Ex Vivo and In Vitro Effect of Human Immunodeficiency Virus Protease Inhibitors on Neutrophil Apoptosis

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Polymorphonuclear leukocytes (PMNL) from human immunodeficiency virus (HIV)-infected patients exhibit accelerated apoptosis and impaired functional activity. HIV protease inhibitor–based therapy produces improvements in both acquired and innate immune responses. Ex vivo and in vitro effects of HIV protease inhibitors on apoptosis and chemotaxis of PMNL were evaluated. After therapy, there was a rapid and significant decrease of PMNL apoptosis, which correlated with increased chemotactic function. These findings were found both in patients with immunological and virological response and in control subjects who showed an increase in CD4+ T cell counts but no concomitant decline in HIV load. After in vitro treatment with ritonavir or indinavir, apoptosis of both HIV-infected and -uninfected PMNL markedly decreased and correlated with significant enhancement of chemotaxis. These results suggest that HIV protease inhibitors may improve the PMNL function by reducing the apoptosis rate and that this effect may, at least in part, be independent of their antiviral activity.

Polymorphonuclear leukocyte (PMNL) function has been reported to be impaired during human immunodeficiency virus (HIV) infection, even in the early stage of the disease [1]. The defective activity of a critical component of antimicrobial immunity, such as PMNL, may contribute to the development of secondary infections in both adults and children with HIV infection. Several PMNL functions are impaired, including chemotaxis, oxidative respiratory burst, phagocytosis, and killing activity against bacteria and fungi. In addition, it recently has been shown that PMNL from patients with AIDS exhibited accelerated apoptosis ex vivo [2]. This observation suggests that apoptosis might be one of the mechanisms causing loss of function and reduction of numbers of PMNL in HIV infection [1, 3].

Highly active antiretroviral treatment (HAART) has been shown to be beneficial in reconstituting T cell responses and in augmenting neutrophil function during HIV infection [4, 5]. Although it has been established that abrogation of T cell apoptosis results in improved T cell reactivity during HIV infection, potential mechanisms and mediators of neutrophil dysfunction during HIV infection have not been clearly elucidated. The current study was conducted to investigate further the basis of HIV-related PMNL dysfunction and to establish whether neutrophil apoptosis may have any bearing on the functional capacity of these cells, particularly in the context of HAART. Therefore, we examined PMNL apoptosis ex vivo in a cohort of patients receiving HAART and compared the results with a marker of functional activity, such as chemotaxis. We also investigated the in vitro effect of HIV protease inhibitors indinavir and ritonavir on PMNL apoptosis and chemotactic function from healthy donors and HIV-infected patients in vitro.

Materials and Methods

Patients and control subjects. Study participants included 17 HIV-infected patients (15 men and 2 women; age range, 21–69 years) from the Department of Infectious and Tropical Diseases of La Sapienza University, Rome, Italy. All patients had a CD4+ T cell count <300 cells/μL and no concomitant infections and were naïve for any antiretroviral drugs. Patients were treated with a protease inhibitor (3 patients with nelfinavir, 9 with indinavir, and 5 with ritonavir) combined with nucleoside or nonnucleoside analogues and were followed up for 48–110 weeks. Ten healthy blood
donors were included as control subjects. Samples from healthy donors were run on different days to determine day-to-day variations in assay and were used as controls more than once. Specimens from patients and donors were tested in parallel, using identical reagents under identical conditions.

**HIV RNA levels and CD4+ and CD8+ lymphocyte counts.** HIV RNA levels were measured by a quantitative polymerase chain reaction (AMPLICOR HIV Monitor; Roche Diagnostics Systems, Branchburg, NJ). The enumeration of CD4+ and CD8+ lymphocyte was performed as reported elsewhere [5].

**PMNL isolation.** PMNL were prepared from heparinized blood by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, followed by dextran sedimentation. Contaminating erythrocytes were removed by a single hypotonic lysis in sterile distilled water for 30 s at room temperature. PMNL were suspended in PBS (pH 7.4) and were adjusted to the desired concentration. The purity of PMNL isolated was always >95%, as determined by Giemsa staining. The PMNL viability was >98%, as estimated by trypan blue exclusion.

**Spontaneous apoptosis.** Isolated PMNL (1 × 10^6/mL) were suspended in PBS and 200 µL of cell preparation, were immediately cytocentrifuged, and were fixed with 4% paraformaldehyde. Apoptosis was evaluated by in situ cell death detection kit, peroxidase (Boehringer-Mannheim, Mannheim, Germany), according to the manufacturer’s instructions. This assay measured DNA fragmentation by immunocytochemistry, using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method [6]. Cell death was confirmed in each sample by annexin V-fluorescein/propidium iodide staining of cell suspensions (Annexin-V–FLUOS; Boehringer-Mannheim) [7]. One hundred viable cells were counted per sample, and results are expressed as the percentage of apoptotic cells.

**Chemotaxis assay.** PMNL migration toward FMLP (10⁻⁷ M; Sigma, St. Louis) was carried out as reported elsewhere [6]. In brief, 200-µL aliquots of chemoattractant were placed in the lower well of a blind-well chamber (Neuroprobe, Cabin John, MD) and were separated by polypyrrolidone-free 3-µm polycarbonate filter (Neuroprobe) from 300 µL of cell suspensions (2 × 10⁵ PMNL) in Dulbecco’s modified Eagle medium placed in the upper well. After 90 min incubation at 37°C in 5% CO₂ in a humidified atmosphere, the filters were removed. After cleaning the upper side to remove nonmigrated cells, they were fixed and were stained by adding DiffQuik (Baxter Diagnostics AG, Dudingen, Switzerland). All assays were carried out in triplicate. Migrated cells were then counted microscopically in 10 randomly selected, oil immersion fields. Cell migration was expressed as the number of neutrophils that migrated per field. Spontaneous migration in the absence of chemoattractant was also calculated and was subtracted from migration in response to FMLP.

**Drug pretreatment of cells.** Indinavir and ritonavir were provided by M. Andreoni (Tor Vergata University, Rome). The 2 drugs were dissolved in distilled water and ethanol (final ethanol concentration, 0.5% vol/vol), respectively, and then were stored at −80°C until use. Isolated PMNL (1 × 10⁶) from both healthy donors and untreated HIV-seropositive individuals were cultured in RPMI 1640 supplemented with 10% fetal calf serum and glutamine (2 mM) in the presence of medium alone or various concentrations of ritonavir or indinavir. On the basis of the therapeutically achievable levels [8], we chose the following target concentrations: indinavir, 50, 100, 150, 200, and 500 nM; ritonavir, 1, 10, and 20 µg/mL. After 18 h of incubation at 37°C under 5% CO₂ atmosphere, PMNL were counted and were evaluated for apoptosis and chemotaxis. PMNL viability was measured by trypan blue exclusion.

**Statistical analysis.** Data at multiple time points in the longitudinal study were compared using the Friedman 2-way analysis of variance by ranks with pairwise comparisons on-treatment versus baseline values (Dunnett’s test). Differences between treated patients at baseline and healthy control subjects were evaluated by the Mann-Whitney U test. The significance of correlation study

**Figure 1.** Spontaneous apoptosis (A) and chemotaxis (B) of neutrophils from the 10 human immunodeficiency virus type 1–infected patients with virological and immunological response during 48 weeks of protease inhibitor–based therapy. Data are medians and ranges. Gray horizontal zone represents ranges in 10 healthy control subjects. Significant differences between multiple time point by Friedman test (*P* < .0001 for apoptosis and *P* < .01 for chemotaxis); *P* < .05 for weeks 2, 8, 16, 24, and 48 vs. baseline (Dunnett’s test for pairwise comparison).
parameters was estimated using the Spearman rank correlation. In the in vitro studies, differences between values at individual drug concentrations and baseline values were assessed with analysis of variance.

Results

**PMNL apoptosis and chemotaxis in HAART-treated patients.** The median pre-HAART CD4+ T cell count and virus load were 92 cells/µL and 5.7 log_{10} copies/mL, respectively. After 48–110 weeks of therapy, there was a sustained increase in CD4+ T cell count (median increase, 170 cells/µL). HIV load was below the assay level of detection in 10 patients (58.8%), whereas 7 subjects remained viremic (median HIV-RNA level, 5.9 log_{10} copies/mL).

Spontaneous apoptosis and chemotactic responsiveness of PMNL in the 10 treated patients with viral suppression and sustained increase in CD4+ T cell count is reported in figure 1. In the treatment group, the baseline percentage of apoptosis was significantly accelerated, compared with that of HIV-negative control subjects (42 vs. 3.5; \( P < .0001 \); figure 1A). After starting HAART, PMNL apoptosis decreased to 12% at week 2, 8.5% at week 8, 2.5% at week 16, 3% at week 24, and 3.5% at week 48 (\( P < .001 \)).

When the ability of PMNL to migrate toward FMLP was analyzed, baseline chemotactic values from treatment group were significantly lower, compared with those of control subjects (median, 27.5 vs. 56; \( P < .001 \); figure 1B). After initiation of HAART, the median chemotactic activity of PMNL increased to 33 at week 2, 41 at week 8, 41 at week 16, 51 at week 24, and 52.5 at week 48. The chemotactic responses of PMNL were inversely correlated with the percentage of apoptotic cells during HAART (\( r = -.54; P < .05 \)). We found a low rate of apoptosis (median, 3%; range, 2%–5%) and an increased chemotactic function (median, 45 migrated cells/field; range, 37–51 migrated cells/field) also in the 7 patients with immunological benefits but persistent detectable viremia after 48–110 weeks of HAART. In the HAART nonvirological responder group, the initial median CD4+ T cell counts (87 cells/µL), viral load (5.8 log_{10} copies/mL), and PMNL functional activity (chemotaxis, 25.5) were comparable at baseline with the responder group.

**In vitro effect of HIV protease inhibitors on PMNL apoptosis and chemotactic function.** In another set of experiments, PMNL from healthy donors and antiretroviral-naive HIV-infected patients were treated in vitro with increased concentrations of indinavir and ritonavir and then were examined for apoptosis and chemotaxis. As reported in table 1, indinavir and ritonavir, in 6 experiments performed with different HIV-seronegative donors, significantly decreased spontaneous apoptosis of normal PMNL within and above the range of therapeutically achievable concentrations. Significant decrease from the baseline rate of apoptosis was observed after drug pretreatment of PMNL from 6 different HIV-seropositive individuals. In these patients, however, the total number of viable cells still present after 18 h of in vitro culture were lower than that of healthy control subjects. Also PMNL chemotactic activity significantly increased in a dose-dependent manner, both in HIV-seronegative and HIV-seropositive individuals.

**Table 1.** In vitro effect of indinavir and ritonavir on apoptosis and chemotaxis of neutrophils from healthy donors and human immunodeficiency virus (HIV)–seropositive individuals.

<table>
<thead>
<tr>
<th>Drug concentration</th>
<th>Percentage of apoptosis</th>
<th>Migrated cells per field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donors</td>
<td>HIV-seropositive</td>
<td></td>
</tr>
<tr>
<td>Healthy donors</td>
<td>HIV-seropositive</td>
<td></td>
</tr>
<tr>
<td>Control (nM)</td>
<td>Healthy donors (µM)</td>
<td>HIV-seropositive (µM)</td>
</tr>
<tr>
<td>0</td>
<td>49.6 ± 5.9</td>
<td>22 ± 1.09</td>
</tr>
<tr>
<td>50</td>
<td>32.6 ± 5.4</td>
<td>31.6 ± 1.2</td>
</tr>
<tr>
<td>100</td>
<td>23.3 ± 3.7</td>
<td>48 ± 2.1</td>
</tr>
<tr>
<td>150</td>
<td>15.6 ± 2.6</td>
<td>55.3 ± 1.8</td>
</tr>
<tr>
<td>200</td>
<td>7.3 ± 0.9</td>
<td>59 ± 4.7</td>
</tr>
<tr>
<td>500</td>
<td>5 ± 0.07</td>
<td>58.6 ± 4.2</td>
</tr>
<tr>
<td>Ritonavir (µg/mL)</td>
<td>Healthy donors</td>
<td>HIV-seropositive</td>
</tr>
<tr>
<td>1</td>
<td>25 ± 3.2</td>
<td>41.3 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>18.6 ± 1.7</td>
<td>52.6 ± 2.6</td>
</tr>
<tr>
<td>20</td>
<td>9.6 ± 1.8</td>
<td>70.3 ± 1.1</td>
</tr>
</tbody>
</table>

NOTE: Data are mean ± SE of 6 different experiments. Significant differences of individual drug concentrations from control (0 concentration; \( P < .05 \)).
chemotaxis and apoptosis, and chemotactic activity increased as apoptosis decreased during therapy.

We recently demonstrated that the administration of HAART in patients with moderately advanced HIV disease induces significant improvements in PMNL chemotactic and fungicidal activity and enhancements in oxidative burst responses by chemiluminescence production [5]. The data presented here indicate that the reversal of neutrophil dysfunction in HIV-infected patients receiving HAART is closely associated with a drastic decrease of PMNL apoptosis rate.

Although the exact mechanisms of apoptosis in HIV infection are still unknown, the susceptibility of cells to apoptosis seems to be dependent on the level of HIV replication, and HIV-related factors, such as soluble HIV proteins, cytokines, and chemokines, may all play an important role. In this respect, it is generally believed that the degree of reduction of apoptosis after HAART is closely associated with an adequate control of HIV viremia; however, a recent article has shown that HAART was able to reduce apoptosis of CD4+ T cells without any relation to plasma viremia [9]. There are data suggesting that protease inhibitors may have clinical and immunological benefits, even in the absence of sustained viral suppression [10, 11]. Our results show that PMNL exhibited a low rate of spontaneous apoptosis and an increased chemotactic function in patients with immunological and virological responses, as well as in control subjects who showed a sustained increase in CD4+ T cell counts but no concomitant decline in plasma HIV viremia. These ex vivo findings also indicate that the improvement of PMNL function during protease inhibitor therapy can be achievable without a satisfactory decrease of HIV load.

On the other hand, a direct effect of protease inhibitors on apoptotic pathways that was independent of HIV inhibition by these drugs cannot be ruled out. To verify this hypothesis, we examined in vitro action of 2 protease inhibitors, indinavir and ritonavir, on PMNL from healthy donors and HIV-infected patients. We found that apoptosis of both HIV-infected and uninfected PMNL markedly decreased after in vitro treatment with ritonavir or indinavir and correlated with significant enhancement of chemotactic function. Recently, it has been shown that HIV protease inhibitors modulate in vitro activation of CD4+ T cells and decrease their susceptibility to apoptosis [12–14]. In an in vitro study, Lu and Andrieu demonstrated that these drugs enhance the survival of HIV-infected T cells by restoring their proliferative responses [15].

Antiretroviral therapies based on protease inhibitors have produced dramatic improvements in both acquired and innate immunity that have not been observed among patients receiving only reverse-transcriptase inhibitors. The immunological recovery of T cell response, as well as the improvement of functional activity of neutrophils induced by protease inhibitors, could be, at least in part, attributed to an immunomodulating effect of these drugs, independent of their antiviral activity.

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References