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Alcohol Enhances HIV Type 1 Infection in Normal Human Oral Keratinocytes by Up-regulating Cell-Surface CXCR4 Coreceptor

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ABSTRACT

Recent studies suggest that normal human oral keratinocytes (NHOKs) can be infected by HIV-1, and alcohol can enhance HIV infection and replication in lymphocytes. In this study, we examined the possibility that alcohol might facilitate HIV-1 infection of NHOKs by up-regulating cell surface expression of the coreceptor, CXCR4. Alcohol enhanced *in vitro* infection of NHOKs by CXCR4-tropic strains of HIV-1 as indicated by synthesis of viral reverse transcripts and production of $p24^{gag}$ protein. Alcohol had no effect on CXCR4 gene expression or on total cellular complements of CXCR4 protein. However, alcohol did enhance the fraction of total CXCR4 expressed on the cell surface relative to intracellular stores. Alcohol-induced up-regulation of cell surface CXCR4 expression and HIV-1 infectivity could be blocked by SDF-1α-mediated internalization. These data suggest that alcohol may influence oral HIV transmission by altering the cellular compartmentalization of CXCR4 in cells of the oral cavity.

INTRODUCTION

EPIDEMIOLOGICAL STUDIES SUGGEST THAT HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) may be transmitted by oral mucosal contact with infected individuals.[1] HIV proviral DNA can be detected in gingival epithelial cells from HIV patients,[2] and *in vitro* studies suggest that cell-free HIV-1 may be taken up and released by primary human gingival cells.[3] Animal studies have also shown that oral exposure to various simian immunodeficiency virus (SIV) strains, including T cell-tropic SHIV, leads to productive infection and disease progression in rhesus macaques.[4,5] In an effort to identify the cellular pathways involved in oral HIV infection, we recently found that normal human oral keratinocytes (NHOKs) can be productively infected by HIV-1 and convey infectious virions to adjacent lymphocytes.[6] These effects depend in part on the presence of the HIV-1 galactosylceramide (GalCer) receptor and CXCR4 chemokine coreceptor on NHOKs, but the signals that regulate the expression of these molecules in the oral mucosa are not well understood.

Alcohol consumption is an established risk factor for oral disease in general[7,8] and is associated with oral lesions in HIV-infected individuals.[9,10] Alcohol consumption has also been linked to a heightened risk of HIV-1 infection.[11,12] The mechanisms that mediate these effects are not known, but the finding that HIV can infect oral epithelial cells suggests that alcohol could conceivably influence cellular vulnerability to infection. In the present study, we examine this possibility by exposing NHOKs to concentrations of alcohol representative of blood level found in heavy drinkers and assessing viral infectivity and replication. To evaluate the possible contribution of altered HIV receptor/coreceptor expression, we also monitored the expression of GalCer and CXCR4 on NHOKs. Results showed that alcohol enhanced cell surface expression of CXCR4, and this increase was associated with enhanced vulnerability to infection by both CXCR4-tropic and dual-tropic strains of HIV-1. No changes in CXCR4 gene expression were observed, but alcohol reduced CXCR4 coreceptor internalization and shifted a substantial portion of the intracellular CXCR4 to the cell surface. Enhanced cell surface expression of CXCR4

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mediated by alcohol appears to increase HIV-1 infection. The natural ligand for CXCR4, stromal-derived factor (SDF)-1α, can block those effects by down-regulating cell surface CXCR4.

MATERIALS AND METHODS

Cell cultures and conditions

Excised retromolar tissue from the oral cavity of healthy volunteers was incubated in Ca^{2+}-free minimal Eagle's medium (MEM, GIBCO/BRL, Grand Island, NY) containing collagenase (type II, 0.3 mg/ml, Sigma, St. Louis, MO) and dispase (Grade I, 2.4 mg/ml, Boehringer-Mannheim, Indianapolis, IN) to separate the epithelium from the underlying mesenchyme. Tissues were incubated for 60–80 min at 37°C in an atmosphere of 95% air and 5% CO_2. Separated epithelial sheets were then dissociated into single cells by incubation in 0.05% trypsin (GIBCO/BRL) with agitation at 37°C for 5–10 min. The cells were resuspended in keratinocyte growth medium (KGM, Clonetics Corp., San Diego, CA) and plated at a density of 5×10^4 cells per 25-cm^2 flask.

Cell proliferation

Viable NHOKs (2×10^3) were cultured in 96-well plates in the presence of different concentrations of alcohol for up to 48 hr. Cellular proliferation was measured using the CellTiter 96 AQ_{ueous} one solution cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's protocol. This assay is a colorimetric method for determining the number of viable cells.

Virus stocks

CXCR4-tropic HIV-1_{NL4-3} and dual-tropic HIV-$1_{89.6}$ stocks were obtained from 24 hr culture supernatants of infected CEM and activated peripheral blood lymphocytes (PBLs). To reduce contamination of viral stocks with HIV-1 DNA derived from lysed cells, supernatants were filtered and treated with 2 μg/ml DNase I (Worthington, Lakewood, NJ) for 1 hr at room temperature in the presence of 0.01 M $MgCl_2$.

HIV infection

NHOKs were seeded onto six-well plates and grown until 60–70% confluent. Cells were incubated with 1 ml of virus stock containing 500 ng of viral $p24^{gag}$ protein for 2 hr at 37°C in the presence of polybrene (10 μg/ml). Cells were then washed three times with phosphate-buffered saline (PBS) to remove residual virus and cultured for up to 9 days. Heat-inactivated virus stocks were prepared by incubation for 1 hr at 65°C. Expression of viral protein in culture supernatants was measured by ELISA specific for the $p24^{gag}$ antigen (Coulter, Hialeah, FL).

PCR detection of HIV-1 DNA and CXCR4 mRNA

Reverse-transcribed HIV-1 DNA structures were quantified in total DNA extracted by urea lysis from infected cells 16 hr postinfection. Quantitative PCR was performed using ^{32}P-end-labeled primers that recognize the R/U5 region of the viral LTR (M667/AA55), with procedures as previously described.[13] Reactions were denatured for 2 min at 94°C and amplified by 25 cycles of denaturation for 1 min at 94°C and polymerization for 2 min at 65°C. Human β-globin sequences were amplified in parallel to control for cellular DNA input.[13] PCR products were fractionated on a 6% polyacrylamide gel and quantified by phosphorimage analysis (Storm, Amersham Biosciences, Piscataway, NJ).

TaqMan real-time PCR was used to quantify viral DNA to show HIV-1 entry. To control the variability of yield and quality of nucleic acid preparation, HIV-1 PCR was performed with β-globin PCR under the same conditions. Primers that recognize the R/U5 region of the viral LTR (M667/AA55) were utilized to detect initiation of reverse transcription. To detect HIV-1 PCR products, a TaqMan fluorescent probe spanning position 584–616 in the HIV-1 LTR was used. Amplification and detection were performed on an Applied Biosystems PRISM 7700 Sequence Detection System using the TaqMan Reagent Kit. Thermal cycling (Perkin-Elmer 7700 Thermal Cycler) was initiated with preincubation at 50°C for 2 min and enzyme activation at 95°C for 10 min. Subsequently, 40 cycles were conducted in two temperature steps: 15 sec at 95°C and 60 sec at 60°C. Postrun manipulations of data were performed according to the manufacturer's instructions (Perkin-Elmer Cetus, Norwalk, CT).

To quantify CXCR4 mRNA levels, total RNA was extracted from 1×10^6 NHOKs (Qiagen RNeasy, Chatsworth, CA) and digested with DNase I (Promega). Reverse transcription was conducted with murine leukemia virus reverse transcriptase (MuLV, Perkin-Elmer Cetus) and a 20-thymidine oligonucleotide primer. One-tenth of the synthesized cDNA was PCR amplified using CXCR4-specific primers, 5′-TCATCTACA-CagTCAACCTCTACA-3′ and 5′-gAACACAACCACCCA-CAAgTCATT-3′.[14] β-Actin was also amplified to quantify cellular RNA input. One primer of each pair was ^{32}P-end-labeled and amplification was performed using Taq DNA polymerase (Perkin-Elmer Cetus) with 34 cycles of denaturing at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min. The amplified products were subjected to electrophoresis on a 6% polyacrylamide gel and quantified by phosphorimage analysis.

Flow cytometry

Cell surface expression of CXCR4 was quantified by flow cytometry. NHOKs (5×10^5) were pretreated with varying concentrations of alcohol for 48 hr. Blocking studies included preincubation with 100 ng/ml SDF-1α for 1 hr at 37°C. Cells were then detached with 0.5 mM ethylenediaminetetraacidic acid (EDTA) at 37°C for 60–90 min and immunostained with monoclonal antibodies (MAb) against either GalCer or CXCR4. CXCR4 was identified by a phycoerythrin (PE)-conjugated MAb (Pharmingen, San Diego, CA). GalCer was identified by anti-GalCer MAb (Galactocerebroside; Chemicon International Inc., Temecula, CA) with secondary staining by a PE-conjugated goat $F(ab')_2$ antimouse IgG (heavy and light chains; Caltag Labotatories, Burlingame, CA). Mouse IgG_{2a} was used as isotype control in analysis of CXCR4 and GalCer, respectively. After staining, cells were washed and fixed in 2% paraformaldehyde. Data were ac-

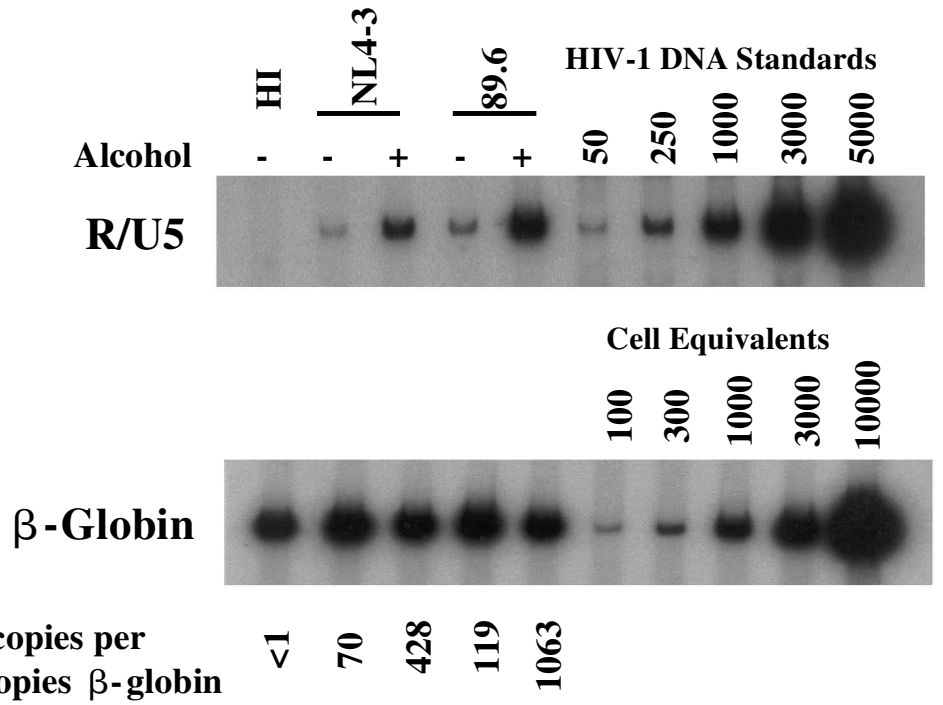

FIG. 1. Effect of alcohol on HIV-1 entry in NHOKs. Cells (1×10^6) were incubated with 0.5% alcohol for 48 hr before being exposed to 500 ng p24 of live HIV-1_{NL4-3}, HIV-$1_{89.6}$, or heat-inactivated virus (HI) for 2 hr at 37°C in the presence of 10 μg/ml polybrene. Viral DNA was PCR amplified with the R/U5 primer pair (M667/AA55) to detect early viral LTR reverse transcripts. β-Globin primers were used in parallel for cell loading control.

quired on a FACScan instrument and analyzed with the CellQuest program (Becton Dickinson Immunocytometry, Mountain View, CA).

To assess the intracellular vs. extracellular compartmentalization of CXCR4, flow cytometry was performed to determine both extracellular levels alone and total CXCR4 levels (intracellular and extracellular). NHOKs (1×10^6) were pretreated with varying concentrations of alcohol for 48 hr and then detached with 0.5 mM EDTA at 37°C for 60–90 min. Cells were then fixed and stained with anti-CXCR4 antibody (Ab) after permeabilization (intracellular and extracellular staining) or left unpermeabilized during staining (extracellular staining alone).[15] Cells were fixed by suspension in 0.5 ml cold 4% paraformaldehyde, pelleted, and resuspended in 0.5 ml of saponin buffer [0.1% (w/v) saponin, 0.05% azide in Hank's balanced salt solution (HBSS)] for permeabilization. Unpermeabilized cells were suspended in 0.5 ml HBSS. All cells were then pelleted, 450 μl of suspension solution was aspirated, and 10 μl of anti-CXCR4 MAb was added. Following 30 min of incubation at room temperature, cells were washed once in either 300 μl of saponin buffer (permeabilized cells) or HBSS (unpermeabilized cells) and then pelleted and resuspended in 200 μl PBS for flow cytometry. Intracellular CXCR4 compartmentalization was quantified by subtracting the mean fluorescence intensity (MFI) of anti-CXCR4 MAb binding on unpermeabilized cells (extracellular CXCR4) from that of permeabilized cells (total CXCR4 = intracellular CXCR4 and extracelluar CXCR4).

RESULTS

Alcohol increased viral entry in NHOKs

We have previously shown that NHOKs can be infected with CXCR4-tropic and dual-tropic HIV-1, whereas infection with CCR5-tropic virus is inefficient.[6] However, CCR5-tropic virus can transmit to PBLs very efficiently. To study the effects of alcohol on HIV-1 entry and replication in oral epithelial cells,

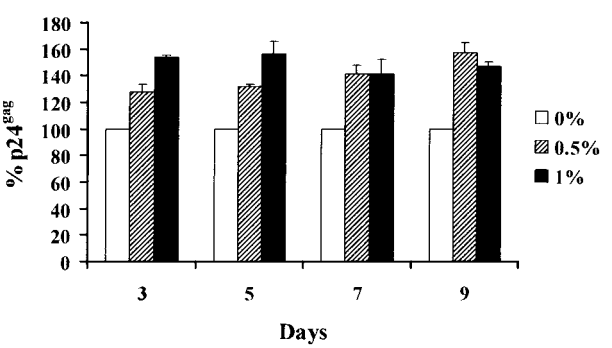

FIG. 2. Effect of alcohol on viral replication. NHOKs were exposed to alcohol before being infected with 500 ng HIV-1_{NL4-3} p24 for 2 hr at 37°C. Postinfection culture supernatant was collected at 3, 5, 7, and 9 days and was tested for $p24^{gag}$ by ELISA. Error bars represent the standard deviation of triplicate values.

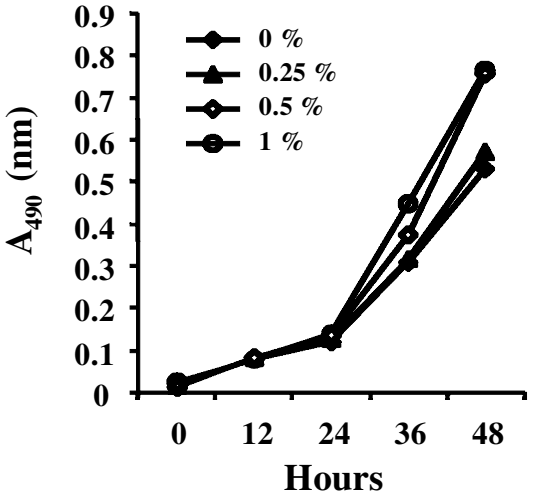

FIG. 3. Effect of alcohol on cellular proliferation of NHOKs. NHOKs (2×10^3) were incubated with or without alcohol for 48 hr and the cellular proliferation was measured by CellTiter 96 AQ_{ueous} one solution cell proliferation assay kit (Promega).

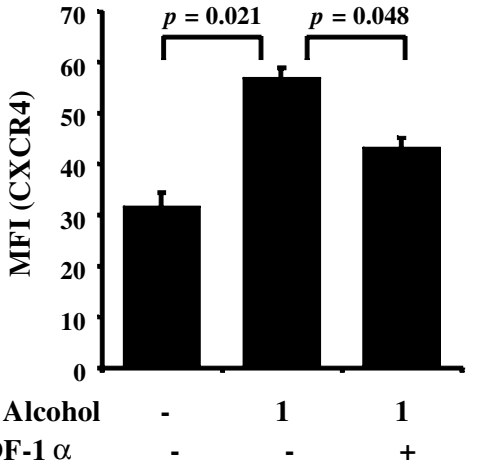

FIG. 5. Effect of alcohol on SDF-1α-induced CXCR4 down-regulation. NHOKs were pretreated with 1% alcohol for 48 hr and then exposed to SDF-1α (100 ng/ml) for 1 hr at 37°C. CXCR4 expression was measured by flow cytometry for immunofluorescence staining with an anti-CXCR4 Ab. Results represent the mean of duplicate experiments, and differences between conditions were analyzed by *t* test.

NHOKs were exposed to virus-containing supernatants (or heat-inactivated negative control) for 2 hr, after which time the cells were washed and cultured for up to 9 days. As shown in Figure 1, quantitative PCR verified the presence of early reverse transcripts of the viral long terminal repeat (LTR) region by 16 hr after exposure to either CXCR4-tropic (NL4-3) or dual-tropic (89.6) strains of HIV-1. Pretreatment of NHOKs with alcohol enhanced entry of both strains of virus by at least 6-fold. Viral DNA was absent in NHOKs exposed to heat-inactivated virus, confirming that the cells were initially free of infection and that contaminating DNA in the virus stock was minimal.

To confirm productive viral replication, HIV-1 $p24^{gag}$ levels were monitored in culture supernatants. As shown in Figure 2, NHOKs treated with either 0.5% or 1% alcohol prior to infection with HIV-1_{NL4-3} strain showed 30–50% elevation in $p24^{gag}$ relative to untreated cells. Alcohol concentrations in the range of 0.5–1% produced significant increases in HIV-1 replication.

To determine whether the alcohol concentrations that were used might be toxic to NHOKs, we monitored cell growth using a proliferation assay kit. As shown in Figure 3, alcohol concentrations below 0.5% did not alter cell growth kinetics and concentrations in the range of 0.5–1% produced a small increase in cell growth at 48 hr.

Alcohol enhanced cell surface expression of CXCR4 chemokine coreceptor on NHOKs

Given recent evidence that HIV infection of NHOKs is mediated in part by the GalCer receptor and CXCR4 coreceptor,[6] we sought to determine whether the effect of alcohol on

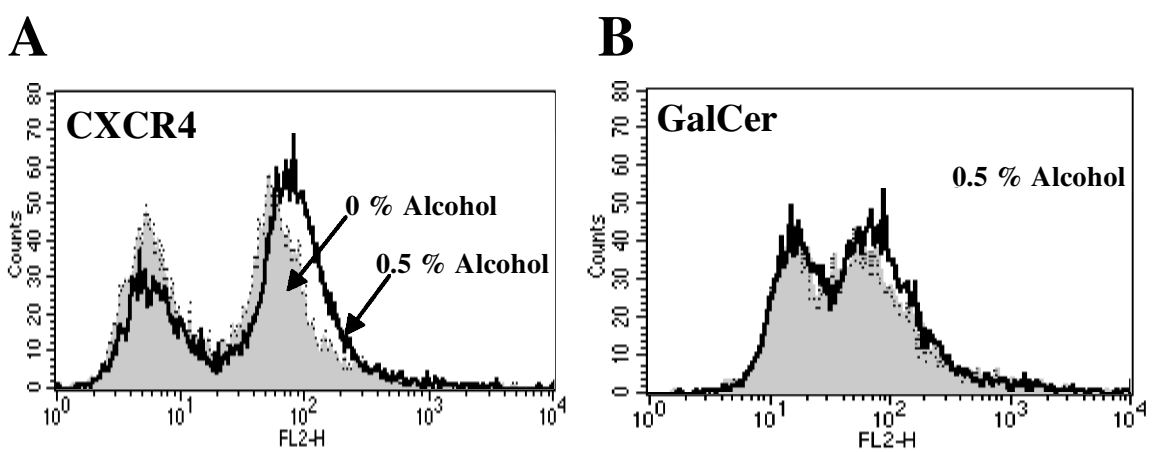

FIG. 4. Effects of alcohol on CXCR4 and GalCer expression on NHOKs. NHOKs were pretreated with or without alcohol for 48 hr before immunostaining with MAb against either CXCR4 (**A**) or GalCer (**B**).

TABLE 1. EFFECT OF ALCOHOL ON LIGAND-INDUCED DOWN-REGULATION IN HIV-1 ENTRY[a]

	HI	*0% Alcohol*	*0.5% Alcohol*	*SDF-1α*	*SDF-1α/0.5% alcohol*
HIV copies	4	110	685	53	88

[a]NHOKs were pretreated with or without alcohol for 48 hr and then exposed to SDF-1α (100 ng/ml) or buffer for 1 hr at 37°C. The cells were subsequently exposed to 500 ng of live HIV-1_{NL4-3} virus for 2 hr at 37°C in the presence of 10 μg/ml polybrene. Viral DNA was detected by TaqMan real-time PCR. The data were analyzed by PCR Prism98 and represented as HIV copies/10^4 cells. HI indicates heat inactivated virus.

HIV-1 infectivity might be mediated by changes in receptor or coreceptor expression. Flow cytometry analyses showed that 0.5% alcohol had little effect on GalCer expression (Fig. 4B), but the intensity of CXCR4 expression increased by approximately 30% (Fig. 4A).

To determine whether alcohol-induced enhancement of HIV-1 infectivity in NHOKs was mediated by up-regulated CXCR4 expression, we sought to block this effect by pretreating the cells with high concentrations of the natural ligand for CXCR4, SDF-1α. Flow cytometric analyses showed that CXCR4 levels significantly increased in the presence of alcohol (Fig. 5). Furthermore, pretreatment of cells with SDF-1α counteracted alcohol-mediated up-regulation to produce CXCR4 levels approximating those observed in untreated cells. PCR analysis of viral entry showed that SDF-1α pretreatment also abrogated the effect of alcohol in enhancing HIV-1 infectivity (Table 1). The vulnerability of NHOKs to HIV-1 infection might be due to alcohol-mediated increase in CXCR4 expression.

Alcohol had no effect on CXCR4 mRNA expression

To determine whether alcohol-induced up-regulation of cell surface CXCR4 was mediated by increased gene expression, RT-PCR was used to assay CXCR4 mRNA levels in NHOKs treated with and without alcohol. No significant difference in steady-state CXCR4 mRNA levels was observed when NHOKs were treated with 0.25, 0.5, and 1% alcohol when compared to untreated cells (Fig. 6). This result suggested that alcohol up-regulation of CXCR4 in NHOKs was controlled at the post-transcriptional level.

Alcohol reduced CXCR4 internalization in NHOKs

Like many G-protein-coupled receptors, CXCR4 circulates between the cell surface and endosomal compartments within the cell.[16,17] To determine whether alcohol might alter cell surface CXCR4 levels by increasing the fraction of total receptors localized to the cell surface, we measured intracellular and extracellular complements of receptors using flow cytometry.[15] Immunofluorescence was analyzed following standard cell surface staining (to assess extracellular receptor pool) followed by parallel staining of CXCR4 in permeabilized cells (detecting both intracellular and extracellular receptor pools). The intracellular receptor pool was quantified as the difference between mean fluorescence intensity (MFI) for anti-CXCR4 Ab in permeabilized cells (intracellular and extracellular) and unpermeabilized cells (extracellular only). Analysis showed that the total complements of CXCR4 protein (intracellular and extracellular) were not significantly altered by alcohol (Fig. 7). However, the fraction of CXCR4 in intracellular compartments decreased by approximately one-third after NHOKs were exposed to alcohol. These results are consistent with the mRNA analyses and suggest that alcohol up-regulates cell surface expression of CXCR4 mainly by shifting CXCR4 molecules from intracellular to extracellular sites, rather than by increasing the size of the total CXCR4 receptor pool.

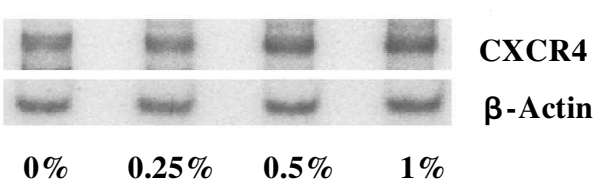

FIG. 6. Effect of alcohol on CXCR4 mRNA expression. RT-PCR was used to demonstrate CXCR4 mRNA expression in NHOKs treated with various concentrations of alcohol. Parallel determination of β-actin mRNA verified equivalent mRNA loading.

DISCUSSION

The data show that alcohol concentration characteristic of those found in alcohol abusers can increase HIV-1 entry in NHOKs *in vitro* by up-regulating cell surface expression of the viral coreceptor CXCR4. The effects are mediated primarily by the redistribution of CXCR4 molecules onto the cell surface,

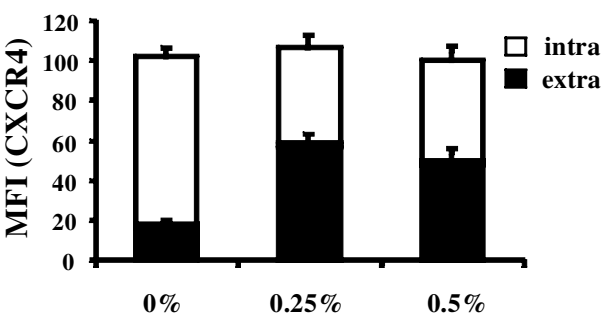

FIG. 7. Effect of alcohol on CXCR4 internalization. NHOKs were pretreated with or without alcohol for 48 hr before immunostaining with anti-CXCR4 Ab. Intracellular CXCR4 compartmentalization (intra) was quantified by subtracting the mean fluorescence intensity (MFI) of anti-CXCR4 Ab binding on unpermeabilized cells with extracellular CXCR4 (extra) from that of permeabilized cells, which include both intracellular and extracelluar CXCR4. Error bars represent the standard deviation of triplicate values.

rather than by transcriptionally mediated increases in the total receptor pool. Concentrations of alcohol ranging between 0.5% and 1% are sufficient to increase viral protein production by 30–50% and to enhance the establishment of reverse-transcribed viral DNA by more than 6-fold. In addition to providing a potential mechanism by which alcohol abuse may increase the risk of HIV-1 infection *in vivo*, these data also illustrate a fundamental biological mechanism by which alcohol can alter the biology of the oral mucosal environment. Although the physiological role of CXCR4 in the oral mucosa remains to be defined, these data clearly suggest that a side effect of its regulation by alcohol might be increased risk of infection by CXCR4-tropic strains of HIV-1.

Oral–genital contact, especially receptive oral intercourse (ROI), has been identified as an independent risk factor for acquiring HIV.[18,19] Epidemiological studies also suggest that alcohol abuse may accelerate the progression of HIV-1 infection.[20,21] The present studies suggest a biological mechanism that may link these two clinical observations by increasing oral mucosal vulnerability to infection. Oral transmission of HIV is difficult to demonstrate in clinical studies due to its strong correlation with other behaviors conferring a high risk of exposure.[3] However, *in situ* PCR studies have shown that HIV-1 proviral DNA can be detected in the oral mucosa of up to 83% of HIV-infected subjects.[22] Recent studies also indicate that oral epithelial cell lines can be productively infected with HIV-1 via GalCer and either CXCR4 or CCR5.[23] In addition, our laboratory has recently demonstrated productive HIV infection of primary oral keratinocytes.[6] The present results reinforce the functional significance of viral coreceptor expression on oral epithelial cells by demonstrating that exogenous factors that regulate their expression can also modulate cellular vulnerability to HIV infection.

The specific signaling pathway by which alcohol modulates CXCR4 compartmentalization is unknown. Several previous studies have shown that major physiological signaling pathways such as the Ca/PKC and cAMP/PKA systems can influence the localization of CXCR4 and other chemokine receptors.[15,16,24] cAMP signaling can enhance HIV infectivity in primary epithelial cells,[3,25] and our studies also showed alcohol can externalize CXCR4 and enhance HIV infectivity in primary lymphocytes.[26] It is not known whether alcohol induces cAMP signaling in NHOKs, but this could be one mechanism underlying the CXCR4 redistribution effects observed here. Studies evaluating this possibility are ongoing. Identification of the signaling pathways that mediate the effect of alcohol on CXCR4 expression could help reduce the risk of oral HIV transmission *in vivo*.

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