RAPID COMMUNICATION

Mitochondrial Dysfunctions in Circulating T Lymphocytes From Human Immunodeficiency Virus-1 Carriers

By Antonio Macho, Maria Castedo, Philippe Marchetti, José J. Aguilar, Didier Decaudin, Neoufal Zamzami, Pierre M. Girard, José Uriel, and Guido Kroemer

In several models of lymphocyte apoptosis, two alterations of mitochondrial function precede advanced DNA fragmentation: (1) a reduction of mitochondrial transmembrane potential (ΔΨm) and (2) an increase in mitochondrial generation of superoxide anion. Here we show that two fluorochromes allow for the identification of analogous mitochondrial perturbations in circulating T lymphocytes from human immunodeficiency virus (HIV-1) donors. The first among these fluorochromes, the cationic lipophilic dye DiOC6(3), measures ΔΨm; the second marker, hydroethidine (HE), is nonfluorescent, unless it is oxidized by superoxide anions to the product ethidium (Eth). CD4+ or CD8+ cells from clinically asymptomatic HIV-1 carriers contain a significantly elevated percentage of cells endowed with enhanced HE → Eth conversion and/or reduced DiOC6(3) uptake as compared with normal controls. Phenotypic characterization of (HE → Eth)low cells from HIV+ donors shows that these cells possess a low ΔΨm, thus demonstrating a functional alteration of mitochondria. In addition, (HE → Eth)high cells display a reduced incorporation of the cardiolipin-specific dye nonyl-deridine orange (NAO), showing a structural defect of the cardiolipin-containing inner mitochondrial membrane. Control experiments involving rotenone, an inhibitor of the respiratory chain complex I, indicate that the reactive oxygen species responsible for HE → Eth conversion is generated during mitochondrial electron transport. In synthesis, it appears that mitochondrial alterations occur in a significant percentage of circulating T lymphocytes from HIV-1 carriers. The extent of ΔΨm reduction, as determined ex vivo, correlates with the frequency of cells undergoing DNA fragmentation after overnight in vitro culture. These observations may be important for the understanding and for the direct ex vivo quantitation of HIV-triggered lymphocyte destruction. © 1995 by The American Society of Hematology.

It is generally assumed that apoptosis or programmed cell death (PCD) is the mechanism by which superfluous or damaged cells are removed in most organ systems. This applies to organogenesis, the maintenance of the status quo in normal tissues, responses to light (subneurogenic) or chemical, or physical damage, as well as acquired immunodeficiency syndrome (AIDS) pathogenesis. The advanced stage of PCD is marked by characteristic alterations of nuclear morphology and a step-wise degradation of chromosomal DNA.1,2 Accordingly, recent techniques for the detection of apoptotic cells are based on the assessment of DNA breaks that create potential acceptor sites for DNA polymerase or terminal deoxynucleotidyl transferase.3,4 However, even during treatments that cause massive cell depletion in vivo and are known to cause apoptosis in vitro, end-stage apoptosis is difficult to be detected in freshly isolated peripheral lymphocytes from the spleen, lymph nodes, or the circulation.3,5,6 This phenomenon can be attributed to the sequestration or phagocytic removal of cells undergoing apoptosis in vivo.10 To show DNA alterations indicative of apoptosis in peripheral lymphocytes, it is indispensable to culture cells during a few hours in vitro. This has been demonstrated for mice treated with lymphocyte-depleting doses of glucocorticoids,7 superantigens,8 or peptide antigens,9 for patients treated with cytotoxic drugs,8 for patients with spontaneous tumor remissions,10 as well as for human immunodeficiency virus (HIV)-infected donors.3,11

We have recently shown that thymocytes or lymphocytes exposed to apoptosis-inducing doses of glucocorticoids demonstrate a step-wise dysregulation of mitochondrial function that precedes signs of nuclear apoptosis. Cells sequentially exhibit a first reduction of the mitochondrial transmembrane potential (ΔΨm), as quantifiable by means of suitable fluorochromes [DiOC6(3), JC-1, rhodamine 123], and then an additional decrease in ΔΨm accompanied by an increased superoxide anion-mediated oxidation of hydroethidine (HE) into the fluorescent product ethidium (Eth).12,14 Disruption of the ΔΨm is not a special feature of glucocorticoid-stimulated peripheral lymphocytes. Rather, it constitutes a general feature of early apoptosis. The early disruption of ΔΨm is observed in all models of PCD tested: (1) withdrawal of trophic factors (e.g., nerve growth factor [NGF] from neurons), (2) hormones (tumor necrosis factor, glucocorticoids), (3) lethal activation (cross-linking of the T-cell receptor or surface Ig in T and pre-B cells, respectively), and (4) second messengers (phorbol ester plus ionomycin in thymocytes, ceramide in different cell types).12,14 At least a fraction of cells with low ΔΨm is irreversibly committed to undergo DNA fragmentation, as shown by experiments in which glucocorticoid-primed ΔΨm cells with yet-intact DNA were purified and cultured in vitro, after withdrawal of the apoptosis-inducing stimulus by means of a glucocorticoid receptor antagonist.15 These data underline the notion that nuclear alterations accompanying apoptosis are relatively late events of the apoptotic cascade16 and that a cytoplasmic (mitochondrial?) control instance can dictate the fate of the cell.16,17

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Stimulated by these observations, we have tested whether perturbations of mitochondrial structure or function might occur among circulating T lymphocytes from individuals infected with HIV-1. Here we show that circulating lymphocytes from HIV-infected donors are characterized by a decreased ΔΨm and/or enhanced mitochondrial reactive oxygen species (ROS) generation, suggesting that HIV-triggered apoptotic lymphocyte depletion obeys the same rules as physiologic PCD. These insights are important for the comprehension of HIV-mediated lymphocyte depletion, as well as for the direct quantitative evaluation of ongoing PCD in AIDS.

MATERIALS AND METHODS

Patients and lymphocyte preparation. Peripheral blood mononuclear cells (PBMC) were obtained from HIV-1+ donors fulfilling the following characteristics: 21 to 50 years of age (mean ± SD; 38 ± 8, n = 22), lymphocyte counts 507 to 2,540 (1,646 ± 549) per μL, 3.2% to 29.5% CD4+ T cells among peripheral blood lymphocytes (12.7% ± 6.4%); 30.0% to 80.5% CD8+ lymphocytes (56.3% ± 12.9%). Patients are listed in Table 1. A total of six patients exhibited clinical or laboratory parameters of AIDS (Centers for Disease Control [CDC] AIDS surveillance case definition), three of whom received treatment with antibiotics and azidothymidine and/or enhanced mitochondrial reactive oxygen species (ROS) generation, as well as ΔΨm, cells were first stained with phycoerythrine (PE)-labeled anti-hCD4 (13B8.2) or anti-hCD8α (B9.11; Immunotech, Marseille, France) antibodies (30 minutes on ice). Cells were washed (5 minutes; 600g; 4°C) in ice-cold staining buffer (phosphate-buffered saline [PBS], pH = 7.2, supplemented with 2% bovine serum albumin [BSA]) followed by exposure during 15 minutes at 37°C to hydroethidine (HE; 2 μmol/L; Molecular Probes, Eugene, OR).8 For the simultaneous determination of one surface marker and mitochondrial ROS generation, as well as ΔΨm, cells were first stained with PE-labeled antibodies and then exposed to DiOC3(3) (3,3’-dihexyloxacarbocyanine iodide; 40 nmol/L; Molecular Probes)9 and HE (2 μmol/L; 37°C, 15 minutes).18 In control experiments, cells were labeled after preincubation with the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (mCCCP; 50 μmol/L; 37°C, 30 minutes; Sigma, St Louis, MO), menadione (1 mmol/L; 37°C, 1 hour; Sigma), or rotenone (1 mmol/L; 37°C, 30 minutes; Sigma). In some tests, HE labeling was combined with 10-N-nonyl-acridine orange (NAO; 100 nmol/L; 37°C, 15 minutes)9 or 2’,7’-dichlorofluorescin diacetate (DCFH-DA; 5 μmol/L; 37°C, 15 minutes; Molecular Probes).22 Annexin V-FTTC conjugate (250 ng/mL, 10 minutes at 4°C; Brand Application BV, Maastricht, The Netherlands) was used to detect phosphatidylserine residues22 exposed at the cell surface of HE-labeled cells. Analyses were performed on an Epics Profile II cytofluorometer (Coulter, Hialeah, FL). Forward and side

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Abbreviation: ND, not determined.
* CDC AIDS data surveillance criteria. All patients falling into the clinical category “C” or possessing <200 CD4+ T lymphocytes/μL blood are considered to be afflicted with AIDS.
† Persistent generalized lymphadenopathy.
‡ Same data (in %) as in Fig 2B.
§ Same data (in %) as in Fig 1B.
MTCHONDRIAL DYSFUNCTIONS IN HIV-1 INFECTION


compensation. desoxyribonucleotidyltransferase dUTP into permeabilized cells ("Tunnel" method), following the DiOC6(3), DCFH-DA, and NAO was recorded in FL-1 (525 nm), PE in FL-2 (575 nm), and HE in FL-3 (600 nm) after suitable compensation.

Cell culture and assessment of DNA fragmentation. Cells (5 × 10^6/mL) were cultured (18 hours, 37°C) in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, and HEPES. DNA fragmentation was assessed among cultured lymphocytes using terminal deoxynucleotidyltransferase to incorporate Fluorescein-12-dUTP into permeabilized cells ("Tunnel" method), following the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis. Results are computed as X ± SEM and differences between groups are calculated by means of the nonpaired Student’s t-test. Correlations were calculated by regression analysis using the least-square difference method.

RESULTS AND DISCUSSION

T cells from HIV-infected donors exhibit a ΔΨm decrease and enhanced ROS generation. It is widely assumed that AIDS-associated lymphopenia is the result of an enhanced apoptotic turnover of peripheral T lymphocytes. However, circulating T lymphocytes from HIV+ donors fail to exhibit signs of DNA fragmentation when analyzed directly ex vivo, and it is necessary to culture such cells in vitro to reveal nuclear signs of apoptosis. To resolve this dilemma, we have measured mitochondrial alterations that are associated with an early stage of the apoptotic process, as it occurs in a number of different systems of physiologic apoptosis induction. As shown for one representative asymptomatic HIV+ donor (Fig 1A), circulating lymphocytes from HIV+ donors contain a fraction of cells that are able of oxidizing the nonfluorescent lipopholic (ie, membrane-permeable) dye HE into the hydrophilic fluorescent product Eth. The percentage of such cells, which bear an (HE → Eth)high phenotype, is elevated when compared to noninfected controls (Fig 1A). This applies to both CD4+ and CD8+ T cells (Fig 1, A and B), which are freshly isolated from peripheral blood. Statistical analysis showed a highly significant (P < .01) difference between HIV+ donors and healthy controls. In addition, we found that, irrespective of the CD4/CD8 phenotype, a relatively high percentage of peripheral blood T lymphocytes from HIV+ donors incorporate low levels of DIOC6(3) (Fig 2A), a dye that incorporates into mitochondria in strict dependence of the ΔΨm. Again, this parameter was significantly different between HIV+ donors and uninfected age- and sex-matched controls (Fig 2B).

No correlation between the clinical status of HIV carriers and HE → Eth conversion or DIOC6(3) incorporation was observed (Table 1). Both asymptomatic HIV carriers and patients with manifest clinical signs of AIDS possess approximately the same percentage of (HE → Eth)high and DIOC6(3)high cells (Figs 1B and 2B). Most of the HIV+ donors characterized in this study (Figs 1A and 2A, open circles in Figs 1B and 2B) were free from clinical signs of secondary infections, yet exhibit enhanced HE → Eth conversion (Fig
1) and reduced DiOC<sub>6</sub>(3) incorporation (Fig 2). These results suggest that perturbed lymphocyte metabolism is a consequence of HIV infection rather than a result of secondary infections by opportunistic microorganisms.

In synthesis, circulating T lymphocytes from HIV-infected yet-asymptomatic individuals display an increase in two functionally aberrant subsets: (1) a major subset of cells that possess a low ΔΨ<sub>m</sub> and (2) a minor population that hypergenerates ROS.

Relationship between ΔΨ<sub>m</sub> decrease and ROS hypergeneration. The mean difference between HIV<sup>+</sup> and HIV<sup>-</sup> groups is less pronounced for HE → Eth conversion (approximately 3%, Fig 1B) than for DiOC<sub>6</sub>(3) staining (approximately 20%, Fig 2B). To unravel the relationship between HE → Eth conversion and DiOC<sub>6</sub>(3) staining, cells from controls and HIV<sup>+</sup> donors were labeled simultaneously with HE and DiOC<sub>6</sub>(3). As shown in Fig 3, all (HE → Eth)<sup>high</sup> cells possess a low ΔΨ<sub>m</sub>. Thus, HIV infection is accompanied by an increase of two subpopulations of ΔΨ<sub>m</sub><sup>−</sup> cells, a first population that still lacks ROS generation and a second one that hyperproduces ROS. Among these populations, the first one has the same phenotype as control cells treated with the protonophor mCCCP (inset in Fig 3), and the second behaves like control cells exposed to menadione, a substance that causes mitochondrial ROS generation as well as ΔΨ<sub>m</sub> disruption. Treatment of lymphocytes from HIV<sup>+</sup> donors with rotenone, an inhibitor of the respiratory chain complex I, greatly reduces labeling with HE (Fig 3) and thus confirms that the ROS responsible for HE → Eth conversion are generated in mitochondria. Accordingly, (HE → Eth)<sup>high</sup> lymphocytes do not stain with DCFH-DA (Fig 3), a fluorescent probe that detects H<sub>2</sub>O<sub>2</sub> formation but is not sensitive to superoxide anions. Therefore, the principal ROS that is produced by T cells from HIV<sup>+</sup> donors is likely to be superoxide anion, an ROS that is preferentially produced in mitochondria and to which HE is particularly sensitive.

Correlation between ΔΨ<sub>m</sub> decrease and nuclear signs of apoptosis in cells from HIV carriers. In a further series of experiments we attempted to correlate mitochondrial perturbations, as assessed by DiOC<sub>6</sub>(3) staining, with nuclear signs of apoptosis. When cultured during 18 hours in vitro, a significant portion of cells from HIV<sup>+</sup> individuals undergo DNA fragmentation, as detected by the incorporation of fluoroscein-12-dUTP catalyzed by the enzyme terminal deoxynucleotidyltransferase. The percentage of cells that spontaneously undergo DNA fragmentation after overnight in vitro culture correlates positively with the percentage of ΔΨ<sub>m</sub><sup>−</sup> cells determined ex vivo (Fig 4). As a consequence, it appears plausible that those cells which are characterized by a low ΔΨ<sub>m</sub> ex vivo are programmed to die from apoptosis.

As described previously, no correlation between nuclear apoptosis occurring after in vitro culture and progression to disease was observed (Fig 4). As a consequence, the lack of correlation between mitochondrial parameters of apoptosis and the clinical state of patients appears to be coherent.

ROS-producing lymphocytes from HIV<sup>+</sup> donors exhibit structural alterations of membranes. To obtain information on the membrane structure of (HE → Eth)<sup>high</sup> lymphocytes
from HIV+ donors, we performed double-staining experiments with two markers of membrane structure, NAO and annexin V (Fig 5). NAO is a fluorochrome that interacts stoichiometrically with cardiolipin in the inner mitochondrial membrane, independently of the $\Delta \psi_m$.13,20 (HE $\rightarrow$ Eth)$^{\text{high}}$ cells exhibit a relatively weak staining with NAO, indicating that ROS hypergeneration is coupled to a structural alteration of mitochondria. Moreover, we found that a minor fraction of (HE $\rightarrow$ Eth)$^{\text{high}}$ cells, especially those with the strongest capacity of HE oxidation, bind an annexin V-FITC conjugate. Conversely, all cells that bind annexin V are (HE $\rightarrow$ Eth)$^{\text{high}}$. The cytoskeleton protein annexin V reacts with phosphatidyl serine residues that are aberrantly exposed on the outer leaflet of membranes and may serve as a marker of early apoptosis.22 Because phosphatidyl serine residues allow for the phagocytic recognition of apoptotic cells,15 it can be speculated that (HE $\rightarrow$ Eth)$^{\text{high}}$ annexin V+ cells represent a stage of early apoptosis that is subject to in vivo purging by the reticuloendothelial system.

In conclusion, two membrane alterations affect (HE $\rightarrow$ Eth)$^{\text{high}}$ cells from HIV-1 carriers. All (HE $\rightarrow$ Eth)$^{\text{high}}$ cells carry a defect of the inner mitochondrial membrane, whereas...
a minor subset of \((\text{HE} \rightarrow \text{Eth})^{\text{high}}\) cells exposes phosphatidylserine residues at the surface.

**CONCLUDING REMARKS**

Lymphocytes undergoing physiological PCD suffer a sequential dysregulation of mitochondrial function early during the apoptotic process.12-14 Cells first exhibit a strong reduction in \(\Delta \Psi_m\), then they manifest a phenotype similar to menadione-treated cells, namely a near-to-complete disruption of \(\Delta \Psi_m\) accompanied by the generation of superoxide anions (Fig 3). Here we present data suggesting that this sequence of early apoptotic changes also applies to lymphocytes derived from HIV-infected individuals (Figs 1 through 3). Thus, cytofluorometric assessment of \(\Delta \Psi_m\) and superoxide anion production show an enhanced frequency of DiOC\(_6\)(3)\(^{\text{low}}\) and \((\text{HE} \rightarrow \text{Eth})^{\text{high}}\) cells among CD4\(^+\) and CD8\(^+\) lymphocytes from HIV\(^+\) donors when compared with age-matched normal controls. These alterations are found among freshly isolated cells that still lack nuclear DNA alterations, without any need of in vitro culture.

The observation that mitochondria from HIV\(^+\) lymphocytes exhibit a \(\Delta \Psi_m\) reduction is compatible with the decrease of \(\Delta \Psi_m\) caused by in vitro infection of cell lines with feline AIDS virus, a retrovirus that shares several functional and structural characteristics of HIV.26 Moreover, antioxidant inhibition of HIV-associated T-lymphocyte apoptosis in vitro27 suggests that elevated superoxide anion production by a fraction of HIV\(^+\) lymphocytes is indeed important for lymphocyte depletion. As to the mechanism of mitochondrial alterations associated with HIV infection, two findings may be relevant. On one hand, HIV-encoded RNA has been found to selectively enrich in mitochondria of infected cells,28 thereby hinting at the possibility that HIV products directly affect mitochondrial function in situ. On the other hand, HIV gene expression has been associated with a depletion of cells from oxidative stress, namely a reduction of glutathione peroxidase activity29 and a Tat-mediated transcriptional suppression of a mitochondrial enzyme responsible for superoxide detoxification, Mn-dependent superoxide dismutase (SOD2).30 Insufficient conversion of superoxide anions into \(\text{H}_2\text{O}_2\) in HIV-infected cells would be compatible with the negative DCFH-DA staining of \((\text{HE} \rightarrow \text{Eth})^{\text{high}}\) cells (Fig 3). Speculatively, deficient detoxification of superoxide anions could further enhance the vulnerability of HIV-infected cells to the apoptosis-inducing effect of endogenous ROS. However, it is premature to extrapolate these published data—that have been obtained in vitro26-30—to lymphocyte populations recovered from HIV-infected donors ex vivo (this report). Indeed, both direct cytopathic effects31,32 and indirect HIV effects (gp120-CD4 interactions, self-destructive Fas/FasL loops, etc)33,34 could give rise to the apoptotic destruction of T cells. The DiOC\(_6\)(3)/HE staining used in this report cannot discriminate between these possibilities. Nevertheless, the assessment of mitochondrial structure and function may allow for a direct estimation of HIV-induced lymphocyte depletion and thus may provide a useful tool for the in vivo/ex vivo evaluation of antiretroviral and/or apoptosis-inhibitory treatments. Indeed, the determination of mitochondrial parameters unravels apoptosis-associated dysfunctions among circulating cells at a moment at which conventional tests addressing nuclear parameters of apoptosis fail to yield positive results.

**ACKNOWLEDGMENT**

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**REFERENCES**


Mitochondrial dysfunctions in circulating T lymphocytes from human immunodeficiency virus-1 carriers [see comments]

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