Apolipoprotein B mRNA–Editing Enzyme, Catalytic Polypeptide–Like 3G: A Possible Role in the Resistance to HIV of HIV-Exposed Seronegative Individuals

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Apolipoprotein B mRNA–editing enzyme, catalytic polypeptide–like 3G (APOBEC3G), a human cytidine deaminase, is a potent inhibitor of HIV replication. To explore a possible role of this protein in modulating in vivo susceptibility to HIV infection, we analyzed APOBEC3G expression in HIV-exposed seronegative individuals, HIV-seropositive patients, and healthy control subjects. The results showed that the expression of APOBEC3G is significantly increased in peripheral blood mononuclear cells (PBMCs)—mainly CD14+ cells—and in cervical tissues of HIV-exposed seronegative individuals. Higher APOBEC3G expression correlated with a reduced susceptibility of PBMCs to in vitro infection with the HIV-1Ba-L R5 strain. APOBEC3G could be important in modulating in vivo susceptibility to sexually transmitted HIV infection.

Apolipoprotein B mRNA–editing enzyme, catalytic polypeptide–like 3G (APOBEC3G) belongs to a family of at least 10 other proteins with broad antiretroviral activity. After the initiation of the reverse transcription of the HIV RNA genome into DNA, the cytidine deaminase activity of APOBEC3G catalyzes the conversion of cytosine to uracil in negative-strand viral cDNA, resulting in the reduction of viral fitness [1, 2]. The viral infectivity factor (Vif) protein of HIV counteracts the activity of APOBEC3G, inducing its degradation by a ubiquitine-proteasome pathway [3].

Susceptibility to HIV is widely different among humans [4, 5]. To verify whether APOBEC3G is involved in modulating susceptibility to HIV infection, we analyzed the expression of this protein in peripheral blood mononuclear cells (PBMCs) and cervical biopsy samples from individuals who, in spite of repeated exposure to HIV, do not become infected (HIV-exposed seronegative individuals). APOBEC3G expression was also evaluated in in vitro HIV infection assays performed on PBMCs from healthy control subjects and HIV-exposed seronegative individuals.

Methods. This study was approved by the institutional review boards of the S. M. Annunziata Hospital; written, informed consent was obtained from all patients. Blood samples were collected from 30 HIV-exposed seronegative individuals. Inclusion criteria were a history of multiple unprotected sexual episodes for ≥4 years at the time of the enrollment, with at least 8 episodes of at-risk intercourse within the 4 months before study entry and an average of 30 (range, 18 to >100) reported unprotected sexual contacts per year. Thirty age-matched HIV-infected patients and 30 age-matched healthy control subjects without any known risk factor for HIV infection were also included in the study. All HIV-seropositive patients were undergoing highly active antiretroviral therapy. All women underwent gynecologic and laboratory evaluation that did not reveal any concomitant infectious or gynecological problems. Cervical biopsy samples from 7 HIV-exposed seronegative individuals, 5 HIV-infected patients, and 7 healthy control subjects were collected in vials containing 1 mL of RNAlater (Ambion). Multiple PBMC samples (average, 3 samples; range, 2–4 samples) but only 1 cervical biopsy sample was collected from each patient over a period of 3 months, to

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minimize sampling errors. The final results were expressed as mean values.

For PBMC and CD4⁺, CD8⁺, and CD14⁺ cell isolation and culture, whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (Becton Dickinson), and PBMCs were separated on lymphocyte separation medium (Organon Teknika). CD4⁺ and CD8⁺ lymphocyte isolation was done with the RosetteSep kit (StemCell Technologies), in accordance with the manufacturer’s instructions. CD8⁺ and CD4⁺ cell purity was assessed by flow cytometry and ranged between 89% and 97% and between 85% and 94%, respectively. Monocytes were isolated with the Monocyte Isolation Kit (Miltenyi Biotec). Purity (range, 88%-94%) was evaluated by flow cytometry. Cells were cultivated in medium alone (RPMI 1640 and 20% fetal bovine serum [FBS]) or in the presence of 400 U/mL recombinant human interferon (IFN)-α (R&D) for either 4 (mRNA extraction) or 8 (protein extraction) h.

For real-time polymerase chain reaction (PCR), total RNA was extracted from cells or cervical biopsy samples with RNaseAqueos (Ambion) and were retrotranscribed as described elsewhere [6]. cDNA quantification for APOBEC3G and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research). Reactions were performed using a SYBR Green PCR mix (Finnzymes), as described elsewhere [6]. Primer sequences were designed to distinguish among the highly homologous sequences of cytidine deaminases (for APOBEC3G, 5′-CCGGCTTGCTGCAGGA-3′ [forward] and 5′-GCTTCCTCCA-CCTGCTGAACCA-3′ [reverse]); for GAPDH, 5′-CAGGATGGT- GTGGATTGGA-3′ [forward] and 5′-GCTTCCTTCTCA-GCTTTG-3′ [reverse]). Results were expressed as ΔΔCt (where “Ct” is the cycle threshold) and presented as ratios between the target gene and the GAPDH housekeeping mRNA.

For quantification of APOBEC3G protein by ELISA, total proteins extracted with an M-Per reagent and Halt Protease Inhibitor Cocktail (Pierce) were quantified by a BCA assay (Pierce). Ten micrograms of sample was coated on a 96-well plate overnight at 4°C. After blocking with 5% bovine serum albumin in Tris-buffered saline, the samples were incubated with 5 µg/mL chicken anti-APOBEC3G antibody, produced and characterized in our laboratory (L. Piacentini, E. Cesana, V. Naddeo, C. Fenizia, G. Bechi, A. Marino, R. Longhi, S. Beltrami, E. Lafone, A. Folci, M. Biasin, M. Clerici, and A. Clivio, manuscript submitted), for 2 h at 37°C. After a wash step, an anti-lgY–horseradish peroxidase conjugate (diluted 1:8000; Promega) was added. The coloring reaction was performed with the TMB Microwell Peroxidase Substrate (KPL).

For IFN-α receptor 1 (IFNAR1) analysis, PBMCs were stained for 1 h at 4°C in the dark with an anti-human IFN-α/β receptor 1 biotinylated antibody (R&D Systems), anti-CD4–phycoerythrin (PE), and anti-CD8–PE–Cy5 or with biotinylated anti-human IFN-α/B receptor 1 and anti-CD14–PE–Cy5 (Caltag Laboratories). Cytometric analysis was performed using an FC500 flow cytometer (Beckman Coulter).

For the in vitro challenge assay, PBMCs (2 × 10⁶ cells/mL) were cultured for 2 days at 37°C and 5% CO₂ in RPMI 1640 containing FBS (20%), phytohemagglutinin (5 µg/mL), and interleukin (IL)-2 (10 ng/mL). After viability assessment and CD4⁺ and CD8⁺ cell percentage evaluation, done as described elsewhere [7], 3 × 10⁶ cells were resuspended in medium containing 0.05 ng of HIV-1 Rev p24 viral input and incubated for 3 h at 37°C. Cells were then washed and resuspended in 3 mL of complete medium with IL-2 (10 ng/mL). Cells were plated in 24-well tissue culture plates and incubated at 37°C and 5% CO₂. PBMCs were then divided into 3 wells to be analyzed on days 2, 3, and 5. Cultures were refed with complete medium plus IL-2 on day 3, and supernatants were collected for batch ELISA of p24 antigen. Absolute levels of p24 were measured using the Alliance HIV-1 p24 ELISA Kit (PerkinElmer), and APOBEC3G protein expression was evaluated by ELISA. HIV-1_Luc was contributed by Drs. S. Gartner, M. Popovic, and R. Gallo (courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program).

Statistical analyses were performed using SPSS (version 11; SPSS). Differences between the groups were assessed using non-parametric analyses (Mann-Whitney U test). The standard t test was used for comparing different conditions within the same group. All P values are 2-tailed.

**Results.** APOBEC3G mRNA expression in unstimulated PBMCs was significantly augmented in HIV-exposed seronegative individuals, compared with that in healthy control subjects (P = .042). IFN-α stimulation resulted in a 3-fold increase in APOBEC3G mRNA levels in PBMCs from HIV-exposed seronegative individuals (P = .001) but had only a modest effect in HIV-infected patients and healthy control subjects. As a result, APOBEC3G mRNA levels in IFN-α-stimulated PBMCs were significantly augmented in HIV-exposed seronegative individuals, compared with those in both healthy control subjects (P = .042) and HIV-infected patients (P = .026) (figure 1A).

ELISA results confirmed that the highest levels of APOBEC3G protein were detected in PBMCs from HIV-exposed seronegative individuals, both in basal conditions (P = .006, vs. HIV-infected patients) and after IFN-α stimulation (P = .001, vs. HIV-infected patients; P = .031, vs. healthy control subjects) (figure 1B).

IFN-α stimulation of CD14⁺ cells resulted in a 20-, 9-, and 6-fold increase in APOBEC3G mRNA levels in HIV-exposed seronegative individuals, healthy control subjects, and HIV-infected patients, respectively (P = .042, for HIV-exposed seronegative individuals vs. healthy control subjects) (figure 1C). A weaker effect was seen for CD4⁺ cells (6-, 4-, and 3-fold increase in HIV-exposed seronegative individuals, HIV-infected patients, and healthy control subjects, respectively), whereas
IFN-α–induced modulation of APOBEC3G mRNA levels was minimal (<2-fold increase) in CD8⁺ cells from all individuals included in the study (figure 1C). ELISA analyses of APOBEC3G protein in CD14⁺ cells confirmed these results by showing higher levels of APOBEC3G in CD14⁺ cells from HIV-exposed seronegative individuals than in healthy control subjects and HIV-infected patients, both in basal condition and after IFN-α stimulation (figure 1D).

Flow cytometry analysis (mean fluorescence intensity) showed that IFNAR1 expression was significantly higher in CD14⁺ cells (mean ± SE percentage of IFNAR1-positive cells, 8.2% ± 3.8% for HIV-exposed seronegative individuals, 7.9% ± 3.6% for HIV-infected patients, and 8.3% ± 3.1% for healthy control subjects) than in both CD4⁺ cells (2.9% ± 0.6% for HIV-exposed seronegative individuals, 3.5% ± 1.3% for HIV-infected patients, and 3.0% ± 1.4% for healthy control subjects) and CD8⁺ cells (3.7% ± 1.1% for HIV-exposed seronegative individuals, 4.5% ± 2.6% for HIV-infected patients, and 4.2% ± 1.9% for healthy control subjects), providing a possible explanation for the enhanced responsiveness of monocytes to IFN-α. Surface levels of IFNAR1 on CD14⁺ cells were, nevertheless, comparable among the 3 groups of individuals.
APOBEC3G mRNA levels were significantly increased in cervical biopsy samples from HIV-exposed seronegative individuals, compared with those in HIV-infected patients and healthy control subjects ($P = .024$, vs. HIV-infected patients) (figure 1E). Unfortunately, protein analyses could not be performed because of a limitation in the amount of available material.

In vitro HIV infection of PBMCs from HIV-exposed seronegative individuals resulted in a lower amount of p24 after 2, 3, and 5 days, compared with that in PBMCs from healthy control subjects. This result was not likely to be due to quantitative differences in CD4$^+$ and CD8$^+$ cells, because the median CD4$^+$ (45% ± 6% for HIV-exposed seronegative individuals and 44% ± 5% for healthy control subjects) and CD8$^+$ (20% ± 3% for HIV-exposed seronegative individuals and 19% ± 4% for healthy control subjects) T cell percentage was comparable between the 2 groups. Quantification of APOBEC3G protein by ELISA showed that PBMCs from HIV-exposed seronegative individuals produced higher levels of APOBEC3G on days 2, 3, and 5 than did those from healthy control subjects. Differences were statistically significant ($P < .05$) at day 5 (table 1).

**Discussion.** In this study, we investigated whether the reduced susceptibility to HIV infection that characterizes HIV-exposed seronegative individuals could be related to differential expression of APOBEC3G and whether the expression of this protein could be differently modulated by exposure to IFN-α. The results showed that APOBEC3G mRNA levels were significantly increased in PBMCs from both HIV-exposed seronegative individuals and HIV-infected patients, compared with those in PBMCs from healthy control subjects. In contrast, levels of APOBEC3G protein were significantly augmented only in PBMCs from HIV-exposed seronegative individuals. The chronic exposure to HIV taking place in HIV-infected patients and HIV-exposed seronegative individuals could explain why APOBEC3G mRNA expression is stimulated in both. The Vif protein of HIV induces the degradation of APOBEC3G by a ubiquitine-proteasome pathway. By definition, HIV infection does not take place in HIV-exposed seronegative individuals [8]; in these individuals, the absence of Vif would prevent APOBEC3G degradation, possibly justifying the discrepancy between mRNA and protein levels.

After IFN-α stimulation, both APOBEC3G mRNA and protein levels increased in the 3 groups studied, with a more pronounced effect in HIV-exposed seronegative individuals than in both HIV-infected patients and healthy control subjects. Although IFN-α–induced modulation of APOBEC3G levels could seem relatively modest (3-fold), it has been demonstrated that even slight changes in the in vitro expression of APOBEC3G result in the reduction of HIV infectivity. These results, thus, suggest that population-level variation in APOBEC3G and Vif levels are likely to deeply influence the outcome of infection with HIV [9]. To verify this hypothesis, we performed in vitro infection assays on PBMCs from HIV-exposed seronegative individuals and healthy control subjects. We decided to use a macrophagotropic HIV-1 strain (R5) to approach the situation seen in vivo primary HIV infection during sexual transmission [10]. Interestingly, the kinetics of changes in p24 and APOBEC3G levels were different in the 2 groups examined. Thus, compared with what was observed in healthy control subjects, in vitro HIV infection of PBMCs from HIV-exposed seronegative individuals resulted in a slower increase in p24 concentration and a much more rapid up-regulation of APOBEC3G. A working hypothesis stemming from these data is that, once exposed to the virus, exposed seronegative individuals would respond to IFN-α production with a faster and more robust increase in the expression of APOBEC3G and a consequent reduced susceptibility to HIV infection.

**Table 1. p24 concentration in supernatants and apolipoprotein B mRNA–editing enzyme, catalytic polypeptide–like 3G (APOBEC3G) protein expression level in peripheral blood mononuclear cells from HIV-exposed seronegative individuals and healthy control subjects.**

<table>
<thead>
<tr>
<th>Day, protein</th>
<th>Exposed/seronegative ($n = 10$)</th>
<th>Healthy controls ($n = 10$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p24, pg/mL</td>
<td>4 ± 0.8</td>
<td>21 ± 7.2</td>
</tr>
<tr>
<td>APOBEC3G, HIV challenge/basal ratio</td>
<td>1.43 ± 0.15</td>
<td>0.98 ± 0.23</td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p24, pg/mL</td>
<td>96 ± 23.0</td>
<td>357 ± 79.5</td>
</tr>
<tr>
<td>APOBEC3G, HIV challenge/basal ratio</td>
<td>2.91 ± 0.63</td>
<td>1.17 ± 0.32</td>
</tr>
<tr>
<td>Day 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p24, pg/mL</td>
<td>4315 ± 114.3</td>
<td>16613 ± 175.6</td>
</tr>
<tr>
<td>APOBEC3G, HIV challenge/basal ratio</td>
<td>4.01 ± 0.54</td>
<td>2.03 ± 0.85</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± SE values. Cultures were infected with limiting amounts (0.05 ng of p24 viral input) of the HIV-1$_{Ba-L}$ R5 strain.

$^*$ $P < .05$. 
The present data show that monocytes are exquisitely responsive to IFN-α–induced up-regulation of APOBEC3G. Monocyte-derived type I IFNs are responsible for the earliest phase of the immune responses against pathogens, and their synthesis is directly triggered by viruses [11]. The higher amounts of APOBEC3G proteins seen in IFN-α–stimulated monocytes from HIV-exposed seronegative individuals could result in an amplification of these early, innate defensive immune mechanisms. Presumably, the peculiar responsiveness to IFN-α and up-regulation of APOBEC3G in CD14+ cells observed in HIV-exposed seronegative individuals would be seen not only in monocytes but, more generally, in nonlymphoid cells such as macrophages and Langerhans cells. It is tempting to speculate that the higher quantity of APOBEC3G produced by monocyte-derived cells, including Langerhans cells and macrophages, that cluster in peripheral tissues at mucosal sites could constitute an important barrier to HIV penetration during sexual exposure. This hypothesis is reinforced by the observation that APOBEC3G mRNA expression was indeed increased in cervical biopsy samples from HIV-exposed seronegative individuals.

Recent findings have demonstrated that APOBEC3G can exist into 2 different forms: an active one (low molecular weight [LMW]) and an inactive one (high molecular weight [HMW]) [12, 13]. It will be interesting to evaluate whether the prevalent localization of APOBEC3G in LMW and HMW complexes differs in HIV-exposed seronegative individuals. Similarly, we will evaluate in our cohort the presence of the C40693T APOBEC3G gene sequence variant recently associated with an increased risk of infection [14].

Given that, worldwide, the vast majority of HIV infections are sexually acquired [15], a potent APOBEC3G-mediated systemic and mucosal antiviral response could offer a formidable barrier against HIV infection. The present results could play an important role in the design of new therapeutic and vaccine strategies.

References


