Different Sensitivity to Apoptosis in Cells of Monocytic or Lymphocytic Origin Chronically Infected with Human Immunodeficiency Virus Type-1

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Apoptotic death of CD4+ T lymphocytes is a major cause of the immunodeficiency caused by human immunodeficiency virus (HIV), but it is still unclear how this process precisely occurs. To characterize a potentially useful cellular model, we have analyzed the tendency of chronically HIV-infected CD4+ human cell lines of different origin to undergo apoptosis. We studied ACH-2 and U1 lines, derived from the CD4+ T-cell A301 and the promonocytic U937 cell lines, respectively, and induced apoptosis *via* several stimuli that trigger different pathways. Their capacity to regulate plasma membrane CD95 expression and to produce soluble CD95 was also analyzed. Using staurosporine, TNF-α plus cycloheximide, and γ-radiations, we observed that ACH-2 were more sensitive to programmed cell death than A301, while U1 were less sensitive than U937. Both infected cell types had a lower sensitivity to CD95-induced apoptosis; the analysis of changes in mitochondrial membrane potential corroborated these observations. Plasma membrane CD95 was similarly regulated in all cell types, which, however, presented a different capacity to produce soluble CD95 molecules.

Our *in vitro* results may offer a new perspective for developing further studies on the pathogenesis of HIV infection. A chronically infected cell line of lymphocytic origin is more susceptible to apoptosis than its parental cell type, while infected monocytic cells are less sensitive than their uninfected counterpart. Thus, it is possible to hypothesize that one of the reasons by which circulating monocytes survive and represent a viral reservoir is the capacity of HIV to decrease the sensitivity to apoptosis of this cell type. However, further studies on *ex-vivo* collected fresh cells, as well as on other cell lines, are urgently needed to confirm such hypothesis. Exp Biol Med 228: 1346–1354, 2003

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Apoptosis plays an important role during human immunodeficiency virus (HIV)-type 1 infection (1). Programmed cell death has been studied under a variety of molecular and cellular perspectives and in different categories of HIV-positive individuals. Several groups, including ours, demonstrated that a positive correlation exists between peripheral blood lymphocytes' (PBL) *in vitro* tendency to undergo apoptosis and the progression/gravity of the infection, from individuals with primary infection to the so-called "long-term non progressors" (2–6).

The interactions between HIV and the host are complex. The increased frequency of apoptosis in PBL from HIV-positive subjects is paralleled by the fact that only a minority of cells that will die are effectively infected. HIV is able to infect either lymphocytes or monocytes and macrophages, but the latter cells do not seem to undergo apoptosis following infection, and represent a potential reservoir for virus production (7).

Many mechanisms can be triggered that provoke the death of immune cells, including a partial, not complete activation (i.e., delivery of the first, activatory signal not followed by the second), the action of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, the activation of receptors that mediate cell death, such as CD95

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(APO-1/Fas), among others (8, 9). However, one of the key problems that awaits solution regards the individual sensitivity of infected cells to apoptosis, and studies in such cells are crucial to understand the interactions between virus and host that cause or inhibit this phenomenon. On one side, apoptosis is a defense mechanism that is activated to clear mutated or infected cells. On the other side, viruses have no advantages in cell death, and have thus developed anti-apoptotic strategies (10, 11).

Recently, using chronically infected cell lines, we studied the expression of a member of the TNF receptor family that is deeply involved in this process (i.e., CD95) along with the expression of its ligand (CD178/FasL) (12). CD95 crosslinking by CD178 or by agonistic monoclonal antibodies (mAbs) resulted in rapid induction of apoptosis in susceptible cells. We have shown that the lymphocytic cell line A301 and its HIV-infected clone ACH-2 displayed similar levels of plasma membrane CD95. Promonocytic HIV-infected U1 cells had instead a consistent decrease in membrane CD95 expression compared with the parental cell line U937. The subsequent analysis of mRNA encoding for both membrane and soluble forms of CD95, with an original approach based upon quantitative competitive reverse transcriptase–polymerase chain reaction (QC RT-PCR), confirmed such observations (13).

In this article, we have further characterized a widely used cellular model for chronic HIV infection. Indeed, we have analyzed the tendency to undergo apoptosis of these cell lines using different stimuli, to gain further insights into modulation of sensitivity to apoptosis determined by HIV in well-defined, controlled cellular models.

Materials and Methods

Cell Lines. The following cell lines of human origin were studied: A301, ACH-2, U937, and U1. The human ACH-2 and U1 chronically HIV-infected cells were derived from the T-cell A301 and the promonocytic U937 cell lines, respectively, by limiting dilution of cells surviving acute *in vitro* infection with HIV-$1_{HTLV\text{-}III/LAV}$ (14, 15). ACH-2 and U1 cell lines are well-characterized models of post-integration latency harboring one and two stable integrated HIV proviruses, respectively, which are carried over by 100% of progeny cells after each cell division (16–21). Restricted HIV-1 expression in these cell lines consists of singly and multiply HIV-1–specific RNA species, with little or no full-length genomic RNA (16–21). The resulting low-level constitutive viral expression (detectable through measurement of p24 Ag or of reverse-transcriptase (RT) activity) is readily inducible by a number of factors including cytokines, which act at transcriptional (i.e., TNF-α) or post-transcriptional (i.e., IL-6, IFN-γ) levels (22). The source of infectious virus for both parental cell lines was the HIV-$1_{LAV/IIIB}$ strain; therefore both cell lines carry the same type of virus, which after the discovery of the HIV co-receptors has been assigned to the X4 viruses (23, 24) (i.e., the ones that use CXCR4 as co-receptor for entry in addition to the CD4 primary receptor).

Indeed, both T lymphocytes and monocyte-derived macrophages express CXCR4 and are able to be infected by CXCR4 using primary strains of HIV-1 (25). Although CCR5 is the major co-receptor involved in HIV transmission, broadening of co-receptor usage, including CXCR4 usage, has been associated with disease progression (26). The U1 and ACH-2 cell lines were kept in complete culture medium (i.e., RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, at 37°C in a humidified atmosphere (5% CO_2 in air)). Cells were collected during the log phase of growth, when viability was more than 95%, and immediately used for the experiments described below.

Chemicals. Staurosporine (STS), propidium iodide (PI), ionomycin, TPA (12-O-tetradecanoylphorbol-13-acetate), and cycloheximide (CHX) were from Sigma (St. Louis, MO). JC-1 was from Molecular Probes (Eugene, OR). Anti-CD95 mAb (clones CH11, IgM, and ZB4, IgG) were from Upstate Biotechnology (Lake Placid, NY). All chemicals were stored and used as suggested by the manufacturers.

Induction of Apoptosis. *Incubation with cycloheximide and TNF-α.* 1×10^6 cells were collected, washed once with cold phosphate-buffered saline (PBS), resuspended in 1 ml of complete medium, pre-treated with 4 μM CHX for 2 hrs and then with 50 IU/ml of TNF-α for 6 hrs, as described (27).

Incubation with staurosporine. 1×10^6 cells were collected, washed once with PBS, resuspended in 1 ml of complete medium and incubated for 6 hrs with 1, 5, or 10 μM STS (28).

Irradiation of cells. 1×10^6 cells were collected and exposed to 5 or 20 Gy of γ-radiations, then incubated in complete culture medium for 3 or 18 hrs, as described (29).

Incubation with anti-CD95 mAbs. 1×10^6 cells were treated with anti-CD95 mAb (clone CH11, 20 ng/mL), which is able to induce apoptosis, or with the mAb produced by the clone ZB4 (100 ng/mL), which binds CD95 but does not induce apoptosis, or with both, as described (3). Cells were incubated for 18 hrs in 1 ml of complete medium, then analyzed by flow cytometry.

Flow Cytometry. *Late apoptosis.* Late apoptosis has been evaluated by the appearance of the hypodiploid peak of propidium iodide (PI) fluorescence, as described (30). Briefly, cells were resuspended in hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml PI, and kept for 20 min at 4°C. Therefore, cells were analyzed: those with low PI fluorescence contained less DNA and were thus considered apoptotic.

Mitochondrial membrane potential. Cells were stained with the Δψ-sensitive probe JC-1 used at the concentration of 10 μM in RPMI 1640 medium containing 10% FCS for 10 min at room temperature in the dark, as de-

scribed elsewhere (31). Briefly, JC-1 is a lipophilic carbocyanine that exists in a monomeric form and is able to accumulate into mitochondria. In the presence of a high $\Delta\psi$, JC-1 can reversibly form aggregates that, after excitation at 488 nm, emit in the orange/red channel (fluorescence-2, FL-2). Monomers emit in the green channel (that of FL1). The collapse in $\Delta\psi$ provokes the decrease in the number of JC-1 aggregates (revealed by a decrease in FL2) and a consequent increase of monomers (increase in FL1) (32). Thus, in a typical dot plot obtained by flow cytometric analysis, cells with depolarized mitochondria move from the upper left to the lower right quadrant of the panel (33).

Plasma membrane expression of CD95. We used an anti-CD95 (DX2, mouse IgG1,κ), directly conjugated with phycoerythrin (PE), from Pharmingen (San Diego, CA), as described (34). Differences in the plasma membrane expression of CD95 were calculated considering the median fluorescence intensity of the populations stained with fluorescent anti-CD95 mAb, from which the background (i.e., the autofluorescence, or the fluorescence due to an irrelevant mAb) was subtracted, as described (35).

All cytometric analyses were performed using a FACScan cytometer (Becton Dickinson, San José, CA) equipped with an argon ion laser tuned at 488 nm. In all cases, a minimum of 10,000 cells per sample were acquired in list mode and analyzed with WinMDI 2.8 software.

Real-Time Polymerase Chain Reaction for Quantitative Analysis of mRNA Expression. Total RNA was extracted from the cell lines using RNeasy Mini-kit by QIAgen, following instructions supplied by the manufacturer. One microgram of RNA was reverse transcribed using Random hexamers, with RevertAid First Strand cDNA Synthesis Kit by Fermentas (Vilnius, Lithuania). The total volume of reverse transcription reaction was 25 μl; 1/25 of cDNA was used for each real-time PCR amplification. The primers and probes for real-time PCR were designed using "Primer3" software, available at the website http://www.-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. Primers for the whole pool of CD95 (Fas) messengers were: Fas Forward: 5′ TGAAGGACATGGCTTAGAAGTTG-3′, Fas Reverse: 5′-GGTGCAAGGGTCACAGTGTT-3′, Fas Probe 5′-(FAM)-AAACTGCACCCGGACCCAGAATACC-(TAMRA)-3′. The primers for CD178 (FasL) were: FasL Forward: 5′-GCAGCCCTTCAATTACCCAT-3′, FasL Reverse: 5′-CAGAGGTTGGACAGGGAAGAA-3′, FasL Probe was: 5′-(FAM)-TCCCCAGATCTACTGGGTGGACAGC-(TAMRA)-3′. The primers for L13/HBP were: HBP Forward 5′-ACCGGTAGTGGATCTTGGCTTT-3′, HBP Reverse 5′-GCTGGAAGTACCAGGCAGTGA-3′, HBP Probe was: 5′-(TexasRed)-TCTTTCCTCTTCTCCTCCAGGGTGGCT-(BlackHole-Quencher2)-3′. These sets of primers amplify 118 base pairs (bp) of CD95, 101 bp of CD178, and 104 bp of HBP. The primers were synthesized by Operon Qiagen (Alameda, CA). A portion of each cDNA was used for quantitative PCR in 50 μl of PCR master mix consisting of PCR buffer 1X (Promega, Madison, WI), $MgCl_2$ 3 mM, 200 nM of each primer for HBP amplification, dNTPs 200 nM, 2U *Taq* polymerase, HBP probe 300 nM, tFas or mFas or FasL probe 300 nM. Each sample was monitored in triplicate for each mRNA. The expression of the messengers was calculated as relative expression referred to 1000 copies of HBP mRNA, on the basis of the difference in the threshold cycle of the mRNA for tFas, mFas or FasL and the threshold cycle of HBP mRNA, as described (36).

ELISA. ELISA for measurement of soluble CD95 (sCD95) was performed by using sFas(S) Elisa Kit (MBL International, Watertown, MA) carefully following instructions recommended by the manufacturer.

Statistical Analysis. To analyze the differences in apoptosis induced by different stimuli, the fluorescence intensity of cells stained with anti-CD95 mAbs, the production of soluble CD95, and the production of mRNA for CD95 and CD178 we used paired or unpaired, two-tailed Student's *t* test or ANOVA for repeated measures with Dunnett's post-test under Graph Pad 3.03 for Windows. A minimum of four experiments were considered for each analysis.

Results

HIV-Infected Cell Lines Have Different Susceptibility to Apoptosis. We analyzed the sensitivity of the chronically HIV-infected promonocytic cell line U1 and of its parental uninfected line U937 to two classical inducers of cell death, such as TNF-α plus CHX (which cause oxidative damage and $\Delta\psi$ alterations, with consequent release of intramitochondrial apoptogenic molecules), and STS (which inhibits protein kinase C activity). The main results are reported in Figures 1 and 2. As expected, addition of TNF-α or CHX alone had no effect. U1 cells were slightly less sensitive than U937 to apoptosis induced by TNF-α plus CHX (P = NS). The analysis of $\Delta\psi$ by a specific, sensitive probe performed in parallel samples supported the data related to late apoptosis. Furthermore, when U1 cells were incubated with STS for 6 hrs, and compared with U937, we observed a slightly lower, not significantly different sensitivity to apoptosis that was dose dependent (Fig. 2).

A completely different behavior was observed when cells of lymphocytic origin were evaluated, (i.e., A301 and its chronically HIV-infected clone, ACH-2). ACH-2 cells showed a higher tendency than A301 to undergo apoptosis in the presence of TNF-α plus CHX ($P < 0.01$). Also in this case, the analysis of $\Delta\psi$ confirmed this observation. In a similar manner, a higher sensitivity to apoptosis in ACH-2 cells versus A301 was also noted after adding STS (Fig. 2, $P < 0.01$ for all STS doses).

We analyzed another apoptotic stimulus that elicits a different pathway, to evaluate whether the different sensitivity to apoptosis was due to an intrinsic proneness of lymphocytic cells to undergo cell death after chronic HIV infection. Treating U937, U1, A301, and ACH-2 cells with γ-radiation can induce DNA damages and apoptosis. Three hours after irradiation, no apoptotic phenomena were ob-

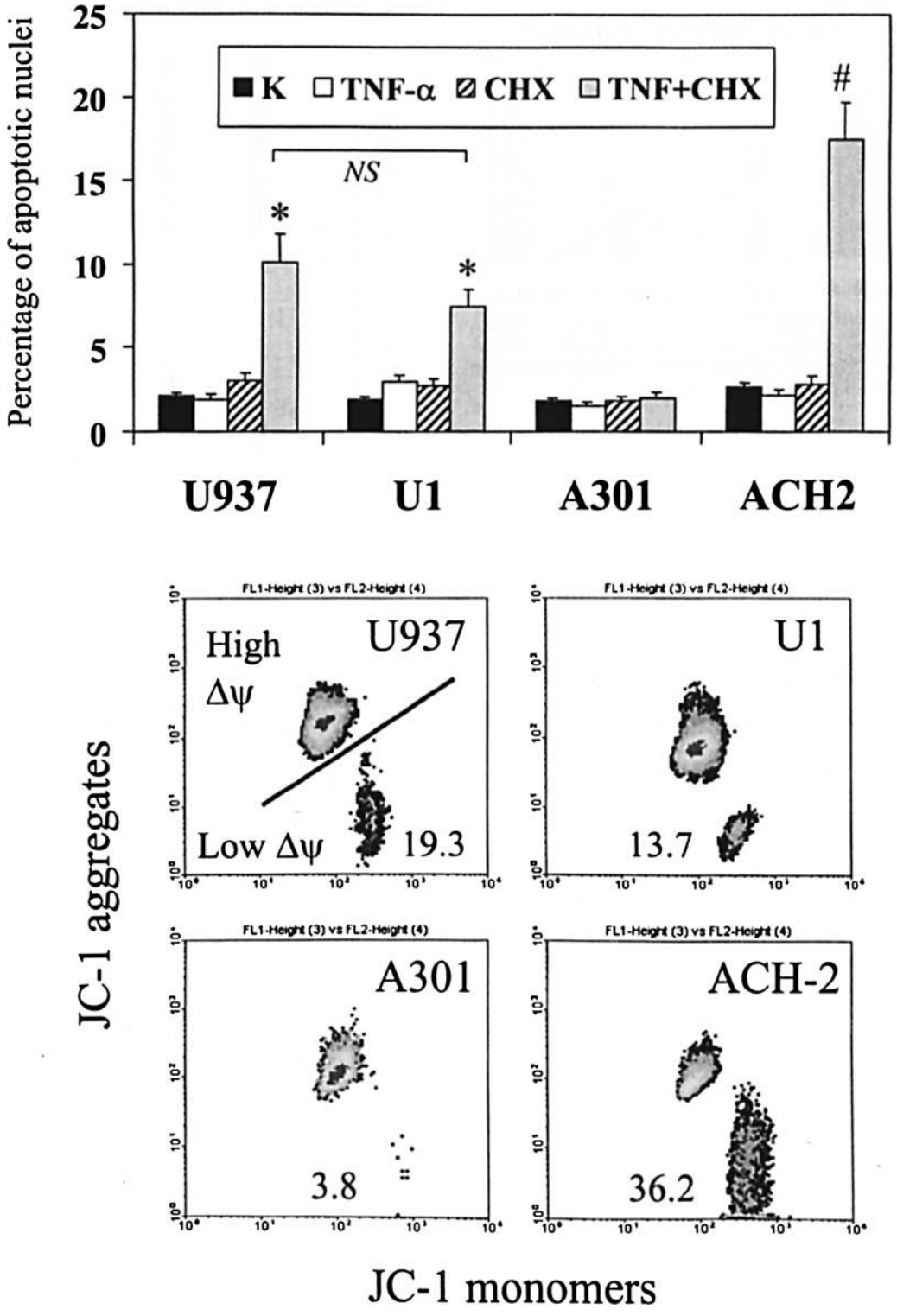

Figure 1. Apoptosis and mitochondrial membrane potential ($\Delta\psi$) in parental or HIV-infected cell lines. Upper part: percentage of apoptotic nuclei after 8 hrs of incubation with TNF-α (TNF), cycloheximide (CHX), or TNF+CHX in control or HIV-infected cell lines. K, control. Data indicate mean + SEM. (*$P < 0.05$ and #$P < 0.01$ vs control). Lower part: analysis of $\Delta\psi$ by fluorescent dye JC-1 in representative samples. Numbers indicate the percentage of cells with low $\Delta\psi$. One representative experiment of four is shown.

served in any cell type (not shown). After 18 hrs, there was a moderate apoptosis in monocytic cells (only at the highest 20 Gy dose), not influenced by HIV infection, since U937 and U1 cells showed a similar behavior (Fig. 3). In the case of lymphocytic cells, a dramatic difference between infected and uninfected cells emerged, as shown by the extremely higher percentage of ACH-2 apoptotic cells compared with A301 (Fig. 3, $P < 0.01$).

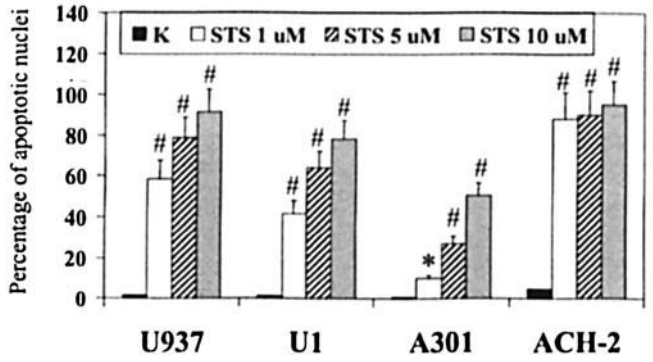

Figure 2. Percentage of apoptotic nuclei after 6 hrs of incubation with different doses of staurosporine (STS) in parental or HIV-infected cell lines. K, control; untreated samples. Data indicate mean + SEM. (*$P < 0.05$ and #$P < 0.01$ vs control).

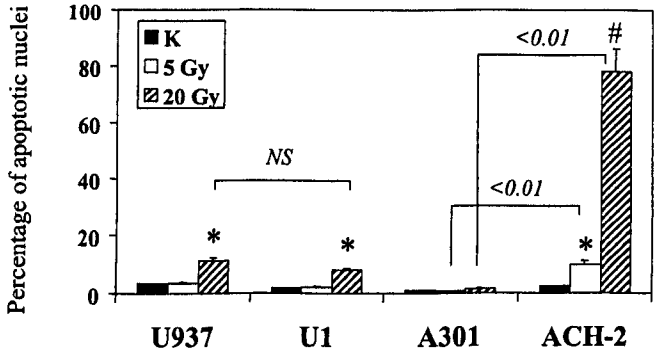

Figure 3. Percentage of apoptotic nuclei in parental or HIV-infected cell lines treated with different doses of γ rays, incubated for 18 hrs. K, control; untreated samples. Data indicate mean + SEM. (*$P < 0.05$ and #$P < 0.01$ vs control).

Modulation of the Expression of Membrane and Soluble CD95. We have previously shown that U1 cells are characterized by a lower expression of plasma membrane CD95 molecule than U937, and ACH-2 by a lower level of CD178 than A301 (13). Thus, we wondered whether a different susceptibility to apoptosis was also present when the apoptotic pathway was triggered by CD95/CD178 interactions. We first analyzed by flow cytometry the amount of CD95 on the plasma membrane of these cell lines after several stimuli that can increase its expression (e.g., ionomycin plus TPA), or induce HIV replication (e.g., IFN-γ, TNF-α). The results are shown in Figure 4. A partial increase of CD95 in U1 cells was obtained after a 12-hr incubation with TNF-α, but the basal amount of CD95 present in U937 cells was not reached. Moreover, CD95 expression in U937 was not significantly modified by any stimulus. A301 and ACH-2 were characterized by similar basal levels of CD95 (slightly higher in ACH-2 cells), which were more elevated than those observed in promonocytic cells (note the different scale in the two panels), but not modified by the stimuli used.

The data we obtained by using real-time PCR are in full agreement with the cytofluorimetric observations. Indeed, as shown in Figure 5, unstimulated U1 had a consistently lower CD95 mRNA production than U937, while no main

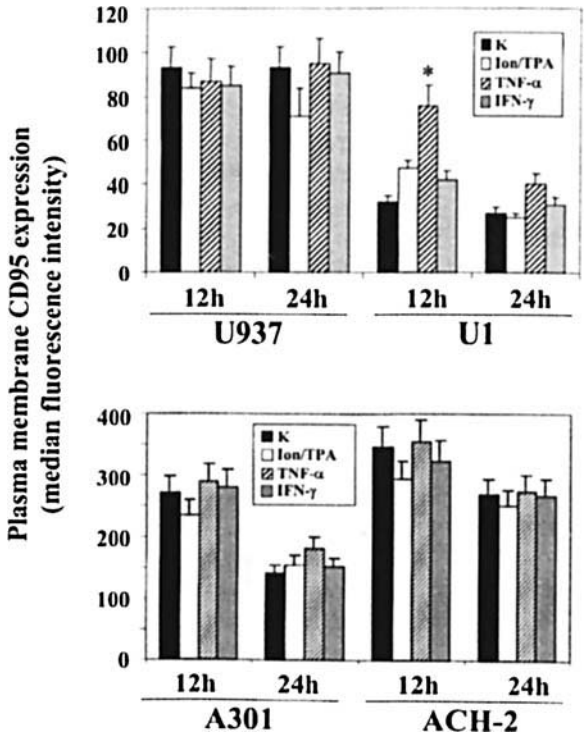

Figure 4. Expression of CD95 on the plasma membrane of parental or HIV-infected cell lines treated with different stimuli. K, control; untreated samples; Ion, ionomycin; TPA, phorbol myristate acetate; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ. Data indicate mean + SEM. (*$P < 0.05$ vs control).

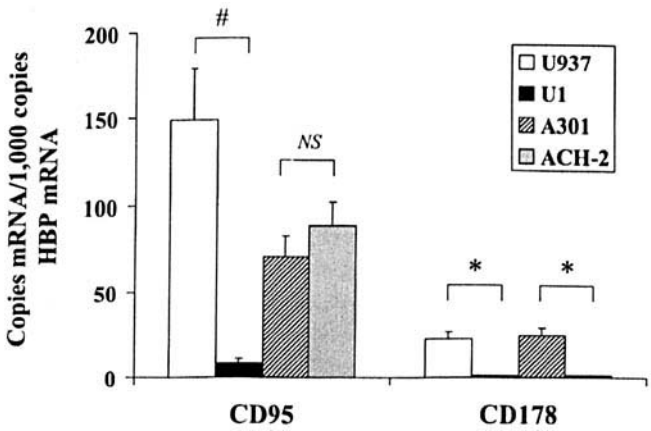

Figure 5. Production of mRNA for CD95 and CD178 by parental or HIV-infected cell lines, analyzed by real-time PCR. Data indicate the production of CD95 and CD178 mRNA per 1000 copies of HBP mRNA, and are reported as mean + SEM. (*$P < 0.05$ and #$P < 0.01$).

differences were present between A301 and ACH-2. Concerning CD178, both uninfected cell lines had significantly higher levels of mRNA than U1 and ACH-2.

We then quantified the production of soluble CD95 molecules by ELISA, to search for a correlation between soluble and membrane CD95 forms. The results are shown in Figure 6. The pattern of production of sCD95 was quite different from that of membrane CD95. The basal production of sCD95 in A301 and ACH-2 was similar, as well as in U937 and U1 cells (P = NS among infected and parental

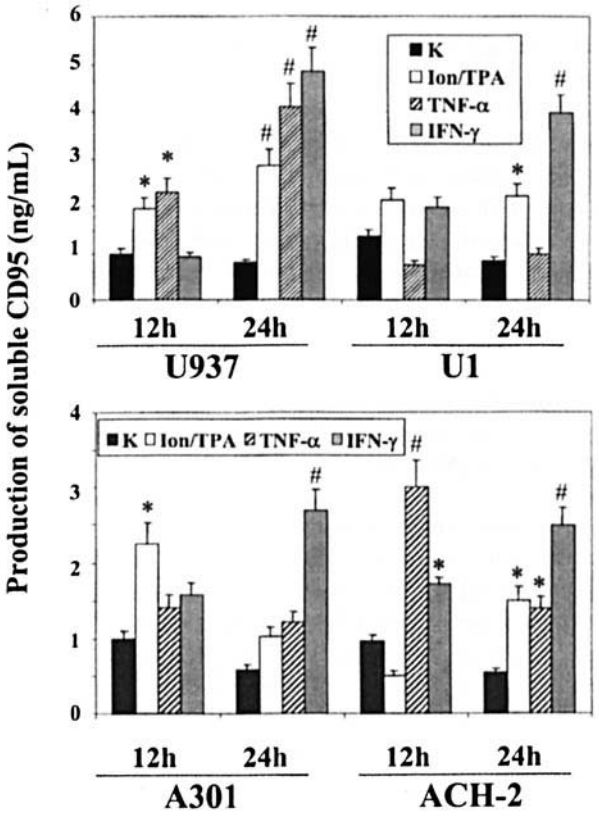

Figure 6. Production of soluble CD95 by parental or HIV-infected cell lines treated with different stimuli. K, control; Ion, ionomycin; TPA, 12-O-tetradecanoylphorbol-13-acetate; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ. Data indicate mean + SEM (*$P < 0.05$ and #$P < 0.01$ vs control).

cells). In all cell lines IFN-γ induced a time-dependent accumulation of sCD95 (P always < 0.01 after 24 hrs). The same effect, even if less evident, was observed with ionomycin plus TPA. In regard to TNF-α, an inductive effect was present in U937 but not in their corresponding infected cells, and vice versa in ACH-2, but not in the uninfected counterpart A301.

CD95 Apoptotic Pathway—Less Functional in Infected than Uninfected Cells. Since we showed that U1 had a lower basal level of plasma membrane CD95 than U937, and a lower production of CD95 mRNA as well, we investigated whether this difference was mirrored by a lower sensitivity to CD95-mediated apoptosis. For this purpose, we incubated U937 and U1 cells for 18 hrs with an agonistic mAb that mimics the binding of CD178 (clone CH11), and is thus capable of triggering apoptosis, or with an mAb that binds but does not activate CD95 (clone ZB4). As shown in Figure 7, U1 cells showed a percentage of apoptotic cells that was not significantly different from untreated controls, whereas in U937 cells CH11 mAb provoked a significant degree of apoptotic cells ($P < 0.01$ vs untreated cells or vs U1).

Surprisingly, also in the lymphocytic cell lines we observed a similar effect (i.e., a decreased susceptibility to CH11-induced apoptosis in ACH-2 as compared with the uninfected A301 cells ($P < 0.02$)).

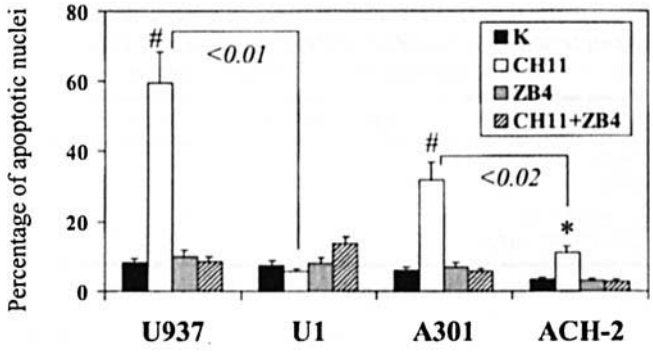

Figure 7. Percentage of apoptotic nuclei in parental or HIV-infected cell lines treated with anti-CD95 mAbs after 18 hrs of culture. CH11 clone is able to trigger apoptosis, ZB4 clone binds CD95 but does not trigger cell death. K, control; untreated samples. Data indicate mean + SEM (*$P < 0.05$ and #$P < 0.01$ vs control).

Discussion

HIV infection is characterized by a progressive loss of CD4+ T cells. Although apoptosis is an important factor in the depletion of CD4+ T lymphocytes, it is still unclear how this process precisely occurs. Indeed, crosslinking of CD4 by gp120, involvement of cytokines and chemokines, activity of superantigens encoded by HIV-1 proteins, and aberrant accessory cell function have all been proposed as potential mechanisms capable of inducing lymphocyte apoptosis (37). Less attention has been paid to the role of monocytes and macrophages in this process, although several studies have evidenced that such cells, as well as dendritic cells, can trigger lymphocyte apoptosis (38, 39). Moreover, monocytes are infected by HIV in the absence of significant virus-induced cytopathicity, which further underlines their role as reservoir for HIV, and for viral persistence and *in vivo* spreading of the infection.

We have characterized the sensitivity to apoptosis of ACH-2 and U1 cell lines, the most widely used models for studies on chronic HIV infection, that can help in understanding cellular modifications provoked by latent infection. In this regard, ACH-2 and U1 cells may resemble latently infected CD4+ T lymphocytes (40, 41) and monocytes (42), respectively. Since *in vivo* both cellular reservoirs appear not to be affected by potent antiretroviral therapy, and are not seen by the immune system (as the expression of viral antigen is lacking), they represent the main problem for viral eradication (43).

In this study, we have used stimuli that trigger cell death through different pathways: inhibition of protein kinase C (STS), oxidative damage and $\Delta\psi$ alterations, with release of intramitochondrial apoptogenic molecules (TNF-α plus CHX), DNA damage (γ-radiations), and CD95/CD178 interactions. As summarized in Table I, monocytic and lymphocytic chronically HIV-infected cells are endowed with opposite sensitivity to apoptosis. Cells belonging to the lymphocytic lineage, such as ACH-2, show an increased tendency to undergo apoptosis; conversely, cells of monocytic origin, such as U1, show a decrease in

Table I. Comparison Among the Sensitivity to Apoptosis of U1, U937, ACH-2, and A301 Cell Lines After Exposure to Different Stimuli

	U1 vs U937	ACH-2 vs A301
TNF-α + CHX	<	>>
STS	<	>
γ-radiations	=	>>
Anti-CD95 mAb	<<	<

this tendency. These data are in agreement with a major *in vivo* feature of HIV infection: although the virus infects CD4+ T lymphocytes as well as monocytes/macrophages, only the former cell type dies and consistently decreases its number. However, studies on other chronically infected cell lines are needed to confirm these observations.

Several authors have shown that the infection of accessory cells such as dendritic cells and monocytes has a great importance for the course of the disease, since infected monocytes are able to kill co-cultured lymphocytes (44). This ability may offer a key in understanding some of the viral strategies. From this point of view, the ability of HIV to protect monocytes from apoptosis can be considered a part of a coordinate strategy to inhibit the immune system, and simultaneously guarantee its own survival. Moreover, this further underlines the observation that monocytes are a crucial reservoir of the virus during the progression of the infection.

We also analyzed apoptosis induced by γ-radiation in all cell lines. Exposure of a wide variety of cells to ionizing radiations results in a division delay, which may be accounted by several components including a G1 block, a G2 arrest, or an S-phase delay. The G1 arrest can be absent in many cell lines and the S-phase delay is typically seen following relatively high doses (>5 Gy), but the G2 arrest is seen in virtually all eukaryotic cells and occurs even after low-dose exposures (<1 Gy). Several pathways can be triggered by such a stimulus, including that of p53 protein, which is, however, absent in U937 (45), and clearly also in its infected clone U1. We found that both cell types of monocytic origin were resistant to γ-ray–induced apoptosis, but that HIV-infected cells of lymphocytic origin were highly sensitive to cell death. This allows us to hypothesize that HIV infection might provoke complex modifications of different pathways in lymphocyte apoptotic machinery, likely including that of p53 protein. Studies are in progress to address this point.

The quantitative analysis of plasma membrane CD95 expression has evidenced that all of the cells we analyzed did not modify significantly the amount of such a pro-apoptotic protein after exposure to different stimuli. With regard to soluble CD95 that has an anti-apoptotic effect being capable of blocking CD178 (i.e., the natural ligand of CD95) (46), only IFN-γ was able to increase its production in all cell lines considered. This increase was not paralleled by an increase in plasma membrane CD95 expression. This surprising difference can be explained considering that sCD95 is measured in the culture medium, and thus represents the cumulative production of the molecule in a given time, so that even little differences in its production can be amplified, whereas membrane CD95 (which is measured on the cell surface) represents the actual measure of CD95 expression in a given moment. Therefore, membrane CD95 is an index of what is happening, while sCD95 indicates what has occurred over the culture period. Another, not mutually exclusive, possible explanation is that a modification of the splicing of CD95 primary transcript exists, that can be skewed towards mRNA for soluble or membrane CD95, in the presence or in the absence of IFN-γ. This hypothesis has to be confirmed by the analysis of mRNA levels, and experiments to test this hypothesis are under way.

It is interesting to note that, in contrast with the general higher sensitivity to undergo apoptosis of ACH-2 in comparison to A301, the infected cells expressed a similar amount of membrane CD95 as the uninfected cells, but showed a reduced tendency to undergo apoptosis in the presence of an antibody that mimics CD178 function. This observation suggests that the apoptotic pathway triggered by CD95 may be partially blocked by HIV. Interestingly, this block is not mediated by binding of soluble CD95 to the apoptosis-activating mAb, as the production of soluble CD95 was not significantly different in uninfected and infected cells. We can hypothesize the existence of an intracellular block in the pathway, and further analyses (e.g., FADD recruitment or caspase activation after CD95 trimerization) are needed to clarify this point.

Several authors have shown an increased expression of CD95 in lymphocytes from HIV-positive subjects, in different phases of HIV infection (47, 48). An increase in the concentration of soluble CD95 was also observed in HIV-positive subjects (49, 50). Our study, using an *in vitro* model of infection, shows that same amounts of CD95 are present on the membrane of infected and uninfected lymphocytic cells, but infected ones are less sensitive to CD95-mediated apoptosis. This apparent discrepancy may be explained by the fact that in HIV-infected subjects only a minority of lymphocytes are infected, while the majority of cells that are characterized by higher tendency to undergo apoptosis are not infected.

In conclusion, we describe a picture where the chronic infection with HIV of lymphocytic cells induces a still uncharacterized sequence of events that increases their tendency to undergo apoptosis. Interestingly, the only exception regards the CD95/CD178-related pathway that appears to be not completely functional. This pathway is considered crucial in the pathogenesis of HIV, since it is claimed as one of the reasons for the loss of CD4+ lymphocytes. Our data suggest that, in a chronically infected cell line, HIV protects from CD95-triggered apoptosis. In the case of monocytic cells, protection from apoptosis appears to be a general condition. Indeed, U1 cells are less sensitive to apoptosis

than U937 cells, express a lower level of plasma membrane CD95, have less CD95 mRNA, and are refractory to stimuli that in other models increase CD95 expression, as well as uninfected cells.

We are well aware that our data have been obtained in two cell lines, and that it is difficult to extrapolate them to primary CD4+ lymphocytes or monocytes. Thus, further studies are needed that investigate other cell lines or primary cell cultures to ascertain whether the phenomena we have described are general, or are characteristics only of the two HIV-infected cell lines we used. In any case, it can be hypothesized that protective, anti-apoptotic mechanisms triggered by HIV may play a role in the pathogenesis of chronic infection. Thus, our data might add new insight in the comprehension of the complex interactions between the host and the virus. The molecular mechanisms are yet unknown, but could represent interesting therapeutic targets.

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