, 2011), leading to formation of multivesicular bodies (MVB) and lysosomes (Dobrowolski and De Robertis, 2012; Hanson and Cashikar, 2012). MVBs contain a network of intraluminal vesicles. The main function of MVB is delivering cargo into lysosomes, where it is degraded (Fader and Colombo, 2009; Piper and Katzmann, 2007). However, MVB compartments can also serve as storage for internalized and recycled proteins, including cell surface receptors and ligands (Cullen and Korswagen, 2012; Kerr et al., 2006; Piper and Katzmann, 2007; Uchil and Mothes, 2005; Vieira et al., 2014). MVB can fuse with plasma membranes, releasing cargo into the extracellular environment (Nickerson et al., 2006; Piper et al., 2014; Piper and Luzio, 2007). The formation of MVB is initiated by fission of early endosomal compartments from their cytoplasmic face, generating a new compartment with intraluminal vesicles (McCullough et al., 2013; Schmidt and Teis, 2012). The establishment and maturation of MVB are mediated by the coordinated effort of more than 30 proteins, which together are called the “endosomal sorting complexes required for transport” (ESCRT) (Schmidt and Teis, 2012). The ESCRT consist of ESCRT-0, ESCRT-I, and ESCRT-II, which are involved in cargo sorting and membrane invagination (McCullough et al., 2013), and ESCRT-III, which cleaves the bud neck from its cytosolic face (Adell and Teis, 2011; Wollert et al., 2009).

We investigated the role of initial HIV-1 internalization in subsequent viral sequestration in the vesicular compartments of polarized tonsil, cervical and foreskin epithelial cells. Monostratified polarized epithelial cells may serve as a model for one of the sheets of stratified mucosal epithelium that are attached to one another by lateral junctions (McCaffrey and Macara, 2011; Muroyama and Lechler, 2012; St Johnston and Sanson, 2011; Tugizov et al., 2003, 2013b, 2011, 2012). Polarized epithelial cells have highly organized and functional vesicular/endosomal compartments (Rodriguez-Boulan and Macara, 2014) and are therefore suitable for modeling HIV-1 transmission via the mucosal epithelium. Our findings show that HIV endocytosis and macropinocytosis lead to sequestration of virions into late endosomes, including MVB and vacuoles. As we showed recently (Yasen et al., 2017), the interaction of activated lymphocytes with epithelia sequestering HIV-1 initiates the spread of virus from epithelial cells into lymphocytes. Thus, intravesicular HIV-1 sequestration may contribute to the molecular pathogenesis of viral spread from mucosal epithelial cells to virus-susceptible immune cells.

2. Results

2.1. HIV-1 internalization into epithelial cells is facilitated by multiple endocytic pathways

To identify the pathways of HIV-1 internalization into epithelial cells, we examined viral penetration in epithelial cells using pharmacologic inhibitors of clathrin- and caveolin/lipid raft-mediated endocytosis and macropinocytosis, which are major entry pathways of multiple viruses. Polarized tonsil epithelial cells were pretreated with increasing concentrations of chlorpromazine and nystatin, which are inhibitors of clathrin- and caveolin/lipid raft -mediated endocytosis, respectively (Ivanov, 2008a, b). Cells were also treated with amiloride, which inhibits macropinocytosis (Ivanov, 2008a; Saeed et al., 2010). These drugs are widely used for blocking endocytosis and macropinocytosis by many viruses, including HIV (Carter et al., 2011, 2009; Daecke et al., 2005; Dorosko and Connor, 2010; Ferreira et al., 2015; Grigorov et al., 2006; Kinlock et al., 2014; Liu et al., 2002; Mikulak et al., 2009; Miyauchi et al., 2009; Platt et al., 2014; Sloan et al., 2013). After 1 h of treatment, dual-tropic HIV-1_{SF33} internalization was examined from the apical (AP) surface of epithelium by p24 ELISA. Data showed that all three drugs reduced viral internalization in a dose-dependent manner (Fig. 1A). The highest concentrations of chlorpromazine (20 μM), nystatin (12 μg/ml) and amiloride (100 μM) were used to examine transepithelial resistance (TER) and the viability of polarized cells. None of the drugs reduced TER or viability (Fig. S1A and B, upper panels), i.e., drug treatment did not alter cell polarity. These concentrations of drugs were used in the rest of the study.

In the next experiments we examined the role of chlorpromazine, nystatin and amiloride in the attachment, internalization and transcytosis of HIV-1_{SF33} from the AP surface of epithelium. Virus attachment to the cell surface was measured at 4 °C, and intracellular and transcytosed virions were evaluated at 37 °C by p24 ELISA. Data showed that ~ 20% of initially inoculated virions were attached to the cell surface (Fig. 1B); none of the drugs significantly changed virus attachment to the AP surface (Fig. 1B, upper panel). Approximately 4% of initially added virions were internalized into cells, and all three drugs reduced viral internalization; inhibitors reduced clathrin- and caveolin/lipid raft-mediated HIV endocytosis by ~ 50%, and the inhibitor of macropinocytosis, amiloride, led to ~ 60% reduction of viral internalization (Fig. 1B, middle panel). HIV-1_{SF33} transcytosis was approximately ~ 0.06% of the initial inoculum, indicating that more than 95% of internalized virions were trapped in the cells. Nystatin or chlorpromazine reduced viral transcytosis by ~ 45%, and amiloride reduced it by ~ 80% (Fig. 1B, lower panel).

Analysis of the internalization of HIV-1_{SF33} in cervical and foreskin epithelial cells showed that virus internalization was significantly (~ 30–40%) reduced by inhibitors of clathrin- and caveolin/lipid raft-mediated endocytosis (Fig. 1C). However, the inhibitor of macropinocytosis, amiloride, had less of an effect on virus internalization in cervical cells (~ 15%). Amiloride did not reduce virus internalization into foreskin epithelial cells.

To verify the functional effect of drugs for endocytosis and macropinocytosis, tonsil TK#15 cells were treated or not treated with nystatin, chlorpromazine or amiloride. After 1 h, nystatin- or chlorpromazine-treated cells were washed and incubated with the endocytosis marker rhodamine-labeled dextran (10 kDa) (Li et al., 2015) (Fig. 1D). Amiloride-treated cells were incubated with Texas Red-labeled dextran (70 kD), which is a macropinocytosis marker (Commisso et al., 2014; Falcone et al., 2006; Li et al., 2015). Flow cytometry analysis showed that both nystatin and chlorpromazine reduced dextran-10 penetration by ~ 50%. Amiloride reduced the penetration of dextran-70 by ~ 45%.

The effect of drugs on HIV-1 internalization was validated in tonsil,