Inverse correlation between CD8+ lymphocyte apoptosis and CD4+ cell counts during potent antiretroviral therapy in HIV patients

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Objectives: We have addressed the relationships between inhibition of CD4+ and CD8+ cell apoptosis and CD4+ cell recovery in HIV patients undergoing potent antiretroviral therapy (PART) by correlating apoptosis levels with virological and immunological parameters detected over a long-term period in HIV patients undergoing therapy.

Patients and methods: Twenty-two HIV-1-infected patients undergoing PART were enrolled in a long-term, open longitudinal study. Data derived from 17 patients with successful response to therapy (TS; median time of follow-up 36 months, range 24–36 months) were used for correlation studies. Apoptosis was evaluated after short-term culture of peripheral blood lymphocytes by flow cytometry analysis of isolated nuclei or of annexin V/CD4, annexin V/CD8 double-stained cells.

Results: Sustained, noticeable levels of apoptosis inhibition in peripheral blood mononuclear cells were measured, in the long-term, in 16 of the 17 TS patients. Levels of total cell apoptosis correlated with levels of CD8+ apoptotic cells more significantly than with levels of CD4+ apoptotic cells. In addition, CD4+ cell counts were correlated inversely with levels of CD8+ apoptotic cells in a highly significant fashion, but not with levels of CD4+ apoptotic cells.

Conclusions: Our data indicate that the increase of CD4+ lymphocytes in HIV patients, as a consequence of successful response to PART, may be related to changes in apoptosis level occurring in the CD8+, and not in the CD4+, cell compartment.

Keywords: HIV, antiviral, CD8+ cells

Introduction

One of the hallmarks of HIV infection is sustained CD4+ lymphopenia. Several mechanisms have been suggested to explain the decrease in CD4+ cells, such as a defect in T cell renewal, cellular sequestration in lymphoid organs, exhaustion due to excess activation and proliferation of lymphoid cells.1,2 In addition, it has been widely documented that apoptotic cell death is among the mechanisms of CD4+ cell destruction during HIV infection.3,4 Nevertheless, it is not clear whether direct infection of CD4+ cells or indirect effects caused by the release of viral or cellular factors, or both, are responsible for CD4+ cell death. The major goals of potent antiretroviral therapy (PART) are the suppression of viral replication and the boosting of immune restoration through the increase of CD4+ cells. Different factors may conspire to cause the latter effect, such as a down-regulation of T cell turnover, a possible recruitment of naive T cells from the...
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lymphoid tissues or the inhibition of lymphocyte apoptosis.5-7 Several studies have shown that inhibition of spontaneous or Fas-induced apoptosis actually occurs following PART in HIV+ patients and is associated with an increase in the CD4+ cell number.8-10 However, the mechanisms involved in this phenomenon await further clarification. In fact, results from us and other authors have demonstrated that following PART, spontaneous or induced apoptosis of short-term cultured peripheral blood mononuclear cells (PBMCs) was significantly correlated, not only inversely with CD4+ cell counts in the periphery, but also directly with viral load (VL) levels, at least in the early phases of treatment.11,12 On the other hand, data from some authors suggest that inhibition of CD4+ cell apoptosis during PART could be related to CD4+ cell recovery during treatment, independently of VL kinetics.13-15 Consistent with this, other studies suggest that CD4+ cell reconstitution could be influenced by the direct effect of some antiviral drugs on the susceptibility of CD4+ cells to apoptosis, and not by virus-related mechanisms.16,17 It has also been hypothesized that CD4+ cell reconstitution reflects a complex interaction between viral infection and CD4+ cell kinetics and activation, also involving different effectors of the immune system.18 In any case, a direct causal effect between CD4+ cell recovery and CD4+ cell apoptosis decrease in response to PART has not yet been demonstrated. Moreover, questions remain regarding the duration of apoptosis suppression during successful PART and the specific contribution of the CD8+ subset in the overall decrease in lymphocyte apoptosis, as a consequence of PART. In order to answer some of the unresolved questions concerning the decrease in lymphocyte apoptosis in response to PART, we extended our previous observations by completing a longitudinal study based on the collection of data to analyse the correlations among lymphocyte apoptosis, T cell counts and VL responses in HIV patients before and during PART. Results concern not only the early phase following therapy initiation, but also long-term treatment, during a 3 year longitudinal study designed for this purpose. Moreover, in a subgroup of patients we specifically investigated the differential contribution of CD4+ and CD8+ cell subsets in the overall decrease in lymphocyte apoptosis during PART.

Materials and methods

Study design and patients

This was a longitudinal, open study in a well-characterized cohort of HIV-1-infected patients starting PART. PART was defined as two nucleoside reverse transcriptase inhibitors (NRTIs) plus a protease inhibitor or, in three patients, a non-NRTI. Patients were enrolled from the Department of Infectious and Tropical Disease, University of Rome ‘La Sapienza’, or from the AIDS Center, S. Giovanni Hospital, Rome. Enrolled patients were: (i) routinely followed up by medical visits; (ii) checked at baseline and monthly for plasma VL during the first 6 months and every 3 months thereafter; (iii) checked at baseline and monthly for CD4+ and CD8+ cell counts; and (iv) checked at baseline and monthly for apoptosis during the first 6 months and every 3 months thereafter.

Eligibility criteria at enrolment required that: (i) the patients were naïve or that they had been off antiretroviral therapy for at least 4 months (one patient only); (ii) their age was ≥18 years; (iii) they had the ability to be compliant with the study; and (iv) they had not been suffering from opportunistic or any kind of severe infection, cancer, autoimmune disorders, or major vascular or neurological diseases. No patient lost eligibility for the last two criteria during the course of the study. Most of the patients remained on their assigned treatment, with the exception of four subjects who changed or briefly interrupted and reinitated the therapy, without modification of the overall response. The study received ethics approval from the institutions participating in the study and informed consent was obtained from patients.

Cell cultures and evaluation of apoptosis

PBMCs were isolated from heparinized blood by Ficoll-Hypaque gradient (Lymphoprep, Nycomed, Oslo, Norway) according to standard methods. In order to evaluate spontaneous apoptosis of short-term cultured PBMCs, as previously described,12 the cells were cultured for 66 h in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% FCS (Life Technologies), 2 mM glutamine, 50 IU/mL penicillin and 50 IU/mL streptomycin (Hycolne, Cramlington, UK). At the end of incubation time, apoptosis of samples from all patients was evaluated by flow cytometry analysis of isolated nuclei following detergent treatment and propidium iodide staining, using a method that distinguishes nuclei from apoptotic, necrotic or viable cells, as previously described.19 Moreover, in order to selectively detect cell death in CD4+ and CD8+ cells, quantitation of apoptosis was also assessed, in additional samples from a subgroup of the patients, by evaluating the percentage of annexin V-positive CD4+ or CD8+ cells. This evaluation was performed by double-fluorescence flow cytometry analysis, following staining with fluorescein-conjugated annexin V (Annexin V-FITC Apoptosis Detection Kit; BD-PharMingen, San Diego, CA, USA) plus phycoerythrin-conjugated CD4 or, alternatively, CD8 monoclonal antibodies (PE-CD4 and PE-CD8; Becton Dickinson, Mountain View, CA, USA). Staining with relevant isotype-matched control monoclonal antibodies (Becton Dickinson) was also performed. Flow cytometry analysis was performed, immediately after staining, on a FACScan or a FACScalibur flow cytometer (Becton Dickinson). Events were gated on forward-scatter versus side-scatter in order to include viable and dead lymphocytes and to exclude debris, doublets and, where present, non-lymphoid cells. As a consequence, presence of CD14+ cells in analysed samples was null or negligible.

VL and T cell counts

HIV-1 RNA VL levels were determined by using the Roche Amplicor HIV-1 Monitor (Roche Molecular System, Branchburg, NJ, USA; limit of detection 400 copies/mL) or by a quantitative ultrasensitive RT–PCR assay (limit of detection 50 HIV RNA copies/mL). An arbitrary value of 200 or 25 HIV RNA copies/mL was assigned to samples below the detection limit of the two assays, respectively, for statistical and graphical management of data. CD4+ and CD8+ cell counts were evaluated on whole blood using flow cytometry analysis according to routine procedures.

Statistical analysis

Preliminary analysis showed that some of the data were not normally distributed, and that no cluster corresponding to different therapy regimens or to gender could be identified within the group of patients with successful response to therapy. As a consequence, data were analysed by non-parametric methods, and therapy and gender subgroups were not considered. Attempts to identify relatively homogeneous subgroups of patients were carried out using k-means cluster analysis. Baseline and follow-up values were compared using non-parametric Wilcoxon matched pairs signed ranks test for related samples, and for a non-parametric correlation, Spearman’s rho correlation coefficients and corresponding two-tailed significances were calculated. For statistical analysis, the numbers of HIV RNA copies/mL were transformed as decimal logarithms, while CD4+ and CD8+ cell counts were transformed...
as corresponding natural logarithms. Statistical analysis was carried out using SPSS software for Windows (SPSS, Chicago, IL, USA).

**Results**

**Virological and immunological response to the therapy**

Twenty-two patients were enrolled in the study. One of them died of acute myocardial infarction 6 months after undergoing PART and was excluded from the study. All the remaining 21 HIV-infected patients were eligible for evaluation. The median time of follow-up was 36 months (range 24–36 months). The Centers for Disease Control classification at enrolment was as follows: 12 patients in class A (three A1, four A2, five A3), four in class B (one B1, one B2, two B3) and five in class C (C3). Patients were classified on the basis of their response to therapy into three subgroups, defined as follows: therapy success (TS; 17 patients), VL <400 copies/mL at the last observation time and overall increasing trend of CD4+ cell count; discordant response (DR, two patients), VL >400 copies/mL at the last observation time with increasing trend of CD4+ cell count (>100 cells/mL with respect to pre-PART); and therapy failure (TF, two patients), VL >400 copies/mL at the last observation time with no increases or increases <100 cells/mL in CD4+ cell count with respect to pre-PART. VL promptly dropped to and remained at undetectable levels in all the patients in the TS groups, except for three patients who experienced transitory viral rebounds concomitant with treatment interruption due to periods of non-adherence or change of therapy. In patients belonging to the DR and TF groups, after a drop in VL during the first 6–12 months, plasma viraemia was not further controlled by PART or showed only transitory decrease in the case of change of therapy. CD4+ and CD8+ cell counts showed a general trend to increase, less pronounced in CD8+ cells, with recurrent fluctuations, both in TS and DR patients. Conversely, in TF patients, CD4+ cells, after an initial increase, slowly, but constantly, declined after virological failure. In particular, this decline was noticeably delayed in one of the two TF patients who had a high CD4+ count at baseline. Differences in the response to therapy and in the trend of apoptosis during PART in the three subgroups of patients we identified suggested the need for a separate analysis of data. However, only the TS subgroup had the numerical requirements for a plausible statistical analysis. As a consequence, hereafter only the data from the TS group are reported and discussed. Characteristics of the patients classified into the TS group as a whole, at baseline and at the last observation time, including VL levels, CD4+ and CD8+ cell counts together with apoptosis levels, are reported in Table 1.

**Long-term variations of apoptosis in TS patients**

Apoptosis showed a prompt, initial inhibition in response to PART and remained at low levels in the long-term in 16 out of the 17 patients of the TS group, with most of the values overlapping those previously reported by us for healthy controls in identical experimental conditions (mean value ~10%). Nevertheless, recurrent minor fluctuations were detected in most of the patients. In fact, major, transitory blips of apoptosis (about a three-fold increase with respect to PART-suppressed baseline levels) were observed concomitant with brief interruptions or changes of therapy with viral rebounds in three of the TS patients. However, no other cluster of data corresponding to the patients with apoptosis blips, except VL rebound, was identified by the k-means cluster analysis. In particular, patients with blips did not differ from the rest of TS patients for NRTI back-bone or clinical manifestations of the disease. Apoptosis levels, as well as VL and CD4+ cell counts, in two representative patients of TS group, one with sustained inhibition (Figure 1a) and one of the three patients who experienced transitory blips (Figure 1b) of apoptosis, during the course of PART, are reported in Figure 1.

**Correlation of PBMC apoptosis with VL, and CD4+ and CD8+ cell count in TS patients**

We investigated, using non-parametric bivariate Spearman’s analysis, whether apoptosis levels detected by flow cytometry, following propidium iodide staining of hypodiploid nuclei, at baseline and during PART, correlated with immunological and virological parameters. Analysis was carried out on values of apoptosis obtained before starting treatment and after different time-points during PART (1, 2, 4, 6, 8, 12, 20, 24 and 36 months) in the group of responder patients, for a total of 160 observations. The data presented in Table 2 show that spontaneous apoptosis of PBMCs was directly and highly significantly correlated with the level of plasma VL (rho = 0.424, P < 0.0001). Moreover, apoptosis was inversely correlated with the CD4+ cell count (rho = −0.409, P < 0.0001) or, less significantly, with the CD8+ cell count (rho = −0.236, P = 0.002). These results extend our previous observation, which was limited to the first 6 months of therapy, to the long-term, confirming an extremely close correlation between proneness to apoptosis in short-term culture of PBMCs from patients responding to PART and established markers of virological and immunological response to therapy over a long period of time.
CD8+ lymphocyte apoptosis and CD4+ cell counts in HIV patients

![Graphs showing therapy success with sustained inhibition and transitory rebound](image)

In a subcohort of seven patients from the TS group, we investigated the relationships between susceptibility to apoptosis of the total lymphocyte population and selectively of the CD4+ and CD8+ PBMC from HIV patients before and during PART. For this investigation, cell death of lymphocytes present in the short-term culture of PBMCs, set up as described above, was assessed in samples stained for double fluorescence in order to selectively distinguish annexin V-positive cells and CD4+ and CD8+ lymphocytes. Figure 2 shows an example of how positivity for annexin V was detected separately in CD4+ and CD8+ cells (Figure 2b and d, respectively) following gating on red fluorescence for CD4 or CD8 positivity (Figure 2a and c, respectively). The death of both CD4+ and CD8+ cells contributed to the percentage of total cells positive for annexin. In fact, levels of total annexin V-positive cells were directly correlated with those of the single annexin-positive CD4+ and CD8+ subsets. However, the correlation of total annexin V-positive lymphocytes with annexin V-positive CD8+ cells was closer than that with annexin V-positive CD4+ cells (rho = 0.891 and 0.474, respectively). As expected, levels of total annexin V-positive cells were highly significantly correlated with levels of apoptosis detected in the parallel samples by means of flow cytometry analysis of hypodiploid nuclei following staining with propidium iodide. In the latter samples, no appreciable levels of nuclei from necrotic cells were detected, thus indicating that cell death by necrosis was negligible in annexin V-positive cells also in the absence of annexin V/propidium iodide double-stained samples.

**Implications of CD4+ and CD8+ cell death on CD4+ cell immune reconstitution**

In order to assess the influence of apoptotic cell death of the distinct CD4+ and CD8+ subsets on the recovery of CD4+ cells in HIV-infected patients successfully responding to PART, analysis was undertaken to investigate the correlation between the absolute number of CD4+ cells and the percentage of annexin V-positive CD8+ and CD4+ cells during the course of the therapy. The results, shown in Figure 3(b) reveal that the log CD4+ cell count was not significantly correlated with the percentage of annexin V-positive CD4+ cells. Conversely, as shown in Figure 3(a), log CD4+ cell count was highly significantly inversely correlated with the percentage of annexin V-positive CD8+ cells (rho = 0.573, *P* < 0.0001). Similarly, no significant correlation was found between changes in CD4+ cell count and the percentage of annexin V-positive CD8+ cells, whereas a highly significant correlation was found between changes in CD4+ cell count and the percentage of annexin V-positive CD8+ cells (rho = 0.471, *P* < 0.0001; data not shown). Moreover, no significant correlation was found between the absolute number of CD8+ cells and the percentage of annexin V-positive CD8+ or CD4+ cells in the same group of patients undergoing therapy (data not shown). These results, together with those showing a highly significant correlation between total lymphocyte apoptosis and CD8+ cell apoptosis, indicate that the inverse relationship we found between CD4+ cell counts and total lymphocyte apoptosis is mainly attributable to the CD8+ subset.

**Table 2. Correlation between apoptosis level, VL, and CD4+ and CD8+ cell count during potent antiretroviral therapy in 17 individuals with successful response to therapy**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sperman’s rho correlation coefficient</th>
<th><em>P</em> value (two-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL (log)</td>
<td>0.424</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD4+ cell count (ln)</td>
<td>−0.409</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD8+ cell count (ln)</td>
<td>−0.236</td>
<td>&lt;0.002</td>
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*All tests were based on 160 observations.

**Discussion**

Although there is general agreement that suppression of apoptosis represents a peculiar, prompt response to PART in HIV patients, its relationship with variations in immunological and infectious parameters during therapy is still highly debatable. The present study is, to our knowledge, the first long-term longitudinal study performed on a selected and homogeneous cohort of patients, focused on the overall variation of apoptosis, VL and CD4+ cell count during PART at numerous time points. It led to the collection of a relatively high number of data for correlations. Data reported show that apoptosis...
levels in short-term cultured PBMCs from HIV patients, even after 3 years of PART, remain noticeably decreased in patients showing positive response to therapy. Moreover, they clearly confirm our previous observation in the short-term, which showed statistical correlations between levels of PBMC apoptosis versus CD4+ counts as well as versus VL in patients undergoing PART.

One of the key aspects of pathogenesis of HIV infection is whether CD4+ cell depletion is mainly due to direct destruction by the virus or rather to selective lymphopenia caused by other, virus-driven mechanisms. Recent reports sustain that the latter phenomenon is very probably involved in CD4+ cell depletion.\textsuperscript{20,21} It has been shown that PART inhibits both CD4+ and CD8+ cell decay rates \textit{in vivo}.\textsuperscript{22} This implies that the death of T lymphocytes during HIV infection is reasonably, or at least partly, independent of direct killing by the virus, as suggested.\textsuperscript{23} Thus, inhibition of PBMC apoptosis during PART could be related to modifications occurring in either CD4+ or CD8+ cell subsets. These could involve indirect mechanisms such as the modulation of pro-inflammatory factors and other mediators, as hypothesized.\textsuperscript{18} However, from existing data it was not clear to what extent modulation of cell death of the two distinct T cell subsets contributes to generate overall PBMC apoptosis inhibition during successful PART. In this context, a major new finding of the present study is the direct evidence that the decrease in CD8+ cell death contributed more than the decrease in CD4+ cell death to determining the inhibition of total PBMC apoptosis in TS patients. Moreover, other evidence comes from our observation that levels of CD4+ cell counts during PART were inversely correlated with spontaneous apoptosis levels in CD8+, but not in CD4+, lymphocytes. This is in agreement with results of a recent cross-sectional observation.\textsuperscript{15} Correlation is not sufficient by itself to infer a causal relationship; in fact, we can simply hypothesize that both decrease in CD8+ cells apoptosis and increase in CD4+ cell counts are secondary phenomena to VL control during PART. In fact, it has been suggested that control of aberrant CD4 signalling as a consequence of reduction of virus burden may be one of the mechanisms of apoptosis decrease in CD8+ cells following antiretroviral therapy.\textsuperscript{24} Another mechanism for reduction of apoptosis in CD8+ cells following PART could be an altered level of cytokines acting as CD8+ cell pro-survival factors, such as interleukin-7 or -15. However, we can also hypothesize that the increase in CD4+ cells during PART directly depends on the inhibition of apoptosis in CD8+ cells. In fact, the rescue of CD8+ cells could play a key role in immune reconstitution during PART. Particularly, it could act on the specific response by developing cytotoxic T lymphocytes,\textsuperscript{25} or on the non-specific response by improving the release of factors exerting an antiviral action, such as the recently identified \(\alpha\)-defensins.\textsuperscript{26} Surprisingly, on the other hand, percentages of annexin V-positive CD4+ cells during PART were not correlated inversely with levels of CD4+ cell counts. This could be explained by the possibility that

\begin{figure}
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\caption{Evaluation of apoptosis selectively in CD4+ or CD8+ cells by detection of annexin V positivity in short-term cultured PBMCs from a representative HIV-infected patient prior to PART. CD4+ or CD8+ cells were gated on red fluorescence (a), gate R2 and (c), gate R3, respectively) and then analysed for positivity for annexin V-FITC (b) and (d), respectively). M1 and M2 indicate the boundaries between cells negative (M1) and positive (M2) for annexin V, which were arbitrarily set on cells stained with relevant control monoclonal antibodies (not shown) and maintained for CD4-PE/annexin V-FITC or CD8-PE/annexin V-FITC double-stained samples. Note that y-axes have different full-scale counts in (b) and in (d). All other axes have the same scale values, which have been omitted.}
\end{figure}
CD8+ lymphocyte apoptosis and CD4+ cell counts in HIV patients

![Figure 3](Image)

CD8+ ANX+/CD4+ ANX+ double-positive cells detected at the same time and in the same patient. Spearman’s rho correlation coefficients are reported in the graphs. The corresponding two-tailed significance was $P < 0.0001$ for (a) and $P = 0.782$ for (b).

a portion of CD4+ cells rescued during PART were nevertheless prone to apoptosis. They could belong to the ‘short-lived’ cells on the basis of the ‘biphasic T-cell die-away kinetic’, in which it has been hypothesized that HIV-infected patients exhibit different proportions of short-lived and long-lived subpopulations that are regulated by their rate of cell death. In any case, absence of correlation seems to exclude the possibility that increase in CD4+ lymphocytes in response to PART is the direct consequence of apoptosis inhibition in CD4+ cells. Moreover, it could be related to the delayed and usually incomplete restoration of CD4+ cell number, even after sustained undetectable VL in PART-treated patients.

Taken together our data show that CD4+ cell rescue during PART is not dependent on the inhibition of CD4+ cell apoptosis, but, rather, it is related to the decrease of apoptosis in the CD8+ subset. Further studies need to be performed in order to understand the mechanisms involved in this phenomenon, and its significance.

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