Cocaine Modulates Dendritic Cell-Specific C Type Intercellular Adhesion Molecule-3-Grabbing Nonintegrin Expression by Dendritic Cells in HIV-1 Patients

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Cocaine Modulates Dendritic Cell-Specific C Type Intercellular Adhesion Molecule-3-Grabbing Nonintegrin Expression by Dendritic Cells in HIV-1 Patients

Madhavan P. N. Nair, Supriya D. Mahajan, Stanley A. Schwartz, Jessica Reynolds, Robert Whitney, Zail Bernstein, Ram P. Chawda, Don Sykes, Ross Hewitt, and Chiu Bin Hsiao

We report that cocaine may act as cofactor in HIV pathogenesis by increasing dendritic cell-specific C type ICAM-3-grabbing nonintegrin (DC-SIGN) expression on dendritic cells (DC). Our results show that cocaine-using, long-term nonprogressors and normal progressors of HIV infection manifest significantly higher levels of DC-SIGN compared with cocaine-nonsing long-term nonprogressors and normal progressors, respectively. Furthermore, in vitro HIV infection of MDC from normal subjects cultured with cocaine and/or HIV peptides up-regulated DC-SIGN, confirming our in vivo finding. Cocaine, in synergy with HIV peptides, also up-regulates DC-SIGN gene expression by MDC. Furthermore, the cocaine-induced effects were reversed by a D1 receptor antagonist demonstrating the specificity of the reaction. Our results indicate that cocaine exacerbates HIV infection by up-regulating DC-SIGN on DC and these effects are mediated via dysregulation of MAPKs. These data are the first evidence that cocaine up-regulates the expression of DC-SIGN on DC. A better understanding of the role of DC-SIGN in HIV infection may help to design novel therapeutic strategies against the progression of HIV disease in the drug-using population. The Journal of Immunology, 2005, 174: 6617–6626.

Dendritic cells (DC) are the critical mediators of various immune responses and are the first line of defense against any infection including HIV (1–4). With respect to HIV-1 infection, DC play a critical role in harborin HIV and possibly infecting neighboring T cells and facilitating virus dissemination through the blood brain barrier, causing CNS dysfunction (5, 6). The first step in the process of HIV infection is the attachment of virus to susceptible target cells. It is known that several host proteins play major roles in this process (2, 7, 8). The recent discovery of the DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) also called CD209, has lead to the observation that it is the first molecule that facilitates HIV infection independent of CD4 or the HIV coreceptors (7, 9). A homologue of DC-SIGN, DC-SIGNR, also has been described as an efficient attachment factor for HIV (3, 9).

Cocaine is one of the most widely abused drugs in the U.S. In 2003, according to the National Household Survey on Drug Abuse, an estimated 2.3 million Americans (1.0%) age 12 or older were active cocaine users, and 604,000 (0.3%) were active crack (smokeable form of cocaine) users (11). The last decade has witnessed a great, entangled epidemic of cocaine abuse and HIV-1 infections. Epidemiologic data demonstrate that drug abuse, particularly with crack cocaine, is a risk factor for contracting infection with HIV-1 (12–14), and has been shown to be independently associated with progression to clinical AIDS (12–14).

Because DC are the first line of defense against HIV infection and serve as HIV reservoirs, and because DC-SIGN facilitates HIV infection independent of CD4 and/or coreceptors, studies on the regulation of DC-SIGN may be important to understand the mechanisms(s) of increased HIV infection in the drug-using population. Several investigators, including ourselves, have shown that cocaine exerts significant immunomodulatory activities on lymphocyte subpopulations (15, 16). However, the role of cocaine use on DC function in subjects with or without HIV infection has not been elucidated. We hypothesize that drugs of abuse significantly up-regulate DC-SIGN to facilitate virus dissemination. Our data are the first evidence on the mechanism(s) of increased immunopathogenesis in cocaine-using, HIV-1-infected subjects. A better understanding of the role of drug use on DC-SIGN may help to design novel therapeutic strategies against HIV disease progression among drug-using patients.

Materials and Methods

Human subjects

Blood donors were apprised of this study, and consents were obtained consistent with the policies of the appropriate local institutions and the National Institutes of Health. Peripheral blood samples from healthy, HIV-negative individuals and HIV-1-infected patients were drawn into a syringe containing heparin (20 U/ml). The HIV-infected subjects were recruited from the Immunodeficiency Services Clinic of the Erie County (NY) Medical Center. HIV-negative subjects who are currently using cocaine were recruited from the Chemical Dependency Clinics of Erie County Medical Center.
HIV-infected and control populations

Normal progressors (NP) of HIV-1 infection are defined as individuals who exhibit a faster decline in CD4 lymphocytes, displaying a declining slope of >50 CD4 T cells per cubic millimeter per semester and an overall CD4 loss >30% with a current CD4 count <500 and plasma viral load >50,000 copies/ml. Long-term nonprogressors (LT-NP) of HIV infection are defined as patients with no HIV-related symptoms for >5 years with a CD4 count >500 and plasma viral load <10,000 copies/ml, who never received any antiretroviral treatment and were generally in good health. HIV-1-seronegative cocaine users were defined as HIV-1 Ab-negative as determined by ELISA. Cocaine use in all the subject groups was confirmed by urine toxicology testing on the day before blood collection. HIV-1-negative controls were healthy volunteers who were age-, sex-, and ethnically matched with HIV-1-infected subjects and cocaine users. A short history was obtained from all normal donors to assure that they were not using illicit drugs and are, at most, moderate users of alcohol (<2 drinks per day) or tobacco (<1 pack per day). Exclusion criteria include use of illicit recreational drugs including opiates, heavy use of alcohol or tobacco, and use of medications known to affect the immune system. Exclusion criteria for all groups were age <18 and >50 years and pregnancy.

Isolation and generation of mature DC (MDC) and immature DC (IDC)

DC were prepared from PBMC as described (17–20). Briefly, PBMC were separated on a density gradient and adhered to plastic culture plates in medium containing serum. Nonadherent cells were removed after 1 h at 37°C, and adherent cells were cultured for 6 days in medium containing 100 U/ml recombinant human GM-CSF and 100 U/ml IL-4 (R&D Systems). After 6 days of culture, IDC were removed by gently swirling the plate to resuspend them for use in the experiments. These IDC were allowed to progress to MDC by incubating for five more days with medium containing 1000 U/ml recombinant human GM-CSF and 1000 U/ml IL-4 as above. Both IDC and MDC were washed in FACS buffer (eBioscience), incubated with nonspecific IgG (20 μg/ml) for 10 min at 4°C to block FcR, stained with specific Abs for DC surface markers, and analyzed by flow cytometry. Both MDC and IDC express CD80, CD86, CD40, HLA-DR, DQ, and CD11c at different levels. However, MDC predominantly express CD83 as described (21–23).

Cell culture

IDC and MDC were cultured at a concentration of 5 × 10^5 cells/ml with HIV proteins (gp120 or tat, 10–100 ng/ml), cocaine (10^{-6}–10^{-12} M) or cocaine plus HIV proteins (gp120 or tat) in six-well plates (19, 20). The HIV-1 tat protein used in the present experiments was obtained from the National Institutes of Health AIDS Reference Reagent Program (catalog no. 2225) as a 25-μg lyophilized protein. This highly purified (>95%) recombinant tat protein encodes 86 aa and HIV-1 tat viral strain was the original source. The functional properties of this protein were confirmed by using a transactivation assay (24, 25). The HIV gp120 protein was obtained from the National Institutes of Health AIDS Reference Reagent Program (catalog no. 4961) as a 100-μg lyophilized recombinant protein. This product was prepared and HIV-1 Isolate, that is prepared from HEK293 cells purified by immunoaffinity chromatography using a mAb specific for a confirmatory epitope of gp120.

Treatment of DC with HIV-1 isolates

The MDC were infected with HIV-1 American Society for Microbiology (ASM)-54 virus (National Institutes of Health AIDS Research and Reference Reagent Program; catalog no. 2794) at a concentration of 10^3.5–50% tissue culture-infective dose (TCID_{50})/0.2 ml for 3 h as described (26, 27), washed, and cultured with or without cocaine (10^{-6}–10^{-12} M) before RNA was extracted. A postinfection period of 24 h was used in the study to amplify the long terminal repeat (LTR)-R/U5 region that represents early stages of reverse transcription of HIV. In separate experiments, MDC were infected with HIV-1 Isolate (National Institutes of Health AIDS Research and Reference Reagent Program; catalog no. 510) at a concentration of 10^4.75 TCID_{50}/ml cells with and without cocaine for 15 days and the culture supernatants were quantitated for p24 Ag.

RNA extraction

Cytoplasmic RNA was extracted by an acid guanidinium-thiocyanate-phenol-chloroform method as described (28). Cultured DC were centrifuged and resuspended in a 4 M solution of guanidinium thiocyanate. Cells were lysed by repeated pipetting, and then phenol-chloroform was extracted in the presence of sodium acetate. After centrifugation, RNA was precipitated from the aqueous layer by adding an equal volume of isopropanol and the mixture was kept at −20°C for 1 h and then centrifuged to sediment the RNA. The RNA pellet was washed with 75% ethanol to remove any traces of guanidinium. The final pellet was dried and resuspended in diethyl pyrocarbone water, and the amount of RNA was determined using a spectrophotometer at 260 nm. DNA contamination in the RNA preparation was removed by treating the RNA preparation with DNase (1 IU/μg RNA) for 2 h at 37°C, followed by proteinase K digestion at 37°C for 15 min and subsequent extraction with phenol/chloroform and NH4OAc/EtOH precipitation. The DNA contamination in the RNA preparation was checked by including a control in which reverse transcriptase enzyme was not added in the PCR amplification procedure. RNA preparation, which is devoid of any DNA contamination, was used in the subsequent experiments in our semiquantitative PCR. The isolated RNA was stored at −70°C until used.

Real-time quantitative RT-PCR

DC-SIGN and DC-SIGNR gene expression were quantitated using real-time PCR. Relative abundance of each mRNA species was assessed using the SYBR green master mix from Stratagene to perform real-time semi-quantitative PCR using the ABI Prism 5700 instrument that detects and plots the increase in fluorescence vs PCR cycle number to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by integrating a fluorescence threshold above background and determining the time point at which each the amplification plot of each sample reaches the threshold (defined as the threshold cycle number (C_{T})). Differences in C_{T} are used to calculate the relative amount of PCR target contained within each tube (29). Relative mRNA species expression was quantitated and expressed as transcript accumulation index (TAI = 2^{-ΔΔC_{T}}), calculated using the comparative C_{T} method. All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, β-actin. In addition, results on RNA from treated samples were normalized to results obtained on RNA from the control, untreated sample.

FACS analysis

Flow cytometry using FACS Calibur instrument (BD Biosciences) was used to identify and quantify DC-SIGN expressing DC. PE conjugated mAbs against DC-SIGN and a appropriately matched isotype control were obtained from BD Pharmingen. Stained cells were subjected to light scatter analysis, and a fixed population of cells was gated after quadrant markers were set, based on the isotype control. Cells positive for DC-SIGN were expressed as a percentage of the total cells gated.

Western blot

DC were cultured with either 10^{-10} M cocaine or gp120 (10 ng) or heat-inactivated gp120 (10 ng) for 48–72 h, and protein was extracted and analyzed by Western blot (30, 31) using Abs specific for DC-SIGN, p38, and ERK MAPKs. The anti-DC-SIGN Ab was an affinity purified, anti-human mAb was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (DC4; catalog no. 5442). The anti-p38 (BD Biosciences; catalog no. 612168) and anti-ERK (BD Biosciences; catalog no. 610103) mAbs were used as recommended by the manufacturer.

Results

Cocaine enhances HIV-1 replication in MDC

Data presented in Fig. 1A show basal levels of expression of different costimulatory molecules on both IDC and MDC as quantitated by flow cytometry. CD40, CD80, and CD86 are expressed at higher levels on MDC compared with IDC, while CD83, a marker for MDC, is expressed significantly greater on MDC (p < 0.0001) but not on IDC consistent with previous reports (32).

Data presented in Fig. 1B show the dose response and kinetics of the effects of cocaine on DC-SIGN gene expression by IDC and MDC. Both IDC and MDC were cultured for 24–72 h with different concentrations (10^{-10}–10^{-12} M) of cocaine. RNA was extracted and reverse transcribed, and cDNA was amplified by PCR using primers specific for the housekeeping gene, β-actin, and DC-SIGN using real-time quantitative PCR (Table I). Results show that cocaine significantly up-regulates the gene expression of DC-SIGN by IDC and MDC in a dose-dependent manner compared with unstimulated controls (p < 0.001).
A, Constitutive/basal expression of costimulatory molecules on MDC and IDC as measured by FACS analysis. Statistical significance was determined by Student’s t test (n = 3). B, Kinetics of the effect of cocaine on the DC-SIGN gene expression by MDC and IDC. MDC/IDC (5 x 10^5 cells/ml) were cultured with and without cocaine (10^{-9}-10^{-12} M) for 24, 48, and 72 h; RNA was extracted and reverse transcribed followed by a quantitative real-time PCR against DC-SIGN primers. Statistical significance was calculated by Student’s t test (n = 3). C, Effect of cocaine (10^{-9}-10^{-12} M) on DC-SIGN expression by MDC as measured by FACS. Statistical significance was determined by Student’s t test (n = 3). D, Effect of cocaine on HIV replication. MDC (1 x 10^5 cells/ml) were infected with HIV-1 ASM-54 at concentration of 10^{5.7} TCID_{50}/0.2 ml and cultured with or without cocaine (10^{-6} and 10^{-8} M) for 48 h. RNA was extracted and reverse transcribed followed by quantitative real-time PCR using primers specific for the LTR region of the HIV genome. Statistical significance was calculated by Student’s t test (n = 3). E, Effect of cocaine on HIV replication in MDC. MDC (1 x 10^5 cells/ml) were infected with HIV-1 Ba-L at concentration of 10^{3.75} TCID_{50}/1.0 ml. After 3 h of incubation at 37°C, MDC were washed and cultured with or without cocaine (10^{-6} M) for 15 days. The culture supernatants were quantitated for p24 Ag using Retrotek, HIV-1 p24 ELISA from ZeptoMetrix (catalog no. 0801111) with a minimum detection range of 1 pg of p24 Ag per milliliter. The values presented are mean ± SD of three independent experiments. Statistical significance was calculated by Student’s t test (n = 3).
with the untreated control culture at 24, 48, and 72 h. However, the effect of cocaine on DC-SIGN gene expression was not significantly different at all the time points studied, for both IDC and MDC, although a slight increase in DC-SIGN expression was observed at 48 h by IDC.

Fig. 1C shows the effect of cocaine on DC-SIGN expression by MDC as analyzed by flow cytometry using PE labeled DC-SIGN-specific Ab. These results demonstrate that cocaine at 10⁻⁶, 10⁻⁸, 10⁻¹⁰, and 10⁻¹² M significantly up-regulates the percentage of DC-SIGN-positive cells: 10.3% (p < 0.0001), 7.4% (p < 0.01), 7.1% (p < 0.01), and 6.4% (p < 0.05), respectively, compared with the untreated control culture (4.7%). These results are consistent with the effect of cocaine on the gene expression of DC-SIGN by MDC (Fig. 1B).

Data presented in Fig. 1D show the stimulatory effect of cocaine on HIV replication in MDC. MDC were infected with a dual tropic HIV-1 strain (ASM-54; National Institutes of Health reagent catalog no. 2794) and treated with or without cocaine. The HIV-LTR-R/U5 region was amplified by real-time quantitative PCR using primers specific for a 180-bp fragment of the region as described (32, 33). This method is designed to detect early stages of reverse transcription of HIV. Our results demonstrate that cocaine at 10⁻⁶ (TAI = 1.25, p < 0.05) and 10⁻⁸ M (TAI = 1.53, p < 0.01) significantly up-regulated the HIV-LTR-R/U5 region compared with the untreated HIV-infected control culture (TAI = 1.0). Data presented in Fig. 1E show the levels of p24 Ag in the culture supernatants of MDC infected with HIV-1Ba-L, in the presence or absence of cocaine (10⁻⁶ M). MDC infected with HIV-1Ba-L, in the absence of cocaine produced 20.3 pg/ml p24 Ag, whereas MDC infected with HIV-1Ba-L, in the presence of cocaine significantly up-regulated the production of p24 Ag (46.7 pg/ml, p < 0.012). Although MDC showed only low level of infection with HIVBa-L, which is consistent with other studies (34, 35), the production of p24 Ag was significantly up-regulated by cocaine and thus supports the LTR amplification as presented in Fig. 1D. This suggests that cocaine can enhance HIV LTR-R/U5 transcript expression as well as p24 Ag production by infected MDC.

Cocaine increases DC-SIGN and DC-SIGNR gene expression by HIV-infected MDC

Data presented in Fig. 2A show the effect of treatment of HIV-1 (dual tropic strain, ASM-54; National Institutes of Health reagent catalog no. 2794)-infected MDC in vitro with different concentrations of cocaine on the gene expression of DC-SIGN and DC-SIGNR as analyzed by real-time quantitative PCR. These results demonstrate that cocaine at 10⁻⁶ (TAI = 2.35, p < 0.001) and 10⁻⁸ M (TAI = 1.4, p < 0.05) significantly up-regulate DC-SIGN gene expression compared with the untreated HIV-infected control culture (TAI = 1.0). Expression of DC-SIGNR was up-regulated by a lower concentration of cocaine (10⁻⁸ M) (TAI = 2.66, p < 0.0001) compared with the control culture (TAI = 1.0).

Cocaine use increases DC-SIGN expression by MDC from HIV-1-infected subjects

Because our in vitro studies show that cocaine up-regulates DC-SIGN expression by both IDC and MDC, we examined the effects of cocaine use in vivo on the phenotypic expression of DC-SIGN by MDC from HIV-infected subjects who were using and not using cocaine. Patients were stratified according to whether they were NP or LT-NP of HIV-1 infection and whether they were currently using or not using cocaine. Results from patients were compared with HIV-1-uninfected cocaine users (non-HIV-drug user (DU)) and normal control subjects. The results show (Fig. 2B) that HIV-seronegative, cocaine-using patients have an increased percentage of DC-SIGN-positive MDC (1.23%, p < 0.05) compared with HIV-negative, cocaine-nonusing normal controls (0.475%). Additionally, NP (who develop disease symptoms within 5 years of HIV-1 infection) demonstrated a significantly higher percentage of DC-SIGN-positive cells (5.21%) compared with LT-NP (1.82%, p < 0.05) who remain symptom free for >5 years after HIV infection. However, NP who are currently using cocaine (NP-DU), demonstrated a significantly higher percentage of DC-SIGN-positive MDC (7.77%, p < 0.001) compared with NP (5.21%) who are currently not using cocaine. Similarly, LT-NP who are currently using cocaine (LTNP-DU), also showed a significantly increased percentage of DC-SIGN-positive MDC (3.34%, p < 0.01) compared with LT-NP who are not using cocaine (1.82%, p < 0.05).

Data presented in Fig. 2C show the results of DC-SIGN gene expression by MDC from normal, non-HIV-1-infected cocaine-using subjects (non-HIV-DU), NP, and LT-NP of HIV-1 infection who are currently using and not using cocaine as determined by quantitative real-time PCR. Our results demonstrate an increase in DC-SIGN gene expression in MDC from HIV-seronegative, drug-using subjects (TAI = 1.93, p < 0.0001) (non-HIV-DU) compared with normal controls (TAI = 1.00). DC-SIGN gene expression was significantly up-regulated in NP-DU who are currently using cocaine (TAI = 2.02, p < 0.012), compared with cocaine-nonusing NP (TAI = 1.904). Similarly, LTNP-DU also showed significant up-regulation of DC-SIGN gene expression (TAI = 1.97, p < 0.007) compared with cocaine-nonusing LT-NP (TAI = 1.74). NP (who develop disease symptoms within 5 years of HIV-1 infection) demonstrated a significantly higher gene expression of DC-SIGN (TAI = 1.904, p < 0.03) compared with LT-NP (TAI = 1.74).

Table I. Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR Product Size (bp)</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>548</td>
<td>5’-5’-TGACCGGGGTTCACCCACACTGTGCCCACATCTA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’-5’-AGTCGATGGCCGCTTAGAAGACTTTGCGGT-3’</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>205</td>
<td>5’-5’-GAAGACTTGCCGGAATTTAGAC-3’</td>
</tr>
<tr>
<td>DC-SIGNR</td>
<td>360</td>
<td>3’-5’-TCAAGGATGGAGAGAAGGAGA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-5’-GAAGAAACCTGGTCTCCCTTG-3’</td>
</tr>
<tr>
<td>P38 MAPK</td>
<td>560</td>
<td>5’-5’-GTCAGCGTCTGACGTTGGGAC3’</td>
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<tr>
<td>INK</td>
<td>407</td>
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<tr>
<td>ERK</td>
<td>186</td>
<td>3’-5’-CCATCAAATCTTCAGGAG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-5’-CATTCACTGACCCATTC-3’</td>
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These real-time quantitative PCR data confirm the flow cytometry data and demonstrate that cocaine use increases the percentage of DC-SIGN-positive DC as well as DC-SIGN gene expression in HIV-infected subjects and these cells likely play a significant role in the progression of HIV disease.

**Synergistic effects of cocaine and HIV proteins gp120 or tat on DC-SIGN gene expression by MDC**

Because MDC from cocaine-using HIV-infected subjects show high expression of DC-SIGN we investigated the in vitro effects of cocaine with and without HIV proteins, gp120, and tat on the gene expression of DC-SIGN by normal MDC using real-time quantitative PCR. The data presented in Fig. 3A show that MDC treated with HIV gp120 protein at 10 (TAI = 1.27, p < 0.006), 50 (TAI = 1.29, p < 0.007), and 100 (TAI = 1.33, p < 0.006) ng/ml significantly up-regulated DC-SIGN gene expression as compared with untreated control cultures (TAI = 1.00).

The data presented in Fig. 3B show that MDC treated with HIV tat protein at 10 (TAI = 1.62, p < 0.001) and 50 (TAI = 1.31, p < 0.014) ng/ml significantly up-regulated the gene expression of DC-SIGN compared with untreated control cultures (TAI = 1.00). However, tat at a higher concentration, 100 ng/ml, did not produce any significant effect (TAI = 1.11, p = 0.17) on the gene expression of DC-SIGN and was similar to the control culture (TAI = 1.00).

Data presented in Fig. 3C show the Western blot analysis of DC-SIGN protein from MDC treated with either cocaine or gp120 or heat-inactivated gp120. The results show that cocaine and gp120 significantly up-regulate DC-SIGN protein expression by MDC compared with untreated or heat-inactivated gp120 treated control cultures. These data confirm our FACS analyses and real-time quantitative PCR results, demonstrating that cocaine and gp120 up-regulate DC-SIGN expression by MDC.

The data presented in Fig. 3D show a synergistic effect of cocaine plus tat on DC-SIGN gene expression by MDC. Normal MDC were cultured alone with either cocaine or tat, or cocaine plus tat. RNA was extracted, reverse transcribed, and real-time quantitative PCR amplified using DC-SIGN primers. Our results show that MDC treated with HIV-1 tat at 10 ng/ml (Fig 3C, lane 3, TAI = 1.21, p < 0.046) or cocaine at 10^{-12} M (lane 4, TAI = 1.39, p < 0.007) significantly up-regulated DC-SIGN gene expression compared with the untreated control culture (lane 1, TAI = 1.0) or heat-inactivated tat (lane 2, TAI = 1.09). However, when cocaine (10^{-12} M) and tat (10 ng/ml) were used in combination, DC-SIGN gene expression was significantly up-regulated (TAI = 4.03, p < 0.002) compared with the additive value of DC-SIGN gene expression by cultures treated with cocaine and tat alone (TAI = 2.60). This suggests that tat in synergy with cocaine up-regulates DC-SIGN gene expression and further supports our clinical data (Fig. 2, B and C) showing that MDC from cocaine-using patients express significantly higher levels of DC-SIGN.

**Effect of dopamine receptor antagonists on cocaine induced DC-SIGN expression by MDC**

The following experiment was undertaken to determine whether the effects of cocaine on DC-SIGN expression were mediated via the dopamine D1 receptor. MDC were cultured separately with cocaine; its metabolite, benzoylecgonine (BE; Sigma-Aldrich; catalog no. B = 8900); the potent dopamine D1 receptor antagonist, statistical significance was calculated by Student’s t test (n = 5).
SCH23390 (Tocris; catalog no. 0925); or cocaine plus D1 antagonist BE plus D1 antagonist. At the end of 48 h of incubation, an aliquot of cells was removed for flow cytometry analysis while the remaining cells were used for RNA extraction. Total RNA was reverse transcribed, and cDNA was amplified by quantitative real-time PCR using specific primers for DC-SIGN. Data presented in Fig. 4A show the effect of cocaine, its metabolite BE, and the D1 receptor antagonist SCH23390 on DC-SIGN expression by flow cytometry analyses. MDC incubated with cocaine showed a significant increase (4.5%, p < 0.006) in the number of DC-SIGN-positive cells compared with the untreated control MDC culture (1.3%). MDC incubated with BE also showed significant up-regulation of DC-SIGN-positive cells (lane 4, 4.9%, p < 0.008) compared with the untreated control culture (1.3%). MDC incubated with SCH23390 alone did not produce any significant effect on the number of DC-SIGN-positive cells (1.9%, p = 0.48) compared with the control culture (1.3%). However, when cocaine and SCH23390 were used in combination, the DC-SIGN-positive MDC were significantly down-regulated (1.5%, p < 0.003) compared with the numerical sum of cocaine- and SCH23390-treated cultures (6.4%). Furthermore, when BE and SCH23390 were used in combination the number of DC-SIGN-positive MDC were significantly down-regulated (1.7%, p < 0.005) compared with the numerical sum of BE- and SCH23390-treated cultures (6.8%). Thus, SCH23390 also completely reversed BE induced up-regulation of DC-SIGN-positive cells. These data indicate that cocaine and its metabolite, BE, up-regulate DC-SIGN expression through the classical dopamine D1 receptor.

Data presented in Fig. 4B show that MDC treated with cocaine (10⁻⁶ M) showed significant up-regulation of DC-SIGN-specific gene expression compared with the control culture (1.3%). Tat (10 ng and 50 ng/ml) treatment significantly up-regulated the gene expression of DC-SIGN compared with untreated control culture (lane 1). Furthermore, when BE and SCH23390 were used in combination the number of DC-SIGN-positive MDC were significantly down-regulated (1.7%, p < 0.005) compared with the numerical sum of BE- and SCH23390-treated cultures (6.8%). Thus, SCH23390 also completely reversed BE induced up-regulation of DC-SIGN-positive cells. These data indicate that cocaine and its metabolite, BE, up-regulate DC-SIGN expression through the classical dopamine D1 receptor.
mRNA (lane 2, TAI = 4.3, p < 0.001) as quantitated by real-time PCR compared with the untreated control culture (lane 1; TAI = 1.0). Similarly, MDC incubated with BE (10^{-6} M) showed significant up-regulation of DC-SIGN-specific mRNA expression (lane 3; TAI = 3.5, p < 0.003) compared with the control culture (TAI = 1.0). MDC incubated with SCH23390 alone did not show any significant effect on DC-SIGN-specific mRNA expression (lane 4; TAI = 1.08, p = 0.39). However, the D1 antagonist completely reversed the cocaine-induced up-regulation of DC-SIGN-specific mRNA expression (lane 5; TAI = 0.89, p < 0.001) compared with cultures treated with cocaine alone (TAI = 4.3).

Similarly, the SCH23390 (10^{-5} M) completely reversed BE-induced up-regulation of DC-SIGN gene expression (lane 6; TAI = 0.90, p < 0.001) compared with cultures treated with BE alone (TAI = 3.5). These data confirm our flow cytometry analyses (Fig. 4A) and indicate that up-regulation of DC-SIGN expression by cocaine or BE is mediated via the dopamine D1 receptor.

Cocaine inhibits signaling molecules of the MAPK pathway

Signal transduction via MAPKs plays a significant role in cellular immune responses. The best-characterized subfamilies of the MAPK superfamily are the ERKs and the two “stress-responsive”
MAPK subfamilies, namely, the JNKs and the p38-MAPKs. We examined the hypothesis that the mechanism of cocaine induced dysregulation of DC-SIGN is mediated through MAPK pathways. MDC (5 x 10^5 cells/ml) were cultured with cocaine (10^{-10} M) for 48 h, and RNA were extracted and reverse transcribed followed by quantitative real-time PCR using p38-, ERK-, and JNK-MAPK-specific primers. Our results show in MDC, cocaine (10^{-10} M) significantly down-regulated the gene expression of p38 (TAI = 0.15, p < 0.001) and JNK-MAPK (TAI = 0.54, p < 0.003) compared with the untreated control culture (TAI = 1.0). However, cocaine (10^{-10} M) significantly up-regulated ERK expression (TAI = 1.29, p < 0.01) in MDC, suggesting that cocaine-induced defects in DC activities may be mediated via suppression of p38 and JNK MAPKs while reciprocally up-regulating ERK MAPKs (Fig. 4C). Data presented in Fig. 4, D and E, show the Western blot analyses for p38 and ERK MAPKs, respectively, of MDC treated with cocaine (10^{-10} M, 48 h). The results (Fig. 4D) show that cocaine significantly down-regulated p38 MAPK (24.6%, p < 0.01) compared with untreated control culture. However, MDC treated with cocaine (10^{-10} M, 48 h) (Fig. 4E) significantly up-regulated ERK MAPKs (47.1%, p < 0.01) compared with control culture. These data confirm our real-time PCR data (Fig. 4C) demonstrating that cocaine differentially modulates MAPKs.

Discussion

The first step in HIV infection is attachment of virus to susceptible target cells. It is known that several proteins play major roles in this process. The recent discovery of DC-SIGN, also called CD209 has been shown to be the first molecule that facilitates HIV infection independent of CD4 or viral coreceptors (8, 21, 22). DC-SIGN affinity for gp120 may exceed that of CD4 (10). After binding of HIV gp120 to DC-SIGN, the virus is not internalized and can promote infection of neighboring T cells (7, 9, 10). DC-SIGN has thus become a molecule of great interest to HIV researchers because it mediates the infection of HIV to permissive cell types, thereby mediating infection with a high efficiency. A homologue of DC-SIGN, DC-SIGNR, also has been described as an efficient attachment factor for HIV (3, 9). DC-SIGN-bound HIV-1 remains infectious over a long period during its transport from the periphery to lymphoid organs. Because DC-SIGN facilitates virus attachment to DC, independent of CD4 and HIV coreceptors, and drug-resistant HIV-infected subjects show increased progression of HIV disease (36, 37), we examined whether drugs of abuse such as cocaine can enhance DC-SIGN expression.

The current study demonstrates that cocaine up-regulates DC-SIGN and DC-SIGNR expression by normal IDC and MDC. We also show that cocaine up-regulates the gene expression of DC-SIGN and DC-SIGNR by HIV-1-infected MDC in vitro (Fig. 2A). Moreover, we found that cocaine use increased the number of DC-SIGN-positive DC in HIV-1-infected subjects (Fig. 2B). Our results also show that DC-SIGN and its homologue DC-SIGNR respond to cocaine in a similar fashion, although a higher concentration of cocaine (10^{-6} M) was required to produce maximum up-regulation of DC-SIGN compared with DC-SIGNR (10^{-8} M). Similar to MDC, IDC also responded to cocaine at a higher concentration (10^{-8} M) to produce maximum DC-SIGN expression as quantitated by flow cytometry (data not shown).

To rule out the possibility that the modulatory effects induced by cocaine were nonspecific, we examined whether cocaine at the concentrations used in our experiments could activate apoptosis or cause direct cell death. MDC were cultured with cocaine at concentrations of 10^{-6} and 10^{-8} M and analyzed for annexin V expression by flow cytometry to measure apoptosis, as well as trypan blue dye exclusion analyses to quantitate cell death. Our results show that cocaine at 10^{-6} and 10^{-8} M did not induce apoptosis of MDC as evidenced by no increase in annexin V-positive cells (17 and 14.9%, respectively) compared with the untreated control culture (16.3%). Furthermore, the number of viable cells measured by trypan blue exclusion assay was similar in cocaine treated and untreated cultures (data not shown). Furthermore, in our real-time PCR assay equal amounts of RNA were used from both treated and untreated cultures for amplification of the housekeeping gene β-actin, which was used as an internal control, further reducing the possibility of error in gene expression assays caused by cell death.

Previous studies suggest that cytokines are known to induce differentiation or maturation of DC (38–41) and depending on the type of DC that were under different stages of maturation stages, conflicting results were obtained regarding the replication of HIV in those cells (42), the IDC being the most susceptible for HIV infection. Frank et al. (34), demonstrated that both MDC and IDC pulsed with HIV-1Ba-L were able to produce significant level of virus production as analyzed by p24 Ag assay; however, in DC- T cell cocultures, the virus replication was three times higher than in the DC culture alone. Ganesh et al. (35) recently showed that DC matured with poly(I-C) treatment could be transduced with luciferase expressing HIV-1ADA vector to a significant level although IDC were more readily transduced with luciferase expressing HIV-1ADA vector. These studies suggest that although IDC are a more susceptible target for HIV infection, MDC also show a basal level of infection with HIV and the rate of infection may be controlled by the stages of DC maturation. In our studies, we have shown that MDC show poor infection with HIV as analyzed by both LTR-R/U5 region amplification (Fig. 1D) and p24 Ag assay (Fig. 1E), which is consistent with the above findings and the infection was significantly enhanced by treatment with cocaine. Our in vitro infection model, when MDC were infected with HIV-1 for 3 h and cultured with cocaine for 48 h, showed a significant up-regulation of DC-SIGN and DC-SIGNR gene expression compared with infected MDC cultured without cocaine (Fig. 2A). Our data also show that tat significantly up-regulated DC-SIGN gene expression (Fig. 3B). However, the highest concentration of tat did not significantly up-regulate DC-SIGN gene expression by DC compared with the significant up-regulation of DC-SIGN mediated by the lower concentration of tat. The reason for this lack of a dose-dependent up-regulation of DC-SIGN is not clearly known. The possibility of toxicity induced by high concentration of tat was ruled out because the viability of the cells treated with the higher concentration of tat was comparable to the viability of cells treated with the lower concentration of tat as determined by annexin V labeling by flow cytometry as well as trypan blue dye exclusion analyses (data not shown). Previous studies demonstrated (43) that the uptake of tat by DC is controlled by two pathways that are different for low, (<100 ng) and high (>100 ng/ml) concentrations of tat. Furthermore, studies show that the low concentration of tat is blocked by specific anti-integrin mAb or competitor ligands while uptake of the high concentration of tat was only partly blocked by anti-integrin mAb suggesting a differential concentration effect in the uptake of tat. In our studies, the lack of up-regulation of DC-SIGN by a high concentration of tat may suggest the participation of two different pathways for tat uptake as suggested by earlier investigators (43). Additionally, this lack of a response may be partly attributed to receptor saturation, steric hindrance, feedback regulation, or nonspecific blocking of receptor, which are yet to be studied. Furthermore, a bell-shaped response is produced with most pharmacologic agents when used at higher concentrations and it is possible that tat also produces similar results.
Although at least five dopamine receptor subtypes have been identified (44), through which cocaine or its metabolite, mediate their effects, the precise roles of these receptors remains unclear. To examine whether the effects of cocaine on DC are specifically mediated through dopamine receptors, MDC were treated separately with a D1 receptor antagonist (SCH23390) to attenuate the effects cocaine or its metabolite BE, had on DC-SIGN gene expression. Our results showed that cocaine and BE mediated up-regulation of DC-SIGN were completely reversed by the SCH23390. These studies further indicate that in addition to the proposed role of the D1 receptor in many of the behavioral effects of cocaine, including stimulation of locomotor activity, interoceptive discriminative-stimulus effects and psychotic actions (45), this receptor also may also be involved in regulating DC cell function. MDC derived from different HIV-infected patient cohorts showed significantly increased percentage of circulating DC-SIGN-positive cells compared with healthy normal controls. It is interesting to note that HIV-uninfected cocaine-using patients also showed significantly increased percentage of DC-SIGN-positive cells compared with non-drug-using control subjects, consistent with our premise that cocaine induces DC-SIGN expression on DC. NP who are using cocaine also showed significantly increased percentage of DC-SIGN-positive cells compared with NP who are not using cocaine. Similarly, LT-NP who are using cocaine also showed increased percentage of DC-SIGN-positive MDC compared with LT-NP who are not using cocaine. This demonstrates that cocaine in synergy with HIV further enhances the gene and protein expression of DC-SIGN on DC. In our in vitro model system, MDC cultured with cocaine plus HIV-1 tat protein showed significant up-regulation of DC-SIGN. This shows that cocaine in synergy with HIV tat protein up-regulate DC-SIGN expression by MDC compared with cocaine alone or HIV tat alone treated cultures. These results further support the premise that HIV-1-infected individuals who are injecting DU (IDUs) may undergo an accelerated rate of HIV disease progression by up-regulating the MDC compared with cocaine alone or HIV tat alone treated cultures. This demonstrates that cocaine induces DC-SIGN expression on DC in HIV-infected patient cohorts who are not using cocaine. Similarly, LT-NP who are using cocaine also showed increased percentage of DC-SIGN-positive cells compared with NP who are not using cocaine. This demonstrates that cocaine in synergy with HIV further enhances the gene and protein expression of DC-SIGN on DC. In our in vitro model system, MDC cultured with cocaine plus HIV-1 tat protein showed significant up-regulation of DC-SIGN. This shows that cocaine in synergy with HIV tat protein up-regulate DC-SIGN expression by MDC compared with cocaine alone or HIV tat alone treated cultures. These results further support the premise that HIV-1-infected individuals who are injecting DU (IDUs) may undergo an accelerated rate of HIV disease progression by up-regulating the CD4 independent virus attachment factor, DC-SIGN. Furthermore, our data support that the mechanism by which cocaine induces dysregulation of DC-SIGN may be mediated by differentially regulating MAPKs. Together these data represent the first evidence of cocaine induced up-regulation of DC-SIGN. A better understanding of this receptor on DC may help to design novel therapeutic strategies against HIV disease progression in the drug-using population.

Disclosures
The authors have no financial conflict of interest.

References


