HIV gp120 Binds to Mannose Receptor on Vaginal Epithelial Cells and Induces Production of Matrix Metalloproteinases

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Abstract

**Background:** During sexual transmission of HIV in women, the virus breaches the multi-layered CD4 negative stratified squamous epithelial barrier of the vagina, to infect the sub-epithelial CD4 positive immune cells. However, the mechanisms by which HIV gains entry into the sub-epithelial zone is hitherto unknown. We have previously reported human mannose receptor (hMR) as a CD4 independent receptor playing a role in HIV transmission on human spermatozoa. The current study was undertaken to investigate the expression of hMR in vaginal epithelial cells, its HIV gp120 binding potential, affinity constants and the induction of matrix metalloproteinases (MMPs) downstream of HIV gp120 binding to hMR.

**Principal Findings:** Human vaginal epithelial cells and the immortalized vaginal epithelial cell line Vk2/E6E7 were used in this study. hMR mRNA and protein were expressed in vaginal epithelial cells and cell line, with a molecular weight of 155 kDa. HIV gp120 bound to vaginal proteins with high affinity, (Kd = 1.2 ± 0.2 nM for vaginal cells, 1.4 ± 0.2 nM for cell line) and the hMR antagonist mannann dote dose dependent inhibited this binding. Both HIV gp120 binding and hMR exhibited identical patterns of localization in the epithelial cells by immunofluorescence. HIV gp120 bound to immunopurified hMR and affinity constants were 2.9 ± 0.4 nM and 3.2 ± 0.6 nM for vaginal cells and Vk2/E6E7 cell line respectively. HIV gp120 induced an increase in MMP-9 mRNA expression and activity byzymography, which could be inhibited by an anti-hMR antibody.

**Conclusion:** hMR expressed by vaginal epithelial cells has high affinity for HIV gp120 and this binding induces production of MMPs. We propose that the induction of MMPs in response to HIV gp120 may lead to degradation of tight junction proteins and the extracellular matrix proteins in the vaginal epithelium and basement membrane, leading to weakening of the epithelial barrier; thereby facilitating transport of HIV across the vaginal epithelium.

Introduction

The global HIV-1 epidemic is fuelled through sexual transmission with women accounting for more than half of the 33 million individuals infected with the virus [1]. The lower female reproductive tract, is the initial site of contact with semen containing cell free and cell-associated virus that have been documented to transmit infection in vivo (in macaque studies) [2–5]. Although HIV can infect the vaginal, ectocervical and endocervical mucosa, the relative contribution of each site to the establishment of infection is not known. The columnar epithelium lining the transformation zone of the endocervix is single layered and thought to be vulnerable to infection [2]; while the stratified squamous epithelium lining the ectocervix/vagina is multi-layered and is believed to offer protection against pathogens when intact [6–8]. However, the greater surface area of the vagina/ectocervical wall provides more potential access sites for HIV entry, particularly when breaches occur in the epithelial-cell layer. This is of importance in light of the observation that HIV transmission can occur solely through the vagina in the absence of the endocervix and the uterus [9,10]. Moreover, anatomically in the vagina, the HIV infected cells include the intraepithelial langerhans cells, T cells [11], as well as dendritic cells, macrophages and T cells that are found in the sub-epithelium or lamina propia below the stratified squamous epithelial layer [12]. While it is plausible that the langerhans cells may extend their projections to the surface, to directly sample HIV from the lumen; HIV must also breach though the robust multilayered vaginal epithelial barrier (25–40 layer thick) to infect the deeply embedded CD4+ immune cells [2,12]. Thus, any aberrations in the integrity of the epithelial...
barrier would increase susceptibility to HIV infection. However, the mechanisms by which HIV gains entry into the sub-epithelial zone is hitherto unknown.

While the epithelial cells are refractory to HIV entry [11,13–15]; the intact epithelial barrier is impermeable to particles above 30 nm diameter, with the HIV virus estimated to have a diameter of ~80–100 nm [9]. However, studies have demonstrated that HIV penetrates interstitially between epithelial cells of the stratified squamous epithelium as early as 2 hr [3,6,14]. These observations rule out the possibility of HIV being transmitted via the classical replication-based mechanisms. Although transcytosis of HIV through the epithelial cells has been reported, the extent is estimated to be very low [16]. Therefore, there must exist alternative mechanisms by which HIV must be able to breach the vaginal epithelial layer.

We and others have previously reported hMR as a CD4 independent receptor playing a role in HIV transmission in different cell types including spermatozoa [17–19]. In human astrocytes, HIV binds to hMR and activates MMPs, which in turn degrade the extracellular matrix proteins [20]. In case of primary genital epithelial cells, HIV has also been reported to decrease the expression of tight junction proteins and increase the leakiness of the epithelial layer towards HIV [21,22]. This led us to hypothesize that hMR may exist on vaginal epithelial cells, which might bind to HIV gp120 leading to production of MMPs, facilitating the degradation of junctional proteins and/or the extracellular matrix in general, thereby inducing a disruption of the epithelial layer organization.

To the best of our knowledge, it is unknown whether human vaginal epithelial cells express hMR that may bind HIV gp120 and induce MMP production. In the present study, we aimed to investigate this hypothesis by studying the presence of hMR, its HIV gp120 binding potential and its affinity constants, and finally the ability of HIV gp120 to induce MMP production via hMR.

Methods

Human vaginal epithelial cell specimens

Ethics Statement. Vaginal epithelial cells from the lateral vaginal wall, were obtained by swab method from women attending the obstetrics and gynecology clinics at NIRRH after written informed consent, and this study and protocol were specifically approved by the ‘National Institute for Research in Reproductive Health Institutional Ethics Committee for Human Subjects’. Women were in the age group of 21–35 years, and had regular menstrual cycles (28–35 days). Purity of the vaginal epithelial cells was confirmed by staining for intracellular cyto-keratin. Viability was assessed using trypan blue, and samples with greater than 80% viability were included in the study. To test for possible contamination of other cells in the vaginal preparations, RT-PCR for markers specific for lymphocytes (CD3), dendritic cells (CD11c) and macrophages (CD14) was performed using RNA derived from pools of vaginal epithelial cells as template.

Culture of human vaginal epithelial cell line - Vk2/E6E7

The cell line was obtained from American Type Culture Collection, originally prepared by R. Fichorova (Fearing Research Laboratory, Boston, MA). Cells were grown as described previously [23] in keratinocyte serum free medium (K-SFM) supplemented with 0.1 ng/ml EGF, 50 μg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA) and 0.4 mM CaCl2 (Sigma, St. Louis, MO), at 37°C and 5% CO2. Cells were split 1:3 at 60% confluence. The Vk2/E6E7 cells express markers of terminal differentiation ( involucrine and cytokeratin 13 ) just as fully differentiated vaginal cells on the surface of vaginal tissues [23]. This cell line represents a valid model that has been used by many others to study HIV - epithelial interaction events [24–28].

HIV gp120 binding to vaginal proteins and competitive inhibition by mannan

As a source of human vaginal proteins, human vagina protein medley comprising SDS solubilized proteins from normal human vagina was purchased from Clontech (CA, USA). Vk2/E6E7 cells were lysed in protein extraction buffer M-Per reagent (Pierce, Rockford, USA). The binding of gp120 to vaginal proteins was tested as described earlier [19] with minor modifications. Briefly, wells of an ELISA plate were coated overnight at 4°C, with 20 μg of proteins in 100 μl of a 0.1 M carbonate buffer, pH 9.2. Wells were washed in TBS (Tris Buffered Saline) and blocked in 0.3% BSA prepared in TBS containing 0.1% Tween for 2 hr at room temperature. Increasing concentrations of HIV gp120-IIIB HRP conjugated (Immunodiagnostics, Woburn, MA USA) (0.2 nM–10 nM) were incubated overnight, followed by six TBS washes containing 0.1% Tween-20. Binding was determined using the substrate 1Mβ/H2O2 and absorbance was read at 450 nm. Wells coated with BSA (bovine serum albumin) or no vaginal proteins served as the negative control. The hMR antagonist mannan (1000 ng/ml to 50 mg/ml) (Sigma) or sucrose (unrelated carbohydrate) was co-incubated with labeled HIV gp120 (100 ng/ml), reacted with vaginal proteins and detected as above. Further, the binding of HIV gp120 (100 ng/ml), was also studied in the presence of increasing doses of the hMR blocking antibody (clone 19.6 BD Pharmingen, San Diego, CA, USA) or an isotype control IgG, and the binding was quantified as described above.

hMR expression by RT-PCR and Western blotting

RNA was extracted from vaginal epithelial cells (n = 22, pooled in groups 3–5 samples) or the Vk2/E6E7 cell line using the Qiagen RNeasy mini kit, (Qiagen, CA, USA). DNase-treated RNA samples were subjected to RT-PCR. RNA was reverse transcribed to cDNA and amplified with the SuperScript One-Step RT-PCR kit (Invitrogen). Briefly, the reaction mixture of 25 μl contained 0.4 μM each of forward and reverse primers, 200 μM dNTP mix and 1 U SuperScript III RT/Platinum Taq enzyme mix. The reverse transcription was carried out at 55°C for 30 min, followed by denaturation at 94°C for 2 min. This was followed by 35 cycles of amplification, each cycle comprising denaturation at 94°C for 15 s, annealing at 58°C for 30 s and extension at 68°C for 30 s. cDNA was PCR amplified using hMR specific primers –FP: 5’-TGT-AAT-CGT-TGC-TGG-AG 3’ and RP: 5’TGT-TTG-AAT-CGT-TGC-TGG-AG 3’ as described earlier [17]. The amplification conditions included denaturation at 94°C for 15 s, annealing at 58°C for 30 s, extension at 68°C for 30 s, with greater than 80% viability were included in the study. To test possible contamination of other cells in the vaginal preparations, RT-PCR for markers specific for lymphocytes (CD3), dendritic cells (CD11c) and macrophages (CD14) was performed using RNA derived from pools of vaginal epithelial cells as template.

HIV gp120 binding and hMR staining by direct fluorescence

Smears of vaginal epithelial cells were processed for HIV gp120-IIIB FITC (Immunodiagnostics) binding (n = 30) and hMR...
HIV gp120 binds to vaginal hMR

A capture ELISA was developed in the laboratory to study the binding of HIV gp120 to hMR. Briefly, anti-hMR polyclonal antibody (1:1000) in 0.1 M carbonate buffer pH 9.2, was immobilized in wells of an ELISA plate, overnight at 4°C. The wells were washed twice with TBS containing 0.1% Tween (wash buffer), and non-specific binding was blocked using 1% BSA. Human vaginal proteins or Vk2/E6E7 proteins (20 μg/well) were incubated to the wells and incubated at 37°C for 1.5 hr, followed by three washes. The ligand HIV gp120 HRP conjugated (100 ng/ml) was added in the presence or absence of the hMR blocking antibody (clone 19.6 BD Pharmingen, San Diego, CA, USA) and detected as described above. Wells coated with normal goat serum and wells containing no vaginal proteins served as controls in the experiment.

**Affinity constants of hMR for HIV gp120**

The affinity constants of HIV gp120 to hMR were determined by competitive ELISA optimized in the laboratory. Briefly, 3.3 nM of HIV gp120-IIIB (Immunodiagnostics) in 0.1 M carbonate buffer pH 9.2, was immobilized in wells of an ELISA plate, overnight at 4°C. The wells were washed twice with TBS and vaginal proteins or Vk2/E6E7 proteins (20 μg/well) were incubated at 37°C for 1.5 hr. To determine the affinity constants, the vaginal or the cell line proteins were mixed with increasing amounts of gp120 (0.083 nM to 40 nM) and then added to the wells. The wells were then washed to remove unbound proteins, and incubated with anti-hMR polyclonal antibody (1:1000) for 2 hr at 37°C to detect the amounts of hMR bound to the immobilized gp120 in the wells. The wells were washed again to remove the excess antibody and then detected using the rabbit anti-goat HRP conjugated secondary antibody (1:5000) (Dako, Glostrup, Denmark). HRP assay was performed as described above and the OD was read at 450 nm. The negative control comprised of wells that had BSA in place of immobilized gp120; wells which did not contain vaginal proteins served as an additional control.

**Measurement of MMP mRNA levels and activity**

For the experiments on MMPs and TIMPs, cells were seeded at density of 1×10^6 cells/well in six well tissue culture plates (Fisher Scientific, Hampton, NH) or 4×10^4 cells/well in 96 well plates. Confluent Vk2/E6E7 cells were treated with HIV gp120 (0.083 nM to 83 nM). These concentrations were chosen based on the affinity constants of hMR determined herein and have been reported to be used by others in different cell types [29-32]. In some experiments in 96 well plates, along with HIV gp120 (83 nM), cells were coincubated with hMR antibody clone 19.2 or isotype matched IgG. Cell supernatants were collected after 24 hr and assayed for MMP activity by zymography using 1 mg/ml gelatin as a substrate as described [33]. Briefly, samples were electrophoresed through a 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, gelatin gels were renatured for 12 h at 37°C in zymogram renaturing buffer (pH 9.2). Then the gels were washed twice with TBS containing 0.1% Tween (wash buffer), and non-specific binding was blocked using 1% BSA. Human vaginal proteins or Vk2/E6E7 proteins (20 μg) were added to the wells and incubated at 37°C for 1.5 hr, followed by three washes. The ligand HIV gp120 HRP conjugated (100 ng/ml) was added in the presence or absence of the hMR blocking antibody (clone 19.6 BD Pharmingen, San Diego, CA, USA) and detected as described above. Wells coated with normal goat serum and wells containing no vaginal proteins served as controls in the experiment.

**Data analysis and statistical methods**

Dissociation constant Kd values were computed using GraphPad Prism software (San Diego, CA). Data were evaluated using unpaired Students t-test. p<0.05 was accepted as statistically significant.

**Results**

**Binding of HIV gp120 to vaginal proteins and effect of mannan on binding**

The binding of HIV gp120 to vaginal proteins, was found to be of high affinity and saturatable (Fig. 1a and b). The affinity of HIV gp120 binding to human vaginal proteins was computed to be 1.2±0.2 nM and 1.4 nM±0.2 nM for the Vk2/E6E7 cell line. BSA at identical or 100× concentrations did not demonstrate gp120 binding. Mannan (an hMR antagonist), dose dependently inhibited the binding of HIV gp120 to vaginal proteins of either sources, while sucrose (unrelated carbohydrate) had no effect. Mannan at doses ranging from 0.1 mg/ml to 50 mg/ml significantly reduced HIV gp120 binding to human vaginal proteins lysates (Fig. 1c) and Vk2/E6E7 lysates (Fig. 1d). At the highest concentration mannans inhibited gp120 binding by almost 75%, indicating that HIV gp120 potentially binds to hMR.

The hMR blocking antibody was found to inhibit the binding of HIV gp120 to vaginal proteins (Fig. 1e), and Vk2/E6E7 proteins (Fig. 1f) by almost 60%. An unrelated isotype matched IgG did not influence the binding, thus demonstrating the specificity.

**Expression of hMR in vaginal cells**

The vaginal epithelial cells were collected by gentle scraping of the lateral vaginal walls and were always observed under a microscope for possibility of other contaminating cells. Most of our preparations were free of any visible contamination; samples where any contaminating cells were visible, were excluded from the study. The purity was also evident by RT-PCR where RNA isolated from 5 independent preparations (and also other pools) did not show presence of transcripts for CD3, CD14 and CD11c which are markers for lymphocytes, macrophages and dendritic cells respectively (Fig. 2a). Bands of expected sizes were however detected in RNA isolated from PBMCs (peripheral blood mononuclear cells) that was used as a positive control. 18S rRNA was detected in all the samples.
RT-PCR using hMR specific primers resulted in the amplification of a product of expected size (201 bp) and sequence that matched with 99% identity to macrophage hMR, when RNA from vaginal epithelial cells or Vk2/E6E7 cells was used as a template (Fig. 2b). In western blots of human vaginal proteins or Vk2/E6E7 cell lysates, probed with goat anti-hMR polyclonal serum, a single band of 155 kDa was detected. No bands were observed when blots were probed with normal goat serum (Fig. 2c).

Localization of HIV gp120 Binding and hMR expression in vaginal cells

HIV gp120-HRBC binding to vaginal proteins and its inhibition by mannann or the hMR blocking antibody. Saturation isotherm of HIV gp120 HRP conjugated binding at the indicated concentrations on the X axis to (a) vaginal protein lysates (Kd = 1.2 ± 0.2 nM) and (b) vaginal epithelial cell line Vk2/E6E7 protein lysates (Kd = 1.4 ± 0.2 nM). Results are representative of three experiments. Inhibition of HIV gp120 binding using mannann in (c) vaginal protein lysates or (d) Vk2/E6E7 protein lysates. Mannann or sucrose concentrations in ug/ml are plotted on a logarithmic scale. Experiments were repeated in triplicates. Binding of HIV gp120 to (e) vaginal protein lysates or (f) Vk2/E6E7 protein lysates in the presence of increasing concentrations of hMR blocking antibody or isotype matched IgG antibody. Results are mean ± SE, n = 3. Statistically significant (p<0.05) when compared with cells treated with HIV gp120 in the absence of antibody.

Figure 1. Binding of HIV gp120 to vaginal proteins and its inhibition by mannann or the hMR blocking antibody. Saturation isotherm of HIV gp120 HRP conjugated binding at the indicated concentrations on the X axis to (a) vaginal protein lysates (Kd = 1.2 ± 0.2 nM) and (b) vaginal epithelial cell line Vk2/E6E7 protein lysates (Kd = 1.4 ± 0.2 nM). Results are representative of three experiments. Inhibition of HIV gp120 binding using mannann in (c) vaginal protein lysates or (d) Vk2/E6E7 protein lysates. Mannann or sucrose concentrations in ug/ml are plotted on a logarithmic scale. Experiments were repeated in triplicates. Binding of HIV gp120 to (e) vaginal protein lysates or (f) Vk2/E6E7 protein lysates in the presence of increasing concentrations of hMR blocking antibody or isotype matched IgG antibody. Results are mean ± SE, n = 3. Statistically significant (p<0.05) when compared with cells treated with HIV gp120 in the absence of antibody.

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The heterogenous punctate staining pattern for HIV gp120 binding as well as hMR expression in vaginal epithelial cells was observed across several samples (n = 30 in each case); with a total
95% cells exhibiting staining in each sample. Experiments on the Vk2/E6E7 cell line were repeated in triplicates.

**Binding of HIV gp120 to vaginal hMR**

Wells coated with anti-hMR polyclonal antibody and incubated with human vaginal proteins or Vk2/E6E7 lysates, exhibited an almost forty fold higher binding of HIV gp120 as compared to negative controls (normal goat serum or no vaginal protein controls). This binding of HIV gp120 to immunopurified hMR was specific, as this binding was reduced to levels comparable to negative controls, in the presence of a neutralizing monoclonal anti-hMR antibody (Fig. 4a).

**Affinity constants of hMR for HIV gp120**

To determine the affinity constant of hMR for HIV gp120, a competition ELISA was utilized. In step 1, hMR from vaginal proteins bound to immobilized gp120 in the well and represents ‘Total hMR binding’. Step 2 involved binding of vaginal proteins with increasing amounts of free gp120 followed by its competition with immobilized gp120. hMR not complexed with free gp120 would bind to the immobilized gp120 in the wells. In step 3, the amount of hMR bound to the immobilized gp120 was estimated. A concentration dependent reduction in hMR binding was observed. To obtain the amounts of gp120 complexed to hMR in step 2, the value from step 3 was subtracted from that observed for Total hMR binding (step 1). These values (obtained at each concentration) were used to compute the affinity constant (Fig. 4b and 4c). HIV gp120 binding to hMR was found to be concentration dependent and saturatable. The Kd value (dissociation constant) was computed to be $2.9 \pm 0.4$ nM (Fig. 4b) from human vaginal proteins and $3.2 \pm 0.6$ nM (Fig. 4c) from Vk2/E6E7 cell line.

**HIV gp120 induces MMP-9 production**

Treatment of Vk2/E6E7 cells with increasing concentrations of HIV gp120, resulted in a dose dependent and significant increase in the mRNA expression of MMP-9 but not MMP-2 (Fig. 5a). As compared to untreated controls, the MMP-9 mRNA levels were two fold higher, in the cells treated with 83 nM of HIV gp120. Culture supernatants were assayed for MMP-9 and MMP-2.
activity. A single band of ~92 kDa and ~72 kDa corresponding to MMP-9 and MMP-2 respectively were obtained on zymograms (Fig. 5c). As compared to the untreated controls, increasing doses of HIV gp120 significantly increased the activity of MMP-9; the activity of MMP-2 was unchanged (Fig. 5b). At 83 nM concentration of HIV gp120, the activity of MMP-9 doubled as compared to untreated controls.

To test if the increase in MMP-9 by HIV gp120, is mediated via hMR, the Vk2/E6E7 cells were incubated with the neutralizing monoclonal blocking antibody (clone 19.2) along with HIV gp120. hMR, the Vk2/E6E7 cells were incubated with the neutralizing monoclonal blocking antibody (clone 19.2) along with HIV gp120. hMR in vaginal cells. The present study demonstrates that human vaginal epithelial cells and the Vk2/E6E7 cell line interact with HIV gp120 via the hMR. Mannose receptors on vaginal epithelial cells were found to exhibit high affinity binding to HIV gp120, demonstrated saturatable binding kinetics and were inhibited by the mannose receptor antagonist mannan. Further, we demonstrate that binding of HIV gp120 to hMR increases MMP-9 transcription and activity in the Vk2/E6E7 cells. To the best of our knowledge, this is the first report demonstrating the presence of hMR on human vaginal epithelial cells, and its ability to bind to HIV gp120 leading to MMP production.

The sexual route of HIV transmission accounts for 70–80% of the HIV epidemic with females being disproportionately affected compared to males [1]; yet HIV transmission across the CD4 negative stratified squamous vaginal epithelium of the lower female reproductive tract remains enigmatic. In women treated with nanoxyinol-9 which damages the vaginal epithelium, a higher risk of HIV infection is reported [35]; micro-aberrations of the vaginal epithelium due to physical insults or inflammation due to STI infections is also known to increase the infectivity [2,6]. In a recent study by Horbul et al., herpes simplex virus induced disruption of the surface epithelium allowed direct access of HIV to sub-mucosal CD4+ T cells [36]. Thus, the epithelial barrier integrity appears to be a crucial determinant for HIV entry via the sexual route. Studies have demonstrated that the normal vaginal epithelium has limited permeability; the vaginal epithelial cells are connected by tight junctions, adherens junctions and desmosomes that form an intercellular seal to restrict paracellular diffusion of molecules across the epithelial sheet [37]. These epithelial cells (25–40 layer thick) rest on a basement membrane comprised of extracellular matrix proteins which are secreted by the epithelial cells [38]. Hence the incoming virus must traverse through several layers of impervious vaginal epithelium and also breach the basement membrane, before it can reach and infect the target CD4+ immune cells in the sub-epithelium. The fact that HIV particles have been detected interstitially between cells in several layers of the stratified epithelium [7,14] and also in the lowermost layer of cells of the vaginal epithelium that rests on the basement membrane [7,11], suggests that there must exist a mechanism by which the virus, would first affect the epithelial cells, and alter their physiology, making them more permeable to eventually allow viral entry and penetration into the sub-epithelial layers. Towards this end, we hypothesized that there must exist some specific receptors for HIV on the human vaginal epithelial cells, which when activated would weaken the epithelial barrier, permitting viral entry to sub-epithelial cells.

Firstly, we studied HIV gp120 binding to human vaginal cell protein lysates and lysates from Vk2/E6E7 cells. HIV gp120 readily bound to proteins from both sources, and this binding was saturatable and of high affinity with Kd values of 1.2±0.2 nM and 1.4±0.2 nM respectively. These results indicate that there exist some high affinity receptors for gp120 on the vaginal epithelium. Vaginal epithelial cells were found to be negative for the classical CD4, CXCR4 and CCR5 receptors (unpublished data). Indeed non-cannonical HIV receptors like syndecans [16] and gp340 [26] have been reported to be present on human vaginal epithelial cells. Beyond these, hMR is a non-cannonical receptor for HIV gp120 [17–19]. To test if hMR could be a putative HIV binding protein in human vaginal cells, we tested the effect of the hMR antagonist mannan on HIV gp120 binding. As reported earlier [17–19], mannan inhibited the binding of HIV gp120 to vaginal proteins leading us to speculate that HIV gp120 may bind to hMR. To test this further, the binding of HIV gp120 was also tested in the presence of an hMR antibody that is known to neutralize ligand binding. This antibody also reduced gp120 binding to vaginal proteins significantly suggesting that HIV gp120 does bind to hMR in vaginal cells.
At present, the existence of hMR in human vaginal epithelial cells has not been reported. In the present study, for the first time we demonstrated that both hMR mRNA and protein are expressed by vaginal cells and also in the epithelial cell line. hMR transcripts were detected by RT-PCR in RNA isolated from vaginal epithelial cells and also from the cell line. The transcript detected in the squamous cells was not from any contaminating lymphocytes, macrophages or dendritic cells indicating that the squamous epithelial cells indeed transcribe hMR. In vaginal squamous epithelial cells, we observed a heterogeneous punctate or speckled pattern of hMR staining by immunofluorescence, and this pattern replicated when HIV gp120 was used as a probe. hMR has been found to exist in equilibrium, in both monomeric and dimeric forms [39,40]. It is possible that in the vaginal epithelial cells too, hMR may exist in a multimeric form and may appear as aggregates. However, such punctate pattern of staining was not observed in cell lines, where both hMR and gp120 binding appeared homogenous. At present it is difficult to explain the reason for such differences in staining pattern; it is possible that in vivo hMR may have already experienced its ligands, and may have dimerized, which may not occur in case of the cell lines.

The observation that mannan inhibited HIV gp120 binding to vaginal cells as well as the Vk2/E6E7 cell line, and hMR is expressed by the same, prompted us to test if HIV gp120 binds to hMR and determine its binding kinetics. Immunopurified hMR from vaginal proteins of either source bound to gp120, and this binding could be displaced by a blocking anti-hMR antibody. The HIV gp120 binding to hMR was concentration dependent and saturatable with a dissociation constant in the range of 2.9 ± 0.4 nM for human vaginal proteins and 3.2 ± 0.6 nM in the case of Vk2/E6E7 cell line proteins; demonstrating it to be a high affinity gp120 receptor. It is interesting to note that this affinity is within the range reported for hMR from other cells [41,42]. However, this dissociation constant is lesser than that observed for HIV gp120 binding to total vaginal proteins observed herein. This is not surprising, as beyond hMR other non-cannonical HIV receptors like syndecans and gp340 are also known to be present in vaginal epithelial cells [16,26]. At present the concentrations of gp120 in the female reproductive tract are not known. In vivo, gp120 exists in both soluble forms and virion bound. Gp120 which is labile due to non-covalent linkage with gp41, sheds spontaneously from the surface.

Figure 5. Effect of HIV gp120 on MMP production. (a) Effect of increasing concentrations of HIV gp120 on mRNA expression of MMP-9 and MMP-2 in Vk2/E6E7 cells. (b) Effect of increasing concentrations of HIV gp120 on MMP-9 and MMP-2 activity. (c) Representative zymograms of HIV gp120 treated Vk2/E6E7 cells (upper panel); lane 1: untreated cells, lane 2: 0.083 nM of HIV gp120, lane 3: 0.83 nM of HIV gp120, lane 4: 83 nM of HIV gp120. Lower panel represents effect of anti-hMR antibody on activity of MMP-9 and MMP-2; lane 1: Cells treated with HIV gp120 (83 nM), lane 2: HIVgp120 (83 nM) + isotype IgG, lane 3: HIV gp120 (83 nM) + anti-hMR IgG, lane 4: untreated cells. (d) Effect of anti-hMR antibody on expression of HIV gp120 induced MMP-9 mRNA. (e) Effect of anti-hMR antibody on MMP-9 activity. (a) and (d) values are mean ± SE of relative expression normalized to 18S rRNA from 3 independent experiments. (b) and (e) are mean ± SE of MMP activity expressed as fold change over unstimulated cells from 3 independent experiments. *Statistically significant (p<0.05) when compared with controls. **Statistically significant (p<0.05) when compared with HIV gp120 treated cells.

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of virions [43–47] and is also released from virus infected cells [44,40]; giving rise to soluble forms of gp120. On the surface of the virus, the gp120 is known to exist in the form of trimers, and is also reported as monomers, with the monomeric form being highly accessible [43,49–52]. While the affinity constants of the trimeric gp120 to hMR have not been determined; considering the high affinity of monomeric gp120 to hMR, hMR could concentrate the virus on the mucosal surface and may act as a local reservoir. Given the fact that each virion would carry around 24 copies of gp120 [53,54], accumulation of virus particles above a threshold would possibly activate a range mechanisms pertaining to epithelial cell damage and transcytosis to gain entry in to subepithelial layers. It is also possible that the affinity of hMR to trimeric gp120 may be higher than the monomeric form (used herein) which may further potentiate accumulation of the virus in the tract.

In vivo, mannosylated proteins and other ligands for hMR are known to trigger a plethora of signaling cascades to elicit biological effects [55]. In astrocytes, it has been demonstrated that under the influence of HIV, hMR activates PI3-kinase signaling leading to production of MMPs [20]. Indeed, an increase in MMPs has been found in various HIV associated pathologies [56]. HIV signaling would largely depend on exposed accessible gp120 at its surface, which contains both trimeric and monomeric forms with the monomeric form though less in quantity is highly accessible [43,49–51]. To test if the binding of monomeric HIV gp120 to hMR may induce the production of MMPs in vaginal epithelial cells, we challenged the Vk2/E6E7 cells with increasing concentrations of gp120 and tested for MMP-2 and MMP-9 transcription and their activities. A dose dependent increase in the mRNA expression and activity of MMP-9 but not MMP-2 in response to HIV gp120, was observed. The levels of TIMP-1, TIMP-2 and TIMP-3 mRNA in gp120 treated and untreated cells were identical (unpublished data). These results imply that HIV gp120 induces MMP-9 production in vaginal epithelial cells. Beyond hMR, the vaginal epithelial cells have other HIV binding proteins like gp340 and syndecans [16,26]. To test if the induction of MMP-9 by HIV gp120 in the epithelial cells is via hMR or other HIV binding proteins, we tested the effects of an hMR neutralizing antibody on gp120 mediated MMP-9 production. The anti-hMR antibody completely abrogated the HIV gp120 mediated increase in MMP-9 production and the levels were identical to untreated controls. These results indicate that the effect of HIV gp120 on MMP-9 production is specifically via hMR. It will be of interest to determine if the other gp120 binding proteins like gp340 also actuate signaling cascades that culminate into activation of proteases. Furthermore, it would be of interest to compare the effects of monomeric and trimeric gp120 on hMR mediated protease production; and determine if the upregulation in proteases observed herein in our study, is further increased by trimeric gp120. These studies using trimeric gp120 merit further investigation.

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Author Contributions
Conceived and designed the experiments: SEF DNM AHB. Performed the experiments: SEF. Analyzed the data: SEF DNM AHB. Wrote the paper: SEF DNM AHB. Principle investigator and took responsibility for the study: AHB. Provided the human vaginal samples for the study: JSG.
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