Role of Calmodulin in HIV-Potentiated Fas-Mediated Apoptosis

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The recently demonstrated extraordinary rate of turnover of T cells in human immunodeficiency virus (HIV)-1-infected patients and the apparently concomitant high rate of viral production and death are consistent with a large amount of cell death directly due to infection. Apoptosis may be one of the major forms of T cell death in HIV-1 infection. Many apoptotic pathways depend on calcium and therefore would be expected to involve calmodulin. As the HIV-1 envelope glycoprotein, gp160, contains two known calmodulin-binding domains, we investigated the possibility that the cytoplasmic domain of the HIV-1 envelope protein gp160 could enhance Fas-mediated apoptosis, the major form of apoptosis in lymphocytes. Our studies have shown that 1) transfection of H9 and MOLT-4 cells with a non-infectious HIV proviral clone, pFN, which expresses wild-type gp160, leads to enhanced Fas-mediated apoptosis, 2) transfection of MOLT-4 cells with a pFN construct pFNΔ147, which expresses a carboxy-terminally truncated gp160 lacking the calmodulin-binding domains, produces less Fas-mediated apoptosis than transfection with pFN, and 3) the calmodulin antagonists trifluoperazine and tamoxifen completely inhibit the pFN enhancement of Fas-mediated apoptosis in MOLT-4 cells. We have replicated all of these results using the vectors pSRHS and pSRHSΔ147, which express wild-type gp160 and truncated gp160, respectively, in the absence of other viral proteins. These investigations provide a mechanism by which HIV-1 may induce apoptosis and a possible intracellular target for future therapeutics. (Am J Pathol 1996, 149:903-910)

Given the massive ongoing T cell destruction even in asymptomatic human immunodeficiency virus (HIV)-1 patients, determination of how HIV-1 destroys cells has become ever more important. Apoptosis is one of the processes by which cells die naturally in the course of normal tissue turnover. Cells undergoing apoptosis display profound structural changes, including rapid blebbing of the plasma membrane, fragmentation of DNA, compartmentation of the degraded chromosomes, and finally nuclear disintegration. Apoptosis may be one of the major forms of T cell death in HIV-1 infection.

Apoptosis of T cells is a frequent event during early T cell development. It is a way of eliminating cells with inappropriate immune specificity. Mature T cells respond to appropriate antigen or to other general stimuli by activation, the result of which is lymphocyte proliferation. T cell homeostasis is maintained by activation-induced apoptosis, which is thought to be mediated by Fas antigen (Fas)-Fas ligand interactions. In contrast, T cells from asymptomatic HIV-1-infected patients do not maintain homeostasis after exposure to activation stimuli in vitro, exhibiting increased apoptosis and T cell depletion.

The human Fas protein (also known as APO1 or CD95) is a 48-kd cell surface glycoprotein that belongs to a family of receptors that include the CD40 antigen, nerve growth factor receptor, and tumor necrosis factor receptors (TNFR). Fas is expressed on a number of lymphoma-derived cells and on activated B and T cells. Exposure of lymphocytes expressing Fas to the anti-Fas antibody or to its natural agonist, the Fas ligand, will induce a fraction to undergo apoptosis. There is evidence that exposure of CD4+ cells to the viral envelope protein gp160 up-regulates expression of Fas, presumably

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predisposing cells to Fas-mediated apoptosis in HIV-1 infection. Investigations of HIV-1-induced apoptosis of peripheral blood mononuclear cells and CD4+ and/or CD8+ T cells have shown that 1) HIV-1 infection increases the percentage of cells dying due to apoptosis, particularly in acute infection and 2) stimulation of Fas by anti-Fas monoclonal antibody selectively kills HIV-1-infected cells.

Calcium and calmodulin have been implicated as participants in various apoptotic signaling pathways. Ionomycin, which allows Ca2+ entry into cells, increases apoptosis in phorbol-ester-treated T cells, and glucocorticoid-induced T cell apoptosis is associated with calmodulin mRNA induction. Although there is some evidence that in some cells apoptotic degradation of DNA may occur via a non-calcium-dependent endonuclease, the characteristic DNA fragmentation process frequently depends on activation of a Ca2+-dependent endogenous endonuclease, and calmodulin inhibitors can reduce the activity of this Ca2+-dependent endonuclease. Thus, calmodulin may play a direct role in regulating the enzyme responsible for the characteristic extensive chromatin and DNA damage of apoptosis.

The envelope glycoprotein gp160 of HIV-1 may interact directly with calmodulin. There are two known calmodulin-binding domains (768 to 788 and 826 to 854) in the intracellular carboxyl-terminal segment of gp41, the transmembrane subunit of the mature envelope glycoprotein. Synthetic peptides made from these sequences competitively inhibit calmodulin-stimulated phosphodiesterase activity, indicating that they bind in vitro. Labeled calmodulin overlays of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels show that the full-length protein binds calmodulin. Deletion of part (even 5 amino acids) of the carboxyl-terminal calmodulin-binding site of the HXB2D genome results in diminished infectivity in H-9 cells, whereas deletion of 67 amino acids including the entire carboxyl-terminal calmodulin-binding site eliminates infectivity. The possibility is thus raised that expression of the full-length cytoplasmic domain of gp160 perturbs calcium and calmodulin signaling in the cell. Therefore, we investigated the hypothesis that expression of gp160 predisposes the cells to Fas-stimulated apoptosis by a Ca2+/calmodulin-dependent mechanism.

**Materials and Methods**

**Cell Culture**

H-9 and MOLT-4 cells were grown in RPMI 1640 supplemented with 15% fetal calf serum in T75 flasks. The cells were split by dilution, usually 1:5 or 1:10.

**Vectors**

The vectors pFN and pSRHS, both wild-type and truncated, have been described previously. In all of the studies performed here, only the truncation mutants PFNA147 or PSRHSΔ147, which express envelope glycoprotein lacking the 147 carboxyl-terminal amino acids (gp160Δ147) were used. These mutants lack both known calmodulin-binding domains (768 to 788 and 826 to 854). PFN contains the entire genome of the HXB2D strain of HIV-1 except for 300 bp of the reverse transcriptase that have been deleted to render it inactive, whereas pSRHS places the envelope glycoprotein or its mutants under control of the SV40 promoter.

**Transfection**

Transient transfections were performed with lipofectin (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's procedures. Briefly, 2×10^6 to 3×10^6 cells were washed with serum-free RPMI 1640 medium twice and resuspended in 800 μL of serum-free medium. Plasmid DNA (5 μg), 2.5 μg of pCH110 β-galactosidase-expressing reporter plasmid for cotransfection, and 10 μL of lipofectin were diluted in 100 μL of serum-free medium, mixed together, and incubated at room temperature for 15 minutes. The DNA-lipofectin complexes were added to cell suspensions and incubated for 8 hours at 37°C in a CO₂ incubator. Cells were then diluted with 3 to 4 mL of complete RPMI 1640/15% fetal calf serum medium and incubated for 2 days. The cells were collected by centrifugation, and transfection efficiency was determined by β-galactosidase activity. At 72 hours after transfection, the β-galactosidase-positive cells varied between 21 and 27% of the total.

**Assays for Apoptosis**

**DNA Fragmentation**

Cells were collected and lysed with 0.5 mL of lysis buffer containing 50 mmol/L NaCl, 10 mmol/L Tris, 5 mmol/L EDTA, and 0.1% SDS. Lysates were subjected to proteinase K digestion at 42°C for 1 hour. Genomic DNA was isolated with phenol-chloroform-ethanol precipitation and separated by electrophoresis on 1% agarose with ethidium bromide staining.
Terminal Deoxynucleotide Transferase (TdT) Staining

Cells were harvested and cytospun onto poly-L-lysine-precoated slides and fixed in 10% formalin for 1 hour at room temperature (24°C). After rinsing with distilled water, proteins were stripped from cells by incubation with 20 μg/ml proteinase K at 24°C for 15 minutes, and the slides were then washed four times with distilled water for 2 minutes each. Slides were immersed in TdT buffer (30 mmol/L Trizma base, pH 7.2, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride). TdT (0.3 μl/slide) and digoxigenin-modified dUTP in TdT buffer were added, and the slides were incubated in a humid atmosphere at 37°C for 1 hour. The reaction was terminated by washing the slides with phosphate-buffered saline (PBS) at 24°C. The slides were dipped in 10% fetal calf serum in PBS at 24°C for 30 minutes and dried, covered with alkaline-phosphatase-conjugated anti-digoxigenin antibody, incubated at 24°C for 60 minutes, washed with PBS, and stained with nitroblue tetrazolium/5-chromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, MO) at 24°C for approximately 30 minutes.

Results

H-9 cells (lymphoblastoid, Fas+, CD3+) were cotransfected with pFN, which expresses gp160 in the viral milieu and the reporter plasmid pCH11021 or transfected with the reporter vector alone. Transfection efficiency was approximately 25% and in any one experiment was comparable in all sets of cells. Transfected cells were incubated in medium alone, medium supplemented with human anti-Fas, medium with anti-CD3 antibody, or medium with phorbol ester (PMA). Only anti-Fas-stimulated cells underwent significant apoptosis by the criterion of intrachromosomal DNA fragmentation (Figure 1). In cells transfected with pFN (Figure 1, right panel, labeled gp160), apoptosis was increased in comparison with that of control cells (Figure 1, left panel, labeled control).

To test the possibility that enhanced Fas-stimulated apoptosis in the pFN-transfected H-9 cells might be related to the presence of calmodulin-binding regions in gp160, cells were transfected with pFN or with pFNΔ147. They were then exposed to a range of Fas antibody concentrations. As can be seen in Figure 2A, DNA fragmentation is increased in both pFN- and pFNΔ147-transfected cells with increasing concentrations of Fas antibody. Quantitation by densitometry of the genomic DNA demonstrated that, at all effective concentrations of anti-Fas, pFN transfection produced greater fragmentation of high molecular weight DNA than pFNΔ147 transfection (Figure 2B). These effects were mirrored by an increase of abundance of the shortest DNA fragment (Figure 2C). At 500 ng/ml anti-Fas, the density of low molecular weight fragments of pFN transfectants was nearly twice that of the pFNΔ147 transfectants.

The greater potentiation of Fas-mediated apoptosis exhibited by pFN transfection when compared with pFNΔ147 transfection was not cell type nor assay specific. A dose dependence of anti-Fas-induced apoptosis performed as in Figure 2 but with another lymphocyte cell line, MOLT-4, produced effects similar to those in Figure 2. Comparison of DNA ladders indicated that transfection with pFN (labeled 160) promoted apoptosis more than transfection with...
pFN\(\Delta\)147 (labeled 147; Figure 3A). Apoptosis was also measured by TdT staining\textsuperscript{22} and counting of apoptotic cells (Figure 3B). The results resembled those obtained by densitometry of DNA fragments from H-9 cells (Figure 2). Again, the difference between transfected vectors was most marked at 500 ng/ml anti-Fas antibody, for which the percentage of apoptotic cells (determined by TdT staining) from pFN transfecitants was more than three times that of pFN\(\Delta\)147 transfecitants. Experiments in which pSRHS and pSRHS\(\Delta\)147 were transfected into MOLT-4 cells (with transfected cells expressing only gp160 and gp160\(\Delta\)147, respectively) replicated the difference in Fas-mediated apoptosis enhancement found between pFN and pFN\(\Delta\)147 transfecitants (data not shown).

Calmodulin inhibitors trifluoperazine (10 \textmu mol/L) and tamoxifen (10 \textmu mol/L) eliminated the pFN promotion of anti-Fas-induced apoptosis (Figure 4, A and C). The microscopic images in Figure 4A show TdT staining of cells. Apoptotic cells appear black and are prominent only in the panel showing cells transfected with pFN (labeled gp160). They are essentially absent from the controls and from samples treated with trifluoperazine and tamoxifen. Quantitative analysis of these results is shown in Figure 4C. Identical results were obtained when MOLT-4 cells were transfected with pSRHS or pSRHS\(\Delta\)147,\textsuperscript{20} so that only gp160 or gp160\(\Delta\)147 was expressed (Figure 4, B and C).

Both DNA degradation and TdT staining methods of estimating apoptosis showed that trifluoperazine and tamoxifen diminished pFN promotion of anti-Fas-induced apoptosis with similar dose dependencies (Figure 5). The half-maximal effect at approximately 5.0 \textmu mol/L corresponds well with known...
calmodulin interaction effects of both inhibitors. Tamoxifen, more commonly known as an anti-estrogenic anti-cancer drug, exhibits anti-calmodulin properties as potent as those of the more commonly used trifluoperazine.23

Discussion
These data provide the first evidence for an important role of calmodulin in Fas-mediated apoptosis in HIV-1 infection. First, elimination of the known calmodulin-binding sites of gp160 decreases the amount of apoptosis. Second, exposure to either of two calmodulin inhibitors, trifluoperazine or tamoxifen, can, in a dose-dependent manner, completely eradicate the potentiation of Fas-mediated apoptosis by pFN.

Figure 3. Apoptosis in MOLT-4 T cells transfected with pFN and pFN\_147 and transfection control. MOLT-4 T cells (1 x 10^6) transfected with pFN (labeled 160) or pFN\_147 (labeled 147) and transfection control (labeled C) without plasmid DNA were stimulated by the indicated concentrations of anti-Fas for 4 hours. After stimulation, genomic DNA was isolated and apoptotic DNA fragmentation (A) analyzed as described in Figure 1. In addition, in situ TdT staining was performed and quantitated by counting 300 cells and calculating the percentage that were apoptotic (B). In B, symbols represent pFN(C), labeled gp160, pFN\_147(A), labeled gp160\_147), and control(X). In situ TdT staining was carried out as described previously27 with slight modification.

Figure 4. Effects of calmodulin inhibitors on Fas-stimulated apoptosis in MOLT-4 cells transfected with pFN and pSHRS. A: MOLT-4 cells transfected with pFN expressing full-length gp160 and therefore labeled gp160, transfection controls (labeled transfection control) and nontransfected controls (labeled control) were stimulated with 1 μg/ml anti-Fas monoclonal antibody for 4 hours. In the experiments with inhibitors, 10 μmol/L trifluoperazine (TFP) or 10 μmol/L tamoxifen (TMX) were preincubated with pFN-transfected cells for 30 minutes before stimulation with 1 μg/ml anti-Fas. The cells (5 x 10^6) were collected by cytopsin on polystyrene-treated slides and stained with TdT terminal polymerase kit. Typical microscopic images of the control, transfection control, cells transfected with pFN (gp160), and cells transfected with pFN exposed to trifluoperazine (gp160 + TFP) or tamoxifen (gp160 + TMX) are shown (70 to 120 cells per field). B: MOLT-4 cells transfected with pSHRS (expressing full-length gp160, labeled gp160), with vector alone (labeled vector alone), or with nothing (labeled control) were stimulated with 1 μg/ml anti-Fas monoclonal antibody for 3 hours. Experiments with inhibitors followed those of A. Typical microscopic images corresponding to those of A are displayed (90 to 150 cells per field). C: Quantitative analysis of apoptosis was obtained by counting the percentage of TdT-staining positive cells in 500 MOLT-4 cells for both pFN and pSHRS transfection protocols (labeled as in A and B, except that control A and B is labeled C, transfection control (A) TC, and vector alone (B) V). The closed bars represent pFN transfectants, and the open bars represent pSHRS transfectants.
Figure 5. Dose-dependency of the inhibitory effect of anti-calmodulin drugs on apoptosis in MOLT-4 T cells transfected with pFN. The MOLT-4 T cells transfected with pFN were exposed to varying concentrations of trifluoperazine and tamoxifen as indicated for 30 minutes before the stimulation with 1 μg/ml anti-Fas. DNA fragmentation and quantitative analysis of TdT apoptotic staining were performed as described in the legend to Figure 3. The dose dependency of the inhibitory effect of trifluoperazine (TFP) and tamoxifen (TMX) on DNA fragmentation (A) and quantitative analysis of TdT apoptotic staining (B) are shown.

It is important to note that the calmodulin-related enhancement of Fas-mediated apoptosis is consistent in two cell lines and that, therefore, the effect may be general.

There are many possible intermediates between a stimulated Fas molecule at the plasma membrane and the final event, DNA fragmentation. Several generic cellular membrane and post-membrane signaling pathways, including sphingomyelinase/ceramide, proteases, phosphotyrosyl phosphatases, and tyrosine kinases, have been implicated as promulgators of Fas-stimulated apoptosis. Nuclear factor of activated T cells (NFAT) translocation to the nucleus may be one key intracellular event that links calmodulin and Fas signaling. The calmodulin-dependent phosphatase, calcineurin, is a strictly calmodulin-dependent enzyme, which regulates movement to the nucleus of the transcription factor NFAT, a critical factor for lymphocyte activation and associated interleukin-2 production. In human lymphocytes, Fas may be expressed both in resting and in interleukin-2-activated cells. However, in lymphocyte-thymocyte hybridomas, Fas expression is dependent on CD3 activation, which generally results in NFAT translocation to the nucleus. Fas ligand expression is also activation dependent (both in hybridomas and T cells) and, more importantly, is inhibited by cyclosporine, which, in conjunction with cyclophilin, specifically inhibits calcineurin. Thus, it appears that the calcineurin control of NFAT may regulate both Fas and Fas ligand expression and could therefore be a site of action for the potentiation of Fas-mediated apoptosis by gp160-induced calmodulin-dependent signaling.

The calmodulin-dependent apoptosis signaling event may also be directly related to a death domain sequence in gp41. The expression of the fruit fly protein, reaper, induces apoptosis. Interestingly, Fas, tumor necrosis factor, Fas associated death domain protein, receptor interacting protein, and tumor necrosis factor receptor/associated death domain protein, and all proteins the activation or over-expression of which induces apoptosis, all also display death domain regions that are homologous to reaper. Deletion of a death domain abrogates the protein’s ability to promote apoptosis. The cytoplasmic region of gp41 contains a sequence that conforms reasonably well to the death domain sequence. Figure 6 compares the gp41 sequence with nine known death domains. The similarity of gp41 to these domains is strong over almost the entire subregion defined by reaper. Near the amino terminus of the death-domain-like region of gp41 can be found one of the known calmodulin-binding sites of gp41 (amino acids 768 to 788). Multimerization of the death-domain-containing proteins has been demon-
strated to be dependent on the death domains. The possibility thus arises that formation of an aggregate of Fas, gp160, and calmodulin may be a key proximal event in apoptosis signaling of HIV-1-infected cells and that modification of one or more of these proteins in the aggregate results in altered (generally reduced) Fas-mediated apoptosis.

We have shown that Ca^{2+}/calmodulin is probably involved in the increased apoptosis exhibited by our model of apoptosis in HIV-1-infected cells, Fas-stimulated pFN-transfected MOLT-4 cells. We have further shown that the Ca^{2+}/calmodulin involvement is likely to be dependent on expression of gp160 which contains calmodulin binding sites on its cytoplasmic tail. In related investigations, using the same in vitro systems as those used here, we have shown that calmodulin expression is increased and its cellular distribution altered in cells transfected with gp160 when compared with cells transfected with gp160A147. Therefore, the possibility is raised that increased calmodulin, pursuant to full-length gp160 expression, is responsible at least in part for HIV-1-enhanced Fas-mediated apoptosis. In other related investigations, we have shown that, in concordance with our model, spontaneous in vitro apoptosis of peripheral blood mononuclear cells from patients with acquired immune deficiency syndrome is inhibited by both trifluoperazine and tamoxifen (unpublished observations). Thus, we believe the observations reported here represent an important step in elucidating one of the cytotoxic mechanisms of HIV-1 and possibly in designing new therapeutic agents to subvert apoptosis.

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References

23. Lam HY: Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. Biochem Biophys Res Commun 1984, 118:27–32