Short Communication: Enhanced CD8⁺ T Cell Apoptosis in HIV-Infected Adolescents with Virologic Failure on Protease Inhibitor-Based Therapy

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Abstract

In this study, we investigated the possibility of differential effects of protease inhibitor (PI)-containing (PI⁺) and PI-sparing (PI⁻) antiretroviral therapies (ART) on CD8⁺ T cell apoptosis. We retrospectively analyzed both PD-1 expression and CD8⁺ T cell apoptosis in a cross-sectional study of HIV-positive adolescents and young adults (mean age = 17.4 years), with perinatally or behaviorally acquired HIV infection. Fifty-one specimens of cryopreserved peripheral blood mononuclear cells (PBMCs) were analyzed using 7-color flow cytometry: 20 from patients receiving PI⁺ ART, 14 from PI⁻ ART, and 17 from the untreated. The results showed that percentages of PD-1⁺ CD8⁺ T cells were strongly correlated with plasma viral loads regardless of treatment ($p = 0.0001$). The percentage of PD-1⁺ CD8⁺ T cells was also positively associated with percentages of Annexin V⁺ CD8⁺ T cells ($p = 0.04$) in the PI⁺-treated group. The fraction of apoptotic (Annexin V⁺) CD8⁺ T cells was associated with viral load in the patients receiving ART that contained one or more protease inhibitors ($p = 0.029$), but not in the PI⁻ or untreated groups. In summary, we found a direct correlation between PD-1 expression on CD8⁺ T cells and HIV levels that was not affected by types of medications used in the ART of those adolescents, suggesting that virological success is necessary for PD-1 downregulation. CD8⁺ T cell apoptosis was linked to high levels of PD-1 expression and HIV viremia. However, there was a higher degree of apoptosis among viremic patients receiving PI therapy, suggesting an immunologically adverse effect of continuing PI⁺ therapy after virological failure.

ANTIRETROVIRAL THERAPY (ART) has been successful in terms of controlling HIV replication, slowing AIDS progression and prolonging survival of many HIV-infected patients. However, drug toxicity and adverse effects of ART and the possibility of exhausting treatment options following the emergence of drug-resistant mutants have generated extensive discussion about the timing and proper selection of antiretroviral drugs for HIV/AIDS treatment.¹ Interestingly, numerous follow-up studies have provided evidence that protease inhibitors (PI) have effects on the maintenance or restoration of CD4⁺ T cell levels that appear to be independent of their antiviral effects.^{2,3} A direct effect of PI on CD4⁺ T cells is to inhibit CD4⁺ T cell apoptosis,⁴ a major cause of CD4⁺ T cell loss during chronic HIV infection.⁵ In contrast, less is known about the immunomodulatory effects of PI on CD8⁺ T cells. CD8⁺ T cells are a crucial component of cellular immu-

nity against viral infections. Unfortunately, protracted exposure to antigens during chronic infections such as HIV infection has been linked to so-called immune exhaustion of CD8⁺ T cells, which poses a great health risk for developing malignancy and opportunistic infection.⁶ Previously, overexpression of programmed death-1 (PD-1) on CD8⁺ T cells, especially HIV-specific CD8⁺ T cells, was reported in HIV⁺ adult patients.⁷⁻⁹ Furthermore, blocking the binding between PD-1 and its ligand PD-L1 presents a potential immunotherapy for chronic HIV infection.¹⁰ This report investigated PD-1 expression on CD8⁺ T cells and CD8⁺ T cell apoptosis in HIV⁺ adolescents who either acquired the virus perinatally or through recent adult behaviors. We attempted to examine whether there was a possibility of differential effects of PI-containing (PI⁺) and PI-sparing (PI⁻) ART on PD-1 expression or CD8⁺ T cell apoptosis.

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We obtained PBMC specimens from an observational study of HIV-1-infected adolescents and young adults (mean age = 17.4 years) with perinatally or behaviorally acquired HIV infection. These subjects were followed from 2000 to 2003, when they received either PI⁺ ($n = 11$) or PI⁻ ($n = 9$) regimens or were not being treated. This study was approved by the institutional review boards of Children's Hospital Los Angeles and UCLA. Their plasma viral loads were determined by Roche Amplicor HIV-1 Monitor assay and virological failure (VL > 400) was common in both groups (PI⁺: 41% vs. PI⁻: 56%, Table 1). Immunophenotyping of cryopreserved PBMCs was performed using 7-color flow cytometry on a BD Biosciences FACSaria. In brief, freshly thawed PBMCs were stained with monoclonal antibodies (mAb): antihuman CD3- Pacific Blue (OKT3), antihuman CD27- APC-Alexa Fluor 750 (O323) (eBioscience, San Diego, CA), antihuman CD45RO- APC (UCHL1), antihuman CD8- PerCP (SK1), and antihuman PD-1- PE (MIH4) (BD Biosciences, San Jose, CA) according to manufacturers' instructions. Surface-stained cells were then stained with Annexin V-FITC (BD). Dead cells were excluded from analysis by using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit for 405 nm excitation (Invitrogen, Carlsbad, CA). Flow cytometric data were then analyzed using FlowJo 7.2 software (Tree Star, Ashland, OR). For independent samples we used the two-sided Kruskal-Wallis test to compare the groups with follow-up Wilcoxon rank sum test for two group comparisons. For the PD-1 and Annexin data we had up to three repeated measures per person. We used a mixed effects model with an unstructured correlation matrix to account for the within-subject correlation.

We analyzed 51 PBMC specimens in total from patients receiving PI⁺ ($n = 20$) or PI⁻ ($n = 14$) therapy or who were untreated ($n = 17$) using 7-color flow cytometric analysis (Fig. 1A). There was a direct correlation between percentages of PD-1⁺ CD8⁺ T cells and plasma viral loads in HIV⁺ adolescents ($p = 0.0001$, Fig. 1B), in agreement with reports involving older adult patients.⁷⁻⁹ We also compared the levels of PD-1⁺ cells in different CD8⁺ subsets based on the phenotypic markers CD27 and CD45RO. PD-1 expression on CD8⁺ T cells in these HIV⁺ adolescents and young adults was mostly upregulated on activated/memory-phenotyped CD8⁺ T cells, similar to the report by Petrovas *et al.*⁸ Furthermore, PD-1⁺ CD8⁺ T cells also frequently expressed another apoptosis-associated receptor Fas (CD95) (data not shown). To reveal a possible treatment effect on PD-1 expression on CD8⁺ T cells, the specimens were further stratified into three categories: no ART, PI⁻ ART, and PI⁺ ART, according to the treatment

received by the patients when the specimen was collected. In all three treatment groups, we found strong and comparable associations between percentages of PD-1⁺ CD8⁺ T cells and plasma viral loads (Fig. 1B), suggesting that PD-1 expression is largely a function of levels of antigen stimulation. A possible cellular effect of PD-1 upregulation is that CD8⁺ T cells become prone to apoptosis.⁸ Therefore, we examined levels of CD8⁺ T cell apoptosis by staining apoptotic cells in PBMCs with Annexin V after exclusion of dead cells for detection of early apoptotic cells. Percentages of PD-1⁺ CD8⁺ T cells were indeed positively associated with percentages of Annexin V⁺ CD8⁺ T cells in the PI⁺ group ($p = 0.04$, data not shown). We further tested whether types of treatment had an effect on the levels of CD8⁺ T cell apoptosis. Our result showed levels of apoptotic CD8⁺ T cells were significantly influenced by types of therapy, with higher viral loads being correlated with higher levels of Annexin V⁺ cells in the PI⁺ ART group ($p = 0.029$) but not in the PI⁻ ART group (Fig. 1C).

Here we first show that PD-1 expression on CD8⁺ T cells is significantly upregulated and levels of PD-1⁺ CD8⁺ T cells are associated with viral loads in HIV-infected adolescents, extending previous findings made in adult patients.⁷⁻⁹ Promising results from an animal model and *in vitro* assays have presented PD-1 and its ligand interaction as a potential immunotherapeutic target for treating HIV infection and other chronic infections. However, factors contributing to PD-1 upregulation on CD8⁺ T cells and immunological effects of high PD-1 levels have not been well understood. This report retrospectively examined whether the HIV medications used affect PD-1 expression on CD8⁺ T cells. A similar association between the levels of PD-1⁺ CD8⁺ T cells and viral loads was found among the untreated group, PI⁻ ART group, and PI⁺ ART group, suggesting that PD-1 expression was a function of the degree of HIV replication. It also implied that positive virological responses to medications may benefit anti-HIV CD8⁺ T cell responses via downregulation of PD-1 expression. Second, we show that numbers of apoptotic CD8⁺ T cells indeed increase with the upregulation of PD-1 expression. Furthermore, numbers of apoptotic CD8⁺ T cells also increased with plasma viral loads ($p = 0.0005$). A possible explanation for this association is that viremia promotes high expression of PD-1, which sensitizes CD8⁺ T cells for apoptosis, as suggested by an earlier report.⁸ We were able to examine CD95 expression on a subset of samples, 27 out of 51, and found that Fas expression on total CD8⁺ T cells or the four subsets of CD8⁺ T cells defined by CD27 and CD45RO markers was not different among three treatment groups. In addition, there was no correlation between Fas expression on

TABLE 1. CHARACTERISTICS OF STUDIED HIV ADOLESCENTS

Treatment	No. of subjects (samples)	Subjects with PN ^a infection	Ages (mean $\pm$ SD)	% of viremic (VL > 400 copies/ml) patients	CD4 ⁺ T cell counts/ μ l	CD8 ⁺ T cell counts/ μ l	CDC classifications			
							N	A	B	C
No ART	11 (17)	2	20.3 $\pm$ 2.3	100	386 $\pm$ 158	837 $\pm$ 391	1	5	4	1
PI ⁻ ART	9 (14)	3	18.3 $\pm$ 3.3	56	447 $\pm$ 307	625 $\pm$ 334	0	3	5	1
PI ⁺ ART ^b	11 (20)	6	17.8 $\pm$ 1.6	41	469 $\pm$ 235	964 $\pm$ 380	0	2	1	8

^aPN = HIV infection acquired by mother-to-child transmission or transfusion in infancy.

^bProtease inhibitor regimens included amprenavir, nelfinavir, lopinavir/ritonavir, saquinavir + lopinavir/ritonavir, saquinavir + ritonavir, and indinavir/ritonavir.

FIG. 1. Correlations of percentages of PD-1⁺ CD8⁺ T cells and percentages of Annexin V⁺ CD8⁺ T cells with patients' viral loads in different treatment groups. (A) Gating scheme of flow cytometric analysis of CD8⁺ T cells in PBMCs. Also, PD-1 expression and Annexin V staining in gated CD8⁺ T cells in Patient 1 (HIV VL = 27,333 copies/ml) and in Patient 2 (HIV VL <50 copies/ml) are shown in comparison. (B) Percentages of PD-1⁺ CD8⁺ T cells were positively associated with patients' viral loads, showing no treatment effect on the correlation. (C) Percentages of Annexin V⁺ CD8⁺ T cells were positively associated with viral loads in the PI⁺ ART group but not in the PI⁻ ART and no treatment groups. Gray triangles represent data of untreated patients. Black circles and open squares represent data of patients in the PI⁻ ART group and the PI⁺ ART group, respectively. Trendlines for all three subgroups are also shown. Viral load values are plotted as the actual value, or the limit of detection on the assay, if HIV RNA was not detected.

total CD8⁺ T cells and viral load ($R=0.067$, $p=0.73$). The inability to identify significant interactions in this secondary analysis may be due to the small size of samples available for Fas staining, but may also indicate that Fas-independent mechanisms may be involved in triggering CD8 apoptosis. Interestingly, a positive association between CD8⁺ T cell apoptosis and viral loads was identified in the group of PI⁺ ART, suggesting an immunologically adverse effect of continuing PI⁺ ART after virological failure. This finding was surprising, given the beneficial effects of PI on CD4⁺ T cell retention and reconstitution previously reported in some HIV⁺ patients.²⁻⁴ However, PI may exert differential cellular effects on different cell types, including proapoptotic effects in some cell types.⁴ Moreover, there were earlier reports of unfavorable clinical outcomes in PI⁺ ART compared to PI⁻ ART.^{11,12}

It is conceivable that these findings may be affected by the higher fraction of individuals with previous advanced HIV-related disease among those receiving PI-containing regimens, although CD4 and CD8 T cells were similar in number at the visits studied (Table 1). To address the possibility that advanced disease (reflected in the CDC classifica-

tion) might have introduced bias, we controlled for disease stage in our mixed regression analysis. After controlling for disease stage classification, log viremia was still a significant predictor of Annexin V, and there was also a significant interaction between viremia and treatment group. We conclude that further, preferably longitudinal, studies are warranted to evaluate the benefits and adverse effects of continuing PI⁺ ART if virological failure occurs.

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Author Disclosure Statement

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