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Advanced Glycation End Products Inhibit Both Infection and Transmission In Trans of HIV-1 from Monocyte-Derived Dendritic Cells to Autologous T Cells

Nadine Nasreddine,*† Chloé Borde,‡†§,‡ Joël Gozlan,†‡§,‖ Laurent Bélec,* Vincent Maréchal,‡†§,‡ and Hakim Hocini*‡

Highly active antiretroviral therapy is associated with carbohydrate metabolic alterations that may lead to diabetes. One consequence of hyperglycemia is the formation of advanced glycation end products (AGEs) that are involved in diabetes complications. We investigated the impact of AGEs on the infection of monocyte-derived dendritic cells (MDDCs) by HIV-1 and the ability of MDDCs to transmit the virus to T cells. We showed that AGEs could inhibit infection of MDDCs with primary R5-tropic HIV-1 Ba-L by up to 85 ± 9.2% and with primary X4-tropic HIV-1 Vl8 variants by up to 60 ± 8.5%. This inhibitory effect of AGEs was not prevented by a neutralizing anti-receptor for advanced glycation end products (anti-RAGE) Ab, demonstrating a RAGE-independent mechanism. Moreover, AGEs inhibited by 70–80% the transmission in trans of the virus to CD4 T cells. Despite the inhibitory effect of AGEs on both MDDC infection and virus transmission in trans, no inhibition of virus attachment to cell membrane was observed, confirming that attachment and transmission of the virus involves independent mechanisms. The inhibitory effect of AGEs on infection was associated with a RAGE-independent downregulation of CD4 at the cell membrane and by a RAGE-dependent repression of the CXCR4 and CCR5 HIV-1 receptors. AGEs induce the secretion of proinflammatory cytokines IL-6, TNF-α, and IL-12, but not RANTES or MIP-1α, and did not lead to MDDC maturation as demonstrated by the lack of expression of the CD83 molecule. Taken together, our results suggest that AGEs can play an inhibiting role in HIV-1 infection in patients who accumulate circulating AGEs, including patients treated with protease inhibitors that developed diabetes. *The Journal of Immunology, 2011, 186: 000–000.

According to the International Diabetes Federation, diabetes currently affects 246 million people worldwide, and it is expected to affect 380 million by 2025. Importantly, the largest increases in diabetes prevalence are expected in developing countries. This epidemic has some common traits with the HIV pandemic. The Joint United Nations Program on HIV/AIDS 2008 report on the global AIDS epidemic indicated that the number of people living with HIV-1 increased to between 30 million and 36 million in 2007, sub-Saharan Africa being the most heavily affected region. Whereas there is now evidence that antiretroviral treatment may be associated with diabetes and insulin resistance, the question of the impact of diabetes on HIV-1 progression—notably in untreated patients—has not yet been addressed. Nonetheless, there are many reasons to raise the question of a possible interplay between diabetes and HIV-1 infection.

Combined antiretroviral therapy dramatically improved the outcome of HIV-1–infected patients, but the chronic administration of these drugs may induce metabolic disorders including dyslipidemia, changes in body fat repartition (1, 2), and insulin resistance ties in carbohydrate metabolism (3–6). Indeed, from 23% to up to 40% of patients treated with protease inhibitors developed intolerance to glucose and hyperglycemia after 1 or 2 y of therapy (7, 8). Factors other than protease inhibitor exposure may account for abnormalities in carbohydrate metabolism during HIV-1 infection. These include adipocyte toxicities induced by other antiretroviral drugs such as reverse transcriptase inhibitors and, just as importantly, other environmental factors, aging, immune restoration, and/or possibly HIV-1 itself (9). Whatever the mechanism(s) involved, these perturbations result in the formation and accumulation both in blood and tissues of advanced glycation end product (AGE) molecules (10, 11), a group of heterogeneous complex molecules produced by the nonenzymatic glycation between aldoses and free amino groups on proteins (12), DNA (13), or lipids (14). Elevated levels of circulating AGEs have been detected in patients with type 1 (15) and type 2 (16) diabetes, including patients infected with HIV-1 (17, 18). Notably, AGEs have also been detected in patients with AIDS even in the absence of carbohydrate metabolism alteration (19).

The biological properties of AGEs have been associated with their ability to interact with several membrane receptors including the receptor for advanced glycation end products (RAGE), the type A macrophage scavenger receptor (20), galectin-3 (21), scavenger

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Abbreviations used in this article: AGE, advanced glycation end product; AGE-BSA, glycated BSA; AU, arbitrary unit; CHX, cycloheximide; DC, dendritic cell; HMGB1, high mobility group box 1; LTR, long terminal repeat; MDDC, monocyte-derived dendritic cell; MFI, mean fluorescence intensity; RAGE, receptor for advanced glycation end products.

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receptors class B type 1 (22, 23), AGE receptor 1 (24–26), and polypeptides potentially present on the cell surface (AGE–receptor complex) (21). The best-characterized receptor for AGEs, RAGE, is expressed on many cells that are targeted by HIV-1, such as monocytes and macrophages (27, 28), CD4 T cells, and dendritic cells (DCs) (29–31). In monocytes and DCs, RAGE activation leads to an increase in proinflammatory cytokines such as IL-1β or TNF-β (32), IFN-γ, and several chemokines and growth factors (31, 33–35). Considering the contribution of these factors to HIV-1 infection, AGE–RAGE interactions are likely to impact the biology of HIV-1 infection in many ways. In the current study, we provide evidence that monocye-derived dendritic cells (MDDCs) exposed to glycated BSA (AGE-BSA) are less sensitive to HIV-1 infection when exposed to both R5-tropic and X4-tropic strains. In addition, we have demonstrated that MDDCs treated by AGEs are also less efficient in transmitting HIV-1 to autologous CD4 T cells. Altogether, these results suggest a role for AGEs in the pathophysiology and transmission of HIV-1 infection in patients who accumulate these molecules in their tissues.

Materials and Methods
Ags and reagents
The PE-conjugated anti-CD4, anti-CCR5, and anti-CXCR4, the FITC-conjugated anti-CD1a and anti-CD83, and the neutralizing Abs to RAGE were from R&D Systems Europe (Abingdon, Oxon, U.K.). The anti-CXCR4–allophycocyanin, anti-mouse–FITC, and FITC–, allophycocyanin–, and PE-conjugated IgG1 were from Immunotech (Marseille, France) and Becton Dickinson (Le Pont de Claix, France), RPMI 1640 (with l-glutamine) was provided by Cambrex (Verviers, Belgium), and penicillin and streptomycin were provided by Invitrogen (Paisley, U.K.). Medium for lymphocytes and FCSs were purchased from Innogenetics (Gent, Belgium).

Preparation and characterization of AGE-BSA
AGE-BSA was prepared as described elsewhere (31) with minor modifications. BSA (Sigma–Aldrich) was added at a concentration of 5 g/l in PBS at pH 7.3 containing 1 M d-glucose. The reaction mixture was incubated at 37°C for 12 wk in the dark. Unincorporated glucose molecules were removed by several dialyses in PBS. The solution was treated with Triton X-114 to remove residual LPS as described (36). LPS concentrations were below the level of detection as verified with an E-toxate assay (Sigma–Aldrich).

The fraction of modified carboxymethyl lysine residues was measured by means of the 2,4,6-trinitrobenzenesulfonic acid method (37) that estimates the proportion of unmodified lysine in an AGE-BSA preparation compared with that of the BSA. By this method, we showed that the extent of lysine modification in our preparation of AGE-BSA was 82%.

HIV-1 strains
The primary X4-tropic HIV-1_Fv44 and the primary R5-tropic HIV-1_Ba-L were provided by Dr. E. Menue and Prof. F. Barre–Sinoussi (Institut Pasteur, Paris, France). Viral stocks were produced in HEK 293T cells (Eurogentec SA, Seraing, Belgium). The LC-PCR master mix contained 9 mM MgCl2, and 0.3 μM of each primer and probe. Cycling conditions were as follows: initial denaturation/FastStart Taq DNA polymerase amplification of 5 μM of each primer and probe. Cycling conditions were as follows: initial denaturation/FastStart Taq DNA polymerase amplification of 5 μM of each primer and probe.

Preparation of immature MDDCs
PBMCs were isolated from buffy coats of healthy adult donors by ficoll density gradient centrifugations. Contaminating lymphocytes were removed from PBMCs (107 cells) by incubation with 2-(2-aminoethyl)iso-thiourea dihydrobromide (Sigma–Aldrich) activated sheep erythrocytes for 20 min at 4°C. Cells were then washed with 4-well plates (Costar, Cambridge, MA) at a concentration of 1 × 106 monocytes/ml and incubated at 37°C for 45 min. Nonadherent cells were removed by four washes. Adherent monocytes were cultured for 6 d in RPMI 1640 medium with 10% FCS, 1% glutamine, and antibiotics in the presence of a combination of rhGM-CSF and IL-4 (both used at 10 ng/ml) to promote differentiation into DCs.

Medium with cytokines was changed every 48 h. After 6 d of culture, adherent cells corresponding with the enriched fraction of MDDCs were washed, then incubated with 50 mg/ml BSA or AGE-BSA for 24 or 48 h. Then, surface markers were analyzed by flow cytometry. For MDDC maturation, cells (106 cells per well) were incubated in the presence of 1 μg/ml LPS at 37°C for an additional 48 h.

To recover the autologous lymphocytes from PBMCs, sheep erythrocytes were lysed by incubation with ammonium chloride at 0.15 M for 2 min at 4°C. After several washings, lymphocytes were cultured in RPMI 1640 supplemented with 10% FCS, 1% antibiotics, PHA (2.5 μg/ml), and IL-2 (10 ng/ml) for 48 h. Then cells were washed and cultured for an additional 24 h in the presence of IL-2.

Cytotoxicity assay
The cytotoxicity of AGE-BSA on DCs was analyzed using the MTT assay (Sigma–Aldrich). MDDCs seeded into 96-well plates at 2 × 104 cells/well were incubated with AGE-BSA at concentrations ranging from 10 to 100 μg/ml for 48 h at 37°C in a humidified 5% CO2 atmosphere. Then, 110 μM MTT mix (0.5 mg/ml final concentration) was added to each well, and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized for 18 h at 37°C according to the manufacturer’s procedure. The absorbance was measured at 570 nm with a spectrophotometer. The percentage of survival cells was calculated using the following formula: % survival = live cell number (stimulated)/live cell number (unstimulated) × 100.

Quantification of cytokine production in culture supernatants
The concentration of IL-6, TNF-α, and IL-12 in the cell culture supernatants was measured using sandwich immunoassays purchased from AbCys (Paris, France) according to the manufacturer’s procedure. RANTES and MIP-1α were measured using a sandwich ELISA purchased from Peprotech (Paris, France).

HIV-1 infection of MDDCs
MDDCs incubated with BSA or AGE-BSA (50 μg/ml) were washed twice and seeded into 96-well culture plates (2 × 105 cells/well) before being incubated with 0.5 ng/ml HIV-1_Fv44 or HIV-1_Ba-L for 3 h at 37°C. The cells were then extensively washed and maintained in complete medium for 6 d. Supernatants were collected every 3 d, and HIV-1 p24 levels in the culture medium were monitored by capture p24 Ag ELISA.

Quantification of HIV-1 DNA by real-time PCR
At 24 h postinfection, HIV-1 DNA was isolated from MDDCs, stimulated or not with AGE-BSA, using an extraction protocol according to the manufacturer’s recommendations (Qiagen DNA mini kit AG; Qiagen, Basel, Switzerland). HIV-1 DNA was quantified by real-time PCR assay using the LightCycler instrument (Roche Applied Science, Indianapolis, IN). DNA (10 μl) was amplified with the sense primer NEC152 (5′-GCCTCAA-TAAAGGCTGGCACTTGA-3′) and the reverse primer NEC131 (5′-GGGG-CCACCTGCAGAATT-3′) in the presence of a dual (FAM and TAMRA) labeled NCT LTR probe (5′-AAGTAGTGTGTGGCCCGTT-CCGTTGCTGACT-3′). Primers and probe were from Applied Biosystems (Foster City, CA).

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an endogenous reference to normalize the variations in cell number as described previously (38). The normalized value of cell-associated HIV-1 DNA load corresponded with the ratio ([HIV-1 copy number − albumin copy numberstimulated cell]/[HIV-1 copy number − albumin copy numberunstimulated cell]).

**HIV-1 entry in MDDCs**

MDDCs incubated or not with AGE-BSA (50 µg/ml) were washed two times and seeded into 96-well culture plates (2 × 10^5 cells/well). Cells were incubated with 5 ng/ml HIV-1_VN44 or HIV-1_BaL for 1 h at 37°C in a humidified 5% CO₂ atmosphere before being washed. Cells were then treated for 20 min with 1 mg/ml pronase at room temperature and extensively washed to remove residual attached virus particles. HIV-1 RNA was extracted from cells using the automated EasyMag method (BioMérieux) according to the manufacturer’s recommendations. HIV-1 RNA was then reverse transcribed as described (39). The TaqMan PCR targeted a conserved consensus region in the LTR region of the HIV-1 major group. The sequences of the forward and reverse primers were 5'-AAAGCTTGCCCTTGA-3' and 5'-GGGCGCCACGTGCTAGAGATT-3', respectively. The internal HIV-1 TaqMan probe LTR was 5’-AAG-TAGTGGTGCGCCCTGCTTGT-3’. This probe carried the 5’ reporter FAM and the 3’ quencher TAMRA (Applied Biosystems, Foster City, CA). All runs were performed in a 20 µl volume containing RNA extract (10 µl), primers (a 500 nM concentration of each), probe (200 nM), 1× PCR buffer, and 1× RT multiscr geb/RNase Inhibitor Mix (TaqMan One-Step RT-PCR Master Mix; Applied Biosystems). Thermocycling conditions were 30 min at 54°C and 5 min at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s.

The HIV-RNA standard was a culture supernatant of an HIV-1_Lai strain containing 2 × 10^6 copies of HIV RNA/ml. For each experiment, one aliquot of this standard was extracted together with the samples and serially diluted to reach concentrations 2 × 10^4, 2 × 10^5, 2 × 10^6, 2 × 10^7, and 2 × 10^8 copies/ml. The number of HIV-RNA copies was normalized toward the number of cells that had been determined by means of estimation of albumin gene by a real-time PCR as described (38).

**HIV-1 adsorption on MDDCs**

MDDCs incubated or not with AGE-BSA (50 µg/ml) were washed two times and seeded into 96-well culture plates (2 × 10^5 cells/well). Then, the cells were incubated with 5 ng/ml HIV-1_VN44 or HIV-1_BaL for 1 h at 37°C in a humidified 5% CO₂ atmosphere. After four washes to remove unattached viral particles, cells were lysed with PBS 1% Triton X-100 and frozen at −70°C. The amount of cell-associated HIV-1 particles was estimated by measuring p24 Ag by capture ELISA.

**HIV-1 transmission in trans from MDDCs to autologous T lymphocytes**

MDDCs incubated or not with AGE-BSA (50 µg/ml) were incubated into 96-well culture plates (2 × 10^5 cells/well) and infected with HIV-1 (0.5 ng p24 Ag) for 1 h at 37°C in a 5% CO₂ atmosphere. Cells were then washed four times, and autologous IL-2–stimulated T cells were added at a 1:5 ratio. Culture supernatants were harvested after 3 d, lysed with 1% Triton X-100, and frozen at −20°C. The viral production by T lymphocytes was evaluated after 3 d of the coculture by measurement of HIV-1 p24 Ag in supernatants using a capture ELISA.

**Statistical analysis**

Measurements were expressed as the mean and SD. An unpaired Student t test or alternate Welch t test was used to determine the statistical significance of the data, and a p value ≤0.05 was considered as the level of statistical significance.

**Results**

*AGEs do not induce toxic effects on MDDCs*

According to the studies and methods used, the concentrations of AGEs in the serum of diabetic patients ranged from 4 to up to 80 µg/ml (10, 16). Therefore, comparable concentrations of AGE-BSA were used in our experiments. We first evaluated the viability of MDDCs in the presence of increasing concentrations (from 10 to 100 µg/ml) of AGE-BSA or BSA for a period of 48 h. Cell viability was unaffected for AGE-BSA concentrations as high as 50 µg/ml and fell slightly to 85% for the highest tested concentration (100 µg/ml). Similarly, significant cell toxicity was observed for incubation periods of 96 h or more. Thus, MDDC stimulations were performed in all experiments with 50 µg/ml AGE-BSA added for 48 h, as it appeared to be both relevant and nontoxic for the cells.

**FIGURE 2.** Effect of AGEs on the production of proinflammatory cytokines by MDDCs. MDDCs were stimulated with AGE-BSA (50 µg/ml), BSA, or LPS for 48 h. The secretion of TNF-α, IL-6, and IL-12 was quantified by ELISA in culture supernatants. Results are expressed as mean ± SD calculated from three independent experiments.

**FIGURE 1.** Effect of AGEs on morphologic characteristics of MDDCs and on CD83 and RAGE expression. MDDCs were stimulated with AGE-BSA (50 µg/ml), BSA, or LPS for 48 h. A, Cells were photographed through an Olympus CK2 (CRI, Boston, MA). Original magnification ×200. B, FACS analysis of CD83 and RAGE expression. Data are expressed as the percentage and the MFI of positive cells (in parentheses) for one representative of three independent experiments.
Effect of AGEs on the phenotype of MDDCs and cytokines release

At the time of collection of immature MDDCs, contamination by CD3+ T lymphocytes was <3% as assessed by FACS analysis. Most cells (80%) expressed the immature MDDC main marker CD1a. MDDC phenotype was also CD86+, CD80+, CCR5+, CD4+, CD83low, CD14−, and DC-SIGN+ (data not shown).

We first checked the effect of AGE-BSA on CD1a expression, a major marker of DCs. No modification in the percentage of expression of CD1a molecule was observed upon AGE-BSA exposure (77% for unstimulated compared with 81% and 70% for BSA-stimulated and AGE-BSA–stimulated cells). Morphological observation, however, showed that MDDCs exposed to AGE-BSA formed cellular clusters that were not observed with untreated or BSA-treated cells. Importantly, similar clusters were observed when MDDCs were stimulated by LPS (Fig. 1A). To determine whether AGE-BSA induced the maturation and/or activation of MDDCs, we looked at the membrane expression of CD83, a major marker of DC maturation. Whereas LPS induced, as expected, a strong increase in CD83 expression, CD83 expression was not enhanced by AGE-BSA suggesting that AGE-BSA did not induce the maturation of MDDCs (Fig. 1B). Investigation of expression of RAGE showed that unstimulated MDDCs express a low but significant level of RAGE molecules (20% and mean fluorescence intensity [MFI] of 7). The RAGE expression was not affected after stimulation with 50 μg/ml AGE-BSA (19% positive cells, MFI of 9) (Fig. 1B).

Activation and/or maturation of MDDCs is often associated with the release of various cytokines. Compared with the cells incubated with BSA, MDDCs incubated with AGE-BSA produced significantly higher amounts of TNF-α (28 ± 4 versus 1155 ± 29 pg/ml; p ≤ 0.034), IL-6 (9 ± 2 versus 145 ± 54 pg/ml; p ≤ 0.033), and IL-12 (165 ± 56 versus 502 ± 192 pg/ml; p ≤ 0.043) (Fig. 2). Altogether, these results demonstrated that AGE-BSA promoted the secretion of proinflammatory cytokines without inducing MDDC maturation.

AGEs inhibit HIV-1 infection of MDDCs

The impact of AGE-BSA on MDDCs was evaluated in the context of HIV-1 multiplication. To this aim we estimated the ability of primary R5-tropic or X4-tropic HIV-1 strains to replicate in MDDCs exposed to AGE-BSA in the presence or absence of neutralizing anti-RAGE Abs. Because MDDCs exposed to LPS have been shown to be resistant to HIV-1 multiplication, LPS-treated MDDCs were used as a positive control (40–43). As expected, LPS largely inhibited viral production after infection of MDDCs with both primary HIV-1VN44 (X4-tropic) and HIV-1Ba-L (R5-tropic) strains (Fig. 3). More surprisingly, virus production was also dramatically reduced when MDDCs were cultivated in the presence of AGE-BSA. Compared with BSA, AGE-BSA stimulation of MDDCs induced up to 60 ± 8.5% inhibition of infection for HIV-1 VN44 (Fig. 3A) and up to 85 ± 9.2% inhibition for HIV-1 Ba-L (Fig. 3B). Incubating the cells with neutralizing anti-RAGE Abs prior to stimulation with AGEs had no effect on their ability to inhibit infection.

AGEs inhibit HIV-1 entry step in MDDCs

To characterize the step at which HIV-1 multiplication was inhibited by AGE-BSA, real-time PCR was used to quantify HIV-1

**FIGURE 3.** Effect of AGEs on HIV-1 infection of MDDCs. MDDCs were stimulated with AGE-BSA (50 μg/ml), BSA, or LPS for 24 h, prior to being infected with primary X4-tropic HIV-1VN44 (A) or R5-tropic HIV-1Ba-L (B). In some conditions, a neutralizing anti-RAGE Ab was added to the cells before AGE stimulation. Viral replication was monitored by measuring HIV-1 p24 Ag in the culture supernatant at day 6. Results are expressed as the mean percentage ± SD of infection inhibition calculated from five independent experiments.
DNA and RNA in BSA-treated or AGE-BSA-treated MDDCs. As shown in Fig. 4A, AGEs-BSA induced a significant decrease in HIV-1 DNA at 24 h postinfection, corresponding with a 49% inhibition (0.51 ± 0.2 versus 1 arbitrary unit [AU] for the control cells) and a 55% inhibition (0.45 ± 0.12 versus 1 AU) for HIV-1 VN44 and HIV-1 Ba-L, respectively.

To measure the putative effect of AGE-BSA on virus entry, MDDCs stimulated or not with AGE-BSA were exposed to both HIV-1 Ba-L and HIV-1 VN-44 for 1 h and extensively washed. The cells were then treated with pronase to remove membrane attached virus particles before the quantification of internalized HIV-1 RNA. As shown in Fig. 4B, the treatment of MDDCs with AGEs-BSA induced a 35% reduction in the level of intracellular HIV-1 RNA for HIV-1 VN44 (0.64 ± 0.1 versus 1 AU for the control cells) and a 60% reduction for HIV-1 Ba-L (0.4 ± 0.02 versus 1 AU). This result demonstrated that AGE-BSA stimulation inhibited an early step of viral entry in MDDCs. Importantly, inhibition of virus entry by AGE-BSA was not associated with an inhibition of virus attachment to cell membrane, as similar amounts of HIV p24 Ag were obtained from virus adsorbed onto nonstimulated or AGE-BSA–stimulated MDDCs (data not shown).

AGEs downregulate CD4, CCR5, and CXCR4 on MDDCs

Our results suggested that HIV-1 infection was blocked at an early step of viral infection, most likely before or during the fusion process. Therefore, we investigated the effects of AGEs on the expression of these molecules at the cell surface. As LPS had been shown to reduce surface expression of CD4 in DCs, it was used in this experiment as a positive control (43). Our experiments confirmed this observation and provided evidence that LPS induced a marked decrease in CCR5 and CXCR4 surface expression as well (Fig. 5). Strikingly, the surface expression of HIV-1 receptor and coreceptors was also significantly lower in MDDCs exposed to AGE-BSA. Indeed, the percentage of CCR5 expression decreased from 71% (MFI of 19) to 3% (MFI of 21), CXCR4 decreased from 94% (MFI of 125) to 38% (MFI of 76), and CD4 expression decreased from 82% (MFI of 35) to 53% (MFI of 22). CD4, CCR5, and CXCR4 down-regulation was reproduced after 24-h and 48-h incubation. For 96-h or longer incubation, cell cytotoxicity increased and did not allow proper analysis of CD4, CCR5, and CXCR4 membrane expression in response to AGEs.

Incubating MDDCs at 4°C for 1 h in the presence of AGEs did not significantly alter the labeling of these cells by anti-CCR5, anti-CXCR4, and anti-CD4 Abs (data not shown), confirming that the observed decrease in labeling corresponds to a true reduced expression of the receptors at the cell membrane rather than a nonspecific inhibition of the Abs binding during the labeling procedure.

CD4 downregulation is RAGE-independent whereas CCR5 and CXCR4 downregulation is RAGE-dependent and requires de novo protein synthesis

RAGE is one of the best-characterized receptors for AGEs although other receptors for AGEs are expressed on DCs. To determine the contribution of RAGE to CD4, CCR5, and CXCR4 downregulation at the cell membrane, MDDCs were incubated with AGEs in the presence of neutralizing RAGE Abs. As shown in Fig. 6, CD4 downregulation was unaffected, whereas CCR5 and CXCR4 downregulation was totally abrogated in the presence of anti-RAGE Abs. Thus, CD4 repression did not depend on RAGE, whereas RAGE was required for CCR5 and CXCR4 downregulation. Because only 20% of the cells expressed RAGE whereas more than 60% of the cells downregulated CCR5 and CXCR4 in response to AGEs, we hypothesized that CCR5 and CXCR4 repression was mediated through a soluble mediator produced by RAGE-expressing cells in response to AGEs. To address this question, we analyzed CCR5 and CXCR4 membrane expression on MDDCs incubated with AGEs in the presence of cycloheximide (CHX), a protein synthesis inhibitor (Fig. 7A). Although CHX treatment had a moderate effect on CXCR4 basal membrane expression by itself, we observed that AGEs could not repress CCR5 and CXCR4 membrane expression in this condition. This demonstrated that AGEs activity was indeed dependent on de novo protein synthesis. Then, we wondered whether the secretion of known ligands of these receptors could be produced by MDDCs in response to AGEs. Most particularly, we measured RANTES and MIP-1α—two well-characterized ligands of CCR5—by ELISA after 24-h and 48-h incubation with AGEs, whereas RANTES and MIP-1α accumulated in the culture medium in response to LPS—which was used as a positive control—we were unable to detect these molecules when MDDCs were exposed to AGEs (Fig. 7B). This led to the conclusion that CCR5 membrane repression was not due to CCR5 internalization after RANTES or MIP-1α secretion.

AGEs inhibit HIV-1 transmission in trans (trans-infection) from MDDCs to autologous CD4 T cells

To evaluate the ability of MDDCs exposed to AGE-BSA to transfer virus particles to autologous T lymphocytes, cells cultured in the presence of AGEs-BSA were incubated with HIV-1 R5-tropic or X4-tropic strains for 1 h. Then, free viral particles were removed by

**FIGURE 4.** Effect of AGEs on HIV-1 entry in MDDCs. A, HIV-1 DNA. At 24 h postinfection, total DNA was extracted from unstimulated or AGE-BSA–stimulated MDDCs, and HIV-1 proviral DNA was quantified by real-time PCR. Results are expressed in arbitrary units as mean ± SD calculated from three independent experiments. B, HIV-1 RNA. Unstimulated or AGE-BSA–stimulated MDDCs were incubated with HIV-1 for 1 h. Cells were washed and treated with pronase to discard membrane attached virus particles. Total cell RNA was then extracted, reverse transcribed, and HIV-1 cDNA was quantified by real-time PCR. Results are expressed as mean ± SD calculated from three independent experiments.
washes, and fresh autologous CD4 T cells were added. The virus released in the supernatant medium after 6 d of coculture was mainly provided from the trans-infection pathway as demonstrated by the very low p24 level detected in the culture medium of infected MDDCs alone. Preincubation of MDDCs with AGE-BSA induced a strong inhibition in the transmission of virus particles to autologous T cells (Fig. 8). Indeed, when compared with BSA-stimulated cells, AGEs stimulation inhibited the trans-infection of CD4 T cells by 82% (4.9 ± 0.3 versus 0.9 ± 0.45 ng/ml p24–HIV-1 Ag) for HIV-1 Ba-L (Fig. 8B) and by 70% (3 ± 0.25 versus 10 ± 0.15 pg/ml p24–HIV-1 Ag) for HIV-1 VN44 (Fig. 8A). This inhibition was not linked to a decrease in DC-SIGN surface expression in AGE-BSA–stimulated MDDCs, as checked by FACS analysis (data not shown).

Discussion

In this study, we demonstrate for the first time to our knowledge the impact of AGE molecules on both HIV-1 infection of MDDCs and transmission of the virus to T cells, one of the major mechanisms of viral dissemination in vivo. The potential consequences of these results must be analyzed with respect to the high incidence of diabetes and/or insulin resistance in the HIV-infected population (44, 45). Furthermore, several studies have already shown the accumulation of circulating AGEs in HIV-infected patients, with or without metabolic syndromes (17–19). Circulating AGEs likely interact with their receptor RAGE in lymphocytes, monocytes/macrophages, and DCs that are also targets and/or reservoirs of HIV-1. Interaction of AGEs with RAGE on macrophages notably increases the secretion of proinflammatory cytokines such as IL-1β and TNF-α (32) that are known to be key players in HIV-1 biology. Because DCs play a major role in the development of immune response against HIV-1 and in its dissemination in vivo, we investigated the effect of AGEs on both cis-infection and trans-infection of MDDCs (46, 47). We show in this study that stimulation of MDDCs with AGE-BSA strongly inhibits their infection with both primary R5-tropic and X4-tropic HIV-1. AGEs induced both a decrease of viral DNA and RNA quantified just after the fusion step.

Because the average amount of membrane bound/attached virus particles was not affected by AGE-BSA, the observed inhibition more likely occurs at the fusion step, which involves gp160 interaction with HIV-1 receptor and coreceptors. This was further confirmed by the strong impact of AGE-BSA on the surface expression of CCR5, CXCR4, and CD4, which decreased by 96, 60, and 35%, respectively. Notably, a similar decrease in CCR5 membrane expression has recently been reported in primary macrophages exposed to high mobility group box 1 (HMGB1), another important ligand for RAGE (48, 49).
We show in this study that RAGE is required for the down-regulation of membrane CCR5 and CXCR4, whereas CD4 decrease is RAGE independent. Because the inhibition of HIV-1 infection in AGEs-treated MDDCs was mainly RAGE independent as well, one may assume that CD4 downregulation had a major impact on infection inhibition compared with CCR5 or CXCR4 downregulation, which are dependent on RAGE. The inhibition of CD4 surface expression mediated by AGEs may involve other receptors for AGEs that are expressed on DCs, such as galectin-3 (50) or the type A scavenger receptors (51). Although AGEs are likely to circulate for a long period of time in the serum, we could not evaluate the long-term effect of AGEs in vitro because a significant cytotoxicity was observed for an incubation time longer than 96 h. Therefore, the expression of CD4, CCR5, and CXCR4 as well as the function of DCs would be very interesting to monitor in subjects that are chronically exposed to AGEs, such as diabetic patients.

The molecular processes controlling CD4, CCR5, and CXCR4 reduction at the cell membrane are currently under investigation. Considering that only a minority of MDDCs express RAGE at the membrane, CCR5 and CXCR4 downregulation most likely relies on the secretion of a secondary mediator. This hypothesis is confirmed by the reversal of CCR5 and CXCR4 downregulation when the cells are incubated with AGEs plus CHX. We initially suspected that the downregulation of CCR5 could result from the AGEs-mediated induction of its natural ligands such as the chemokines RANTES or MIP-1α. Indeed, such observations have previously been made in latently HIV-1–infected primary macrophages grown in the presence of HMGB1, which is another known ligand for RAGE (49). However, neither RANTES nor MIP-1α could be detected after exposure of MDDCs to AGEs. This result is compatible with another recent study reporting that

**FIGURE 7.** Downregulation of HIV-1 coreceptors by AGEs requires de novo protein synthesis but does not involve RANTES or MIP-1α secretion. MDDCs were treated by AGE-BSA (50 μg/ml) for 24 h in the presence or absence of CHX (10 μg/ml). A, FACS analysis of CCR5, CXCR4, and CD4 expression. Data are expressed as the MFI. B, Levels of RANTES and MIP-1α secretion, quantified by ELISA. Results are representative of four independent experiments. LPS was used as a positive control.

**FIGURE 8.** Effect of AGEs on HIV-1 transmission in trans from MDDCs to T cells. MDDCs were stimulated with AGE-BSA or BSA for 24 h before being incubated with HIV-1 Ba-L (A) or HIV-1 VN44 (B) for 1 h. After five washes, autologous IL-2–stimulated T cells were added to MDDCs at a ratio of 1.5. The amount of HIV-1 p24 Ag was measured by ELISA at day 6 postinfection. Results are expressed as the mean ± SD of three independent experiments.

A

HIV-1 VN44

B

HIV-1 Ba-L

P ≤ 0.003

P ≤ 0.004
HMGB1 inhibits the replication of HIV-1 in primary macrophages by decreasing CCR5 surface expression without inducing the release of HIV-1 modulatory chemokines (48). Notably, the effect of HMGB1 on R5-tropic HIV-1 replication was also demonstrated to be dependent on RAGE (48). Altogether, these observations suggest that both AGEs and HMGB1 could lead to the secretion of one of several soluble factors that is capable of downregulating CCR5 and CXCR4 membrane expression. These factors may be growth factors or cytokines such as GM-CSF or TNF-α that are known to decrease surface expression of HIV-1 coreceptors on macrophages (52–55).

AGEs molecules may inhibit the replication of HIV-1 in MDDCs through the induction of these factors as well, as we demonstrated their significant production when MDDCs were exposed to AGEs. Reduction of CCR5 and CXCR4 membrane expression might notably be related to a heterologous desensitization of these receptors. This process has been described in another model (56) that provided evidence that activation of the formyl peptide receptors of DCs by the bacterial chemotactic peptide fMLF resulted in a dramatic decrease in CCR5 and CXCR4 membrane expression through a protein kinase C-dependent pathway. As expected, fMLF was also able to reduce HIV-1 multiplication in DCs. To this respect, let us note that another study already provided evidence that several ligands of RAGE, including AGEs, have been proved to activate protein kinase C-dependent pathways in monocytes (57).

In contrast to LPS, AGE stimulation does not induce MDDC maturation as demonstrated by the lack of expression of the CD83 marker. The effect of AGE molecules on DC phenotype still remains controversial. Indeed, Price et al. (58) observed a dose-dependent inhibition of CD83 expression on DCs exposed to AGE peptide, associated with the loss of their capacity to stimulate primary proliferation of allogeneic T cells. In contrast, Ge et al. (31) have shown that AGE induced a dose- and time-dependent maturation of DCs as indicated by the expression of CD83. This apparent discrepancy could be explained by the use in this study of anti-CD14 Ab-coated beads to purify monocytes and, above all, much higher AGE concentrations (up to 200 μg/l). In our hands, excessive AGE concentrations led to increased cell mortality, a process that may favor the release of soluble molecules such as HMGB1, which are prone to stimulate DC maturation. Notably, both AGE and LPS induce MDDC activation but only LPS induced their maturation confirming that DC maturation and activation are two independent states (40–42).

During HIV-1 infection, DCs and CD4 T lymphocytes synergize in facilitating initial viral replication (46, 59, 60). DCs concentrate viral particles before transmitting them to CD4 T lymphocytes. HIV-1 free particles attach to DCs through several molecular viral particles before transmitting them to CD4+ T lymphocytes and X4-tropic virus to the autologous T cells. This inhibition was not related to a downregulation of expression of DC-SIGN molecules on AGE-treated MDDCs. However, although initial observations suggested that DC-SIGN was the major molecule involved in HIV-1 trans-infection, subsequent studies demonstrated that this phenomenon can also occur through DC-SIGN-independent mechanisms (47). Hence, AGE molecules could disturb the interaction of HIV-1 gp160 with other molecules implicated in the transmission of HIV-1 in trans without inhibiting attachment of the virus on DCs. Similarly, we have previously shown that Abs to DC-SIGN carbohydrate recognition domain, which is involved in the binding of HIV-1 to DCs, inhibited the transmission in trans of HIV-1 from DCs to CD4+ T lymphocytes but did not have any effect on the attachment of the virus to the cells (60). These observations suggest that the attachment of HIV-1 to DCs and transmission in trans to autologous CD4+ T lymphocytes occur through two independent mechanisms.

Altogether, our results demonstrate that AGE molecules display a remarkable activity against HIV-1 by inhibiting both viral multiplication in MDDCs and transmission of the virus from MDDCs to CD4 T cells, one major mechanism of the spread of the virus in vivo. These data bring the first biological evidence to our knowledge of an interplay between AGEs and HIV-1 infection of MDDCs in vitro. However, further experiments will be necessary to determine whether accumulated AGEs in the blood of diabetic patients have the same inhibitory effect on HIV infection of primary myeloid DCs, which also express the HIV-1 receptors CD4/CCR5/CXCR4 and are susceptible to infection by HIV-1 (61).

Effects of AGEs on the immunological status of DCs could be instrumental in the pathophysiology of HIV-1 infection and address the question of their clinical implications.

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Disclosures
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