Interleukin-1β Converting Enzyme-like Protease Involvement in Fas-induced and Activation-induced Peripheral Blood T Cell Apoptosis in HIV Infection. TNF-related Apoptosis-inducing Ligand Can Mediate Activation-induced T Cell Death in HIV Infection

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Summary

Apoptosis of peripheral blood T cells has been suggested to play an important role in the pathogenesis of human immunodeficiency virus (HIV) infection. Spontaneous, Fas (CD95)–induced and activation-induced T cell apoptosis have all been described in peripheral blood mononuclear cell cultures of HIV-infected individuals. We have previously shown that activation-induced T cell apoptosis is Fas independent in peripheral blood T cells from HIV+ individuals. In this study, we extend and confirm these observations by using an inhibitor of interleukin-1β converting enzyme (ICE) homologues. We show that z-VAD-fmk, a tripeptide inhibitor of ICE homologues, can inhibit Fas-induced apoptosis of peripheral blood CD4+ and CD8+ T cells from asymptomatic HIV+ individuals. z-VAD-fmk also inhibited activation (anti-CD3)–induced CD4+ and CD8+ T cell apoptosis (AICD) in some but not all asymptomatic HIV+ individuals. Apoptosis was measured by multiparameter flow cytometry. The z-VAD-fmk inhibitor also enhanced survival of T cells in anti-Fas or anti-CD3 antibody-treated cultures and inhibited DNA fragmentation. AICD that could be inhibited by z-VAD-fmk was Fas independent and could be inhibited with a blocking monoclonal antibody to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a recently described member of the TNF/nerve growth factor ligand family. The above findings show that Fas-induced T cell apoptosis is ICE dependent in HIV infection. AICD can be blocked by ICE inhibitors in some patients, and this AICD is mediated by TRAIL. These results show that TRAIL can be a mediator of AICD in T cells. These different mechanisms of peripheral blood T cell apoptosis may play different roles in the pathogenesis of HIV infection.

Several studies have shown that spontaneous, Fas- and activation-induced T cell apoptosis occurs in PBMCs and purified T cells from HIV-infected individuals (1–5). This apoptosis has been proposed as an important mechanism in the pathogenesis of HIV disease involved in both the functional defects and depletion of CD4+ T cells (6).

Previously, a number of investigators have shown that activation-induced cell death in human T lymphocytes is mediated by Fas–Fas ligand (FasL)1 interactions (7–10). Signaling through Fas, a member of the TNF/nerve growth factor (NGF) receptor superfamily (11), has been shown to induce apoptosis of T cell clones and lines (12–14), to costimulate proliferation and cytokine production of T cells from healthy individuals (14), and to be involved in cytotoxic T lymphocyte-mediated killing (15, 16). We and others have recently shown that peripheral blood CD4+ and CD8+ T cells from HIV-infected individuals are especially susceptible to Fas-induced apoptosis and that this apoptosis correlates with disease progression and severity (4, 5).

TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L (17, 18) has been recently cloned and shown to be a member of the TNF/NGF family of ligands. Although TRAIL, similar to Fas, has been shown to induce apoptosis in a number of cell lines, it does not induce apoptosis in...
normal peripheral blood T and B cells. Thus, the biological function of T R A I L has yet to be determined.

Our initial study on the role of Fas in T cell apoptosis of HIV disease raised the question of whether Fas-FasL interactions are involved in the activation-induced T cell apoptosis observed in HIV infection. Using reagents that block either Fas antigen or FasL, we recently showed that this activation-induced T cell apoptosis is Fas/FasL independent (19). In the present study, we confirm and extend these observations by using z-VAD-fmk, a tripeptide inhibitor of interleukin-1β converting enzyme (ICE) protease homologues. We show that although Fas-induced apoptosis of peripheral blood T cells can be abrogated by z-VAD-fmk in all asymptomatic HIV+ patients, activation-induced CD4+ and CD8+ T cell apoptosis (AICD) of T cells can be inhibited in some but not all patients. We report here that T R A I L can mediate AICD of T cells. AICD of peripheral blood T cells from HIV-infected individuals that could be effectively inhibited by z-VAD-fmk could also be blocked by a neutralizing monoclonal antibody to T R A I L, but not to FasL. Our findings show that multiple mechanisms of T cell apoptosis are operative in HIV infection and may play different roles in the pathogenesis of HIV disease.

Materials and Methods

Samples and Materials. Heparinized blood samples were obtained after informed consent of asymptomatic HIV+ individuals. Mouse monoclonal IgM antibody to Fas antigen (CD95) CH-11 (Immunotech, Westbrook, ME) was used for Fas-induced apoptosis experiments. For AICD experiments, the anti-CD3 monoclonal antibody OKT3 was used. Blocking mouse monoclonal antibody to FasL (NOK1; IgG1 isotype) was a gift by Dr. H. Yagita (Juntendo University, Tokyo, Japan). For T R A I L blocking, the neutralizing monoclonal antibody M180 was used (IgG1 isotype, Immunex, Seattle, WA). The monoclonal anti-CD8 antibody 107.3 was used as an IgG1 isotype control (Pharmingen, San Diego, CA). ICE inhibitors were commercially purchased Cbz-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk; Enzyme Systems Products, Dublin, CA), Cbz-Asp-Glu-Val-Ala-Asp-fluoromethyl ketone (z-DEVD-fmk; Enzyme Systems Products, Cbz-Tyr-Val-Ala-Asp-chloromethyl ketone (z-YVAD-cmk; Bachem, King of Prussia, PA), and Cbz-Phe-Ala-fluoro methyl ketone (z-FA-fmk; Enzyme Systems Products). The IL-1β ELISA was a gift from Dr. J.S. Kenney (Syntex, Mountain View, CA). RPMI-1640 (GIBCO BRL, Gaithersburg, MD) and FCS (GIBCO BRL) contained <0.03 EU/ml and 0.3 EU/ml, respectively.

Anti-Fas and Anti-CD3-induced Apoptosis and Inhibition Experiments. PBMCs from asymptomatic HIV+ individuals were isolated by density centrifugation on Ficoll-Hyphaque. Freshly prepared PBMCs were cultured for 24 h at 10^6 cells/ml in RPMI-1640 plus 5% FCS in 24-well plates coated with or without monoclonal anti-Fas antibody CH-11 or anti-CD3 antibody OKT3. Cultures were performed at 37°C in a 5% CO2 humid atmosphere. Plates were coated with 500 μl/well of 5 μg/ml of antibodies in PBS, pH 7.4, for 2 h at 37°C. Plates were then briefly washed twice with 500 μl/well RPMI-1640 plus 5% FCS and then washed one more time as above for 30 min at 37°C. In the inhibition experiments, PBMCs were preincubated with ICE inhibitors for 3 h at 37°C in a 5% CO2 humid atmosphere before being transferred to antibody-coated plates. Apoptosis inhibition experiments using the Jurkat T cell line were performed by preincubating with ICE inhibitors for 3 h and then stimulating with 200 ng/ml CH-11 in solution. The effect of the ICE inhibitors on IL-1β production was examined by preincubating PBMCS from HIV- individuals with inhibitors for 3 h, and then stimulating for 24 h with 1 μg/ml LPS. Supernatants were then assayed in a specific IL-1β ELISA.

A apoptosis measurement and DNA Fragmentation. Apoptosis was determined by staining cells with 1 μg/ml Hoechst 33342 (H O 342; Molecular Probes, Eugene, OR) for 7 min on ice as previously described (4). Cells were stained with anti-CD8-PE (Becton Dickinson, Mountain View, CA), anti-CD4- FITC (PharMingen), and anti-CD5-Cy5 (PharMingen) or anti-CD3-Cy5 (PharMingen) for 15 min on ice. Anti-CD3-Cy5 was used for staining in the anti-Fas-treated cultures, whereas anti-CD5-Cy5 was used in the anti-CD3-treated cultures. Basic dyes and fixing in paraformaldehyde, 30,000 cells were analyzed by flow cytometry using a Facstar® (Becton Dickinson) and Desk software (20). Apoptosis was determined by measuring HO 342/PI 342 staining gated on forward and side scatter for lymphocytes. Absolute numbers of live CD4+ and CD8+ T cell in culture were determined by measuring the percent of live nonapoptotic cells by flow cytometry and counting the total number of PBMCs in culture (Coulter Counter model ZMA; Coulter Corp., Hialeah, FL).

DNA fragmentation analysis was performed on PBMCs (2 x 10⁶ cells) incubated with immobilized antibodies for 5 h. DNA was extracted by centrifuging and then resuspending cells in 20 μl of 50 mM Tris- HCl pH 8.0, 10 mM EDTA, 0.5% sodium lauryl sarcosylate, 0.5 mg/ml proteinase K, and incubating for 1 h at 50°C. RNAase at 100 μg/ml was then added and the extract was further digested for 1 h at 50°C. DNA was run on a 2% agarose gel in 25 mM Tris-borate, 1 mM EDTA, pH 8.0, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light.

Statistical Analysis. Data were analyzed by Wilcoxon’s signed-rank test for paired data, Mann-Whitney U test, and analysis of variance using JMP Statistics Guide (SAS Institute, Inc., Cary, NC).

Results

ICE Inhibitors and Anti-Fas-induced Apoptosis. Preincubation of cultures for 3 h with z-VAD-fmk tripeptide at 50 μM inhibited 72% of anti-Fas-induced peripheral blood T cell apoptosis in asymptomatic HIV-infected individuals measured at 24 h by multiparameter flow cytometry (n = 11; Fig. 1A). z-VAD-fmk (50 μM) inhibited 24 h Fas-induced apoptosis of both CD4+ and CD8+ T cell (67 and 78%, respectively; Fig. 1B). z-VAD also increased the survival of live cells in anti-Fas-treated cultures and inhibited DNA fragmentation (Fig. 1, C and D). At 10 μM, z-VAD-fmk inhibited 32% of Fas-induced apoptosis of CD3+ T cells (data not shown). Spontaneous 24 h CD4+ and CD8+ T cell apoptosis could not be inhibited by 50 μM z-VAD-fmk. The control peptide z-FA-fmk (50 μM) had no effect on either Fas-induced or spontaneous T cell apoptosis. The z-YVAD-cmk and z-DEVD-fmk tetrapeptides at 50 μM did not inhibit anti-Fas-induced T cell apoptosis of HIV+ PBMCs (Fig. 1, A - C) and concentrations as high as 200 μM still failed to inhibit apoptosis (data not shown). Although anti-Fas-induced apoptosis of the Jurkat T cell line could also be inhibited with z-VAD-fmk down to
concentrations of 10 μM (86% inhibition), higher concentrations of z-YVAD-cmk and z-DEVD-fmk were required to inhibit this apoptosis (29 and 41% inhibition, respectively, at 100 μM) (Fig. 2 A). Both z-VAD-fmk and z-YVAD-cmk at 10 μM inhibited 77 and 73% of IL-1β production by LPS-stimulated PBMCs (Fig 2 B). z-FA-fmk had no effect on either Fas-induced apoptosis of the Jurkat T cell line or on IL-1β production by LPS-stimulated PBMCs.

ICE Inhibitors and Anti-CD3–induced Apoptosis. Previously, we have shown that AICD of peripheral blood T cells in HIV-infected individuals is Fas/FasL independent (19). To extend these studies, we examined the effect of the z-VAD-fmk tripeptide ICE inhibitor on anti-CD3–induced 24-h AICD in HIV-1-infected PBMCs (Fig. 3 A). z-VAD-fmk inhibited CD4+ and CD8+ T cell apoptosis for anti-CD3 alone, anti-CD3 plus z-VAD-fmk, and anti-CD3 plus z-FA-fmk was 32.3 ± 1.8, 22.9 ± 1.9, and 29.7 ± 2.4%, respectively (n = 25; Fig. 3 A); spontaneous CD5+ T cell apoptosis was 14.1 ± 1.8%. Inhibition of AICD by z-VAD-fmk in asymptomatic HIV+ patients correlated with the level of AICD (P < 0.01; Spearman’s Rho), with greater inhibition occurring in patients with higher levels of AICD (Fig. 3 B). Inhibition of AICD by z-VAD-fmk ranged from 0 to 87.2% (Fig. 3 B). In those patients in which z-VAD-fmk inhibited AICD, both CD4+ and CD8+ T cell apoptosis was inhibited (data not shown). When AICD was inhibited, z-VAD-fmk could inhibit DNA fragmentation and enhance survival of anti-CD3 treatment cultures (data not shown). In all the above experiments, the anti-FasL antibody failed to block both ICE-dependent and independent AICD (Fig. 4, A and B; data not shown) confirming our previous findings with HIV+ PBMCs (19). Addition of 100 U/ml of recombinant IL-2 to these cultures also failed to inhibit 24-h AICD (data not shown).

TRAIL Mediates ICE-dependent AICD in HIV-infected Individuals. In patients with AICD that could be inhibited
by z-VAD-fmk, all of CD5+ T cell apoptosis could be inhibited by a neutralizing monoclonal antibody to TRAIL (Fig. 4, A and B). Anti-CD3-induced 24-h apoptosis of CD5+ T cells was 15.9 ± 2.7, 26.3 ± 3.1, 14.1 ± 1.8, 23.6 ± 3.6, 25.9 ± 3.1% for untreated, anti-CD3 antibody–treated, anti-CD3 antibody plus anti-TRAIL–treated, anti-CD3 antibody plus anti-FasL–treated, and anti-CD3 antibody plus isotype control–treated cultures, respectively. Anti-TRAIL inhibited 24-h AICD in both CD4+ and CD8+ T cells. Anti-TRAIL enhanced survival and inhibited DNA fragmentation in these anti-CD3–treated cultures (data not shown). The blocking anti-FasL monoclonal antibody and the isotype control antibody had no effect on T cell AICD (Fig. 4, A and B). In patients in which z-VAD-fmk had little or no effect on AICD (<15% inhibition) neither anti-

TRAIL nor anti-Fasl antibody had any effect on T cell AICD. However, the anti-Fasl blocking antibody could block anti-CD3–induced AICD of a T cell line generated with PHA/IL-2 from a healthy HIV– individual (Fig. 4 C). Neither anti-TRAIL nor anti-Fasl antibody had any effect on spontaneous T cell apoptosis in HIV+ PBMC cultures (data not shown).

**Discussion**

T cell apoptosis may play an important role in the pathogenesis of HIV disease. We and others have previously shown that peripheral blood T cells from HIV-infected individuals are especially susceptible to Fas-induced apoptosis (4, 5). On the other hand, we have shown that activation-induced peripheral blood T cell apoptosis (anti-CD3 stimulation) in HIV+ individuals, is Fas independent (19). To elucidate further T cell apoptosis in HIV infection in the present study, we examined whether inhibiting ICE family homologues can inhibit Fas- and activation-induced T cell apoptosis.

Fas-induced apoptosis involves the activation of members of both the ICE and CPP32 subfamilies of cysteine proteases (21–23). The tripeptide z-VAD-fmk is an irreversible inhibitor of ICE protease and is expected to inhibit other members of the ICE subfamily of cysteine proteases (24). z-VAD-fmk can inhibit apoptosis induced by Fas (25), overexpression of R EAPER (26) and FLICE/MACH (27), and a variety of other stimuli (28, 29). We found that z-VAD-fmk inhibits Fas-induced peripheral blood CD4+ and CD8+ T cell apoptosis in asymptomatic HIV+ individuals, thus showing that members of the ICE subfamily of proteases are involved in this apoptosis. Tetrapeptide inhibitors of ICE (z-YVAD-cmk) or CPP32 (z-DEVD-fmk) could not inhibit Fas-induced T cell apoptosis in PBMC cultures. Previously, z-YVAD-cmk was shown to inhibit Fas-induced apoptosis of purified CD4+ and CD8+ T cells from HIV+ individuals with CD4+ T cell apoptosis being inhibited more efficiently than CD8+ T cells (30). In our study, the tripeptide z-VAD-fmk inhibitor was equally efficient in inhibiting Fas-induced CD4+ and CD8+ T cell apoptosis. The lack of effect of tetrapeptides in our hands is possibly due to differences in cellular uptake of these inhib-
itors and this may explain why IL-1β production by monocytes was inhibited by z-YVAD-fmk, whereas T cell apoptosis was not.

In our studies, activation (anti-CD3)–induced CD4+ and CD8+ T cell apoptosis could be inhibited in some patients but not all, thus subdividing the HIV+ patients into two groups with ICE-dependent and ICE-independent AICD, respectively. Spontaneous T cell apoptosis could not be inhibited by z-VAD-fmk. The ICE-dependent peripheral blood T cell AICD in HIV+ individuals was mediated by TRAIL, as a blocking anti-TRAIL monoclonal antibody inhibited this apoptosis. TRAIL, a member of the TNF/NGF ligand family, has been shown to induce apoptosis of transformed cell lines (17, 18) and TRAIL-induced apoptosis requires ICE-like cysteine proteases (31). Our findings show an involvement of TRAIL in AICD of peripheral blood T cells from HIV+ individuals. An involvement of TRAIL in T cell AICD has not been previously reported. Recently, three TRAIL receptors have been cloned (DR4, TR2, and TR3; references 37–39), two of which can mediate apoptosis (37, 38). It will be important to determine which one receptor or combination of receptors is involved in AICD during HIV infection. Future studies should address these questions.

In some HIV+ individuals, z-VAD-fmk had little or no effect on AICD of peripheral blood T cells. It is not clear at the moment whether this failure to inhibit AICD is due to limitations in our experimental approach or whether this AICD is truly ICE independent. If ICE independent, this AICD may be mediated by other members of the ICE family of cysteine proteases, such as members of the CPP32 subfamily that are not inactivated by z-VAD-fmk (25). Apoptosis of T cells that cannot be inhibited by z-VAD-fmk but can be blocked by inhibitors of CPP32 have been previously reported (40). We have previously described a similar ICE-independent T cell apoptosis in HIV-1 Tat protein–enhanced AICD (41). This raises the possibility that the ICE-independent peripheral blood T cell AICD we

Figure 4. TRAIL, but not FasL, mediates z-VAD-fmk–inhibitable AICD of peripheral blood T cells from HIV+ patients. (A) Blocking monoclonal antibody to TRAIL inhibits AICD in patients that display z-VAD-fmk inhibitable AICD. Multiparameter flow cytometry shown of representative experiment. (a–d) Spontaneous apoptosis (e–h) anti-CD3-induced apoptosis. (b and f) Anti-TRAIL antibody; (c and g) anti-FasL antibody; (d and h) isotype control. Percent apoptosis is shown in upper right corner. Representative experiment is shown. (B) Anti-TRAIL antibody inhibits AICD (n = 7; mean and standard errors of pooled data shown; P < 0.05; Wilcoxon's signed-rank test for paired data). (C) Anti-FasL antibody inhibits anti-CD3–induced AICD of a human T cell line generated from an HIV− individual. Representative experiment of two performed shown.
found in HIV+ individuals may be due to Tat protein-induced apoptosis (42). IL-2 withdrawal from the murine CTLL cell line can also induce apoptosis, which cannot be inhibited by z-VAD-fmk but is abrogated by CPP32 inhibitors (40, 43), thus raising the possibility that the ICE-independent AICD in HIV+ PBMCs may be due to insufficient IL-2 production in these cultures. However, this was shown not to be the case, because recombinant IL-2 added to these cultures failed to inhibit T cell apoptosis. Another possible explanation for the ICE-independent AICD we describe is that it may be due to a bystander killing induced by cytotoxic CD8+ T cells. Such cytotoxicity cannot be inhibited by z-VAD-fmk, because granzyme B cannot be directly inhibited by z-VAD-fmk and during the protease activation cascade granzyme B circumvents the activation of ICE by directly activating CPP32 and Mch3 (40, 44–47). In our study, spontaneous T cell apoptosis of HIV+ PBMCs could not be inhibited by z-VAD-fmk; whether this is the result of T cells having received the lethal hit in vivo or this apoptosis belonging to ICE-independent AICD is not clear at the moment.

The present report extends and confirms our previous studies (19) in which anti-CD3-induced AICD in HIV+ PBMCs was shown to be Fas independent. AICD of purified CD8+ T cells in HIV infection has previously been shown to be Fas independent, although purified CD4+ T cell AICD was shown to be Fas mediated (30). This apparent discrepancy may be the result of using purified versus mixed cell populations. We believe our studies using PBMCs may be more relevant to what may be happening in vivo since they address the interactions of different cellular subsets. Purified CD4+ or CD8+ T cells may reflect a more artificial system, because T cells are restricted to interacting exclusively to cells of the same subpopulation. On the other hand, different modes of T cell stimulation may result in different mechanisms of AICD; this has been reported for superantigen-induced apoptosis, which has been shown to require Fas-FasL interactions, whereas anti-CD3-induced T cell death can proceed in the absence of Fas (48). In our experiments, we chose to examine AICD induced by immobilized anti-CD3 antibody, since this would reflect antigen stimulation of T cells more closely. Recently, Fas ligand regulation on monocytes was shown to be deficient during HIV infection (49). If also true for T cells, this FasL deficiency may explain the paradox of having Fas/FasL-independent T cell AICD in HIV+ individuals whose peripheral blood T cells are sensitive to Fas killing.

Our findings show that in HIV+ individuals Fas-induced apoptosis of peripheral blood CD4+ and CD8+ T cells requires the activation of cysteine proteases of the ICE subfamily. Activation-induced T cell apoptosis in some patients could be inhibited by z-VAD-fmk. This AICD in HIV+ individuals was mediated by TRAIL, showing a role for TRAIL in T cell AICD. Our findings indicate that multiple mechanisms of T cell apoptosis are operative in HIV infection and, thus, possibly contributing differently to disease pathogenesis.

The authors would like to thank Drs. T. Merigan and M. Winters for use of facilities at the Center for AIDS Research (Stanford Medical School, Stanford, CA).

M.E. Garcia-Ojeda was supported by a National Science Foundation fellowship. J.F. Torres-Roca was supported by Stanford Immunology Training grant AI-07290. This work was supported by National Institutes of Health grants CA 42509, LM 04836, and AI 31770, and a grant from the Unicorn Foundation.

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Received for publication 6 June 1997 and in revised form 1 August 1997.

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