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Rapid Induction of Apoptosis in CD8$^+$ HIV-1 Envelope-Specific Murine CTLs by Short Exposure to Antigenic Peptide[1]

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During primary viral infection, in vivo exposure to high doses of virus causes a loss of Ag-specific CD8$^+$ T cells. This phenomenon, termed clonal exhaustion, and other mechanisms by which CTLs are deleted are poorly understood. Here we show evidence for a novel form of cell death in which recently stimulated CD8$^+$ HIV-1 envelope gp160-specific murine CTLs become apoptotic in vitro after brief exposure to free antigenic peptide (P18-I10). Peak apoptosis occurred within 3 h of treatment with peptide, and the level of apoptosis was dependent on both the time after initial stimulation with target cells and the number of targets. Using T cell-specific H-2D^d/P18-I10 tetramers, we observed that the apoptosis was induced by such complexes. Induction of apoptosis was blocked by cyclosporin A, a caspase 3 inhibitor, and a mitogen-activated protein kinase inhibitor, but not by Abs to either Fas ligand or to TNF-α. Thus, these observations suggest the existence of a Fas- or TNF-α-independent pathway initiated by TCR signaling that is involved in the rapid induction of CTL apoptosis. Such a pathway may prove important in the mechanism by which virus-specific CTLs are deleted in the presence of high viral burdens. ***The Journal of Immunology*****, 2002, 169: 6588–6593.**

Although the CTLs play a central role in the control of viral infection, a high viral burden can result in deletion and/or inactivation of Ag-specific CTLs (1, 2). It has been shown in mice infected with lymphocytic choriomeningitis virus that overwhelming virus infection results in the exhaustion of antiviral CTL responses (3). A similar phenomenon may occur in HIV-1-infected patients, as some evidence suggests that HIV-1-specific CTLs are preferentially eliminated during disease progression (4, 5). Furthermore, Pantaleo et al. (6) reported that a significant number of HIV-1-specific CTLs were lost in some patients during the course of primary HIV-1 infection, a loss that appeared to be due to the continuous activation of the CTLs. This loss was not due to escape of virus that was not recognized by the CTL clones, but the precise mechanism of the CTL deletion remains poorly understood.

In studies of peptide-specific CTLs in a murine model, Alexander-Miller et al. (7, 8) demonstrated that stimulation of high avidity CD8$^+$ CTLs with APC bearing supraoptimal densities of peptide/MHC complexes resulted in the apoptotic deletion of the CTLs. They speculated that when large doses of virus were challenged in vivo, viral Ags might be presented at an increased level on the cell surface of APC that might cause the deletion of high avidity CTLs and inadequate control of viral spread by low avidity CTLs. Moreover, they observed that the process of cell death was dependent on the avidity of the CTLs, but not their initial activation state, and was distinct from activation-induced cell death (AICD)[3] observed in CD4$^+$ T cells.

AICD in mature T cells has been thought to result from the restimulation of cycling T cells by Ag and to be mediated by Fas-Fas ligand (FasL) or TNF-α-TNF receptor interaction (9–12). In this study we have examined the behavior of recently stimulated CTLs specific for the HIV-1 gp160 envelope glycoprotein following brief exposure to antigenic peptide. We observed rapid onset of apoptosis within 3 h of peptide treatment, and the level of apoptosis was dependent on both the time after initial stimulation and the number of stimulator cells. Moreover, this kind of cell death required a signal through the TCR, but not through FasL or TNF receptor. Such early apoptosis of the CTLs might be even further increased if targets infected with high titer virus released large amounts of viral Ag. This might allow representation of viral Ags over a short time scale, leading to increased apoptosis of available CTLs. This type of apoptosis, occurring within a very short time of initial infection, probably contributes to the deletion of virus-specific CTLs in the primary viral infection with high viral burden. It may significantly contribute to virus persistence in vivo. Therefore, we performed the present study with a view toward clarifying the mechanisms responsible for induction of apoptosis in such recently stimulated CTLs. Such understanding might allow the development of strategies to protect CTLs from cell death during viral infection and to maintain an effective internal surveillance system.

Materials and Methods

Mice and synthetic peptides

Female BALB/c mice, 6–8 wk of age, were purchased from Charles River Japan (Tokyo, Japan). Peptides were synthesized on a PE Applied Biosystems (Foster City, CA) model 430A peptide synthesizer, using conventional t-Boc chemistry and were cleaved from the resin by liquid. Synthetic

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[3] Abbreviations used in this paper: AICD, activation-induced cell death; FasL, Fas ligand; MAPK, mitogen-activated protein kinase; MMC, mitomycin C; SAPK, stress-activated protein kinase.

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peptides were purified by gel filtration on Bio-Gel P-4 and analyzed by HPLC on a C_{18} reverse phase column. Peptide fractions containing >90% of the desired product were used for the experiments. Peptide I-10 (RGPGRAFVTI) (13) and peptide MNT10 (IGPGRAFYAT) (14) represent the immunodominant CTL epitopes, both presented by the same murine class I MHC molecule, D^d, in the V3 loop of HIV-1 gp160 glycoprotein found in strains IIIB and MN, respectively.

Generation of the CTL lines

BALB/c mouse spleen cells (5×10^6) from mice previously immunized with 1×10^7 PFU of vSC25 (recombinant vaccinia virus expressing HIV envelope glycoprotein gp160 of the IIIB isolate) (15) were stimulated with mitomycin C (MMC)-treated HIV-1-IIIB gp160 gene-transfected BALB/c3T3 fibroblasts (1×10^5 cells, termed 15-12 cells) (16) in vitro in 24-well plates containing 1.5 ml of complete T cell culture medium composed of RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, a mixture of vitamins, 1 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, heat-inactivated 10% FCS, and 10% rat T-STIM (Collaborative Biomedical Products, Bedford, MA). The CTL lines were established and maintained by biweekly stimulation with MMC-treated 15-12 cells, termed LINE-IIIB cells.

Detection of DNA fragmentation

For induction of apoptosis, 1×10^6 CTLs were stimulated with 1×10^5 MMC-treated 15-12 cells. After 1-day incubation, disrupted target cells were removed, and the CTLs were treated with peptide I-10 for 30 min, followed by washing to remove free peptide and further culturing. At 2.5 h after addition of peptide I-10, cells were harvested, and cell viability was estimated by the trypan blue dye exclusion test. Then cells were lysed with hypotonic lysing buffer (10 mM Tris, 10 mM EDTA, and 0.5% Triton X-100, pH 7.4) at 4°C for 10 min and centrifuged at 20,400 $\times$ *g* for 10 min to remove unfragmented DNA and cell debris (17, 18). RNase A (200 μg/ml) and proteinase K (200 μg/ml) were added to samples, and fragmented DNA were recovered by centrifugation after precipitation overnight at −20°C in 1 vol of isopropanol in the presence of 0.5 M NaCl. Electrophoresis was conducted on 2% agarose gels in 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA, and DNA was visualized by ethidium bromide staining.

Abs and reagents

The following Abs and reagents were used for blocking of apoptosis induction: rat anti-mouse TNF-α mAb (clone MP6-XT3; BD PharMingen, San Diego, CA), hamster anti-mouse FasL mAb (clone MFL1; BD PharMingen), rat anti-mouse CD8 mAb (clone 53-6.7; BD PharMingen), caspase 3 inhibitor (Asp-Glu-Val-Asp-fluoromethyl ketone; MBL, Nagoya, Japan), cyclosporin A and FK506 (gifts from Fujisawa Pharmaceutical, Tokyo, Japan), calcinurin autoinhibitor (BIOMOL Research Laboratories, Plymouth Meeting, PA), and mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor U0126 and p38 MAPK-specific inhibitor SB203580 (Promega, Madison, WI).

Cell staining

Cells were pelleted and resuspended at a concentration of 5×10^5 cells in 100 ml of PBS with 0.1% NaN_3 containing FITC-labeled rat anti-mouse Fas mAb (clone Jo2; BD PharMingen), FITC-labeled rat anti-mouse IL-2Rα (clone 7D4; BD PharMingen), or hamster anti-mouse FasL mAb, followed by FITC-labeled goat anti-hamster Ab (Southern Biotechnology Associates, Birmingham, AL). To detect intracellular Bcl-2 expression, cells were fixed and permeabilized with Cytofix/Cytoperm (BD PharMingen) before staining with FITC-conjugated hamster anti-mouse Bcl-2 (clone A19-3; BD PharMingen). After 30-min incubation on ice, cells were washed and resuspended in PBS for analysis by FACScan (BD Biosciences, Mountain View, CA).

Tyrosine phosphorylation assay

Untreated or treated CTLs ($2–4 \times 10^6$) were lysed in 20 μl of lysis buffer (1% Nonidet P-40, 140 mM NaCl, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and 50 mM monoiodoacetamide) on ice for 15 min. After centrifugation at 20,400 $\times$ *g* for 15 min, proteins in cell lysates were separated by 13% SDS-PAGE under reducing conditions and transferred to nylon membrane. The blots were probed with a peroxidase-conjugated mouse mAb against phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY) or hamster anti-mouse CD3ζ (clone H146.956; supernatants of hybridoma), followed by peroxidase-conjugated goat anti-hamster Ab (Jackson ImmunoResearch, West Grove, PA). After visualization of bands using a tetramethylbenzidine substrate kit (Vector, Burlingame, CA), quantification of bands was performed using the National Institutes of Health Image program.

Results

Treatment of stimulated CTLs with free antigenic peptide induces rapid onset of apoptosis

To explore the induction of apoptosis by restimulation of recently activated T cells, we exposed LINE-IIIB cells (that had been last stimulated 14 days before the experiment) to graded concentrations of antigenic peptide. As shown in Fig. 1, the CTLs that had been stimulated 24 h previously showed DNA fragmentation indicative of the initiation of apoptosis. This DNA fragmentation was dose dependent on the amount of antigenic peptide provided. DNA fragmentation was not detected in resting CTLs or in those stimulated with either target cells or peptide alone. The induction of apoptosis following exposure to peptide Ag was also observed in another CTL line (LINE-MN cells) (19) that differed in its specificity for the HIV-1 envelope of the MN isolate (gp160 MN; data not shown), suggesting that this kind of apoptosis is a general phenomenon of the CTLs.

We estimated the activation level of LINE-IIIB cells by the IL-2Rα expression level after stimulation with 15-12 cells. The level of IL-2Rα expression on the CTLs was highest 1 day after stimulation and decreased with time. Moreover, the level of both Fas and IL-2Rα expression after 1-day stimulation was similar to that in cells stimulated with PMA (5 ng/ml) and calcium ionophore (1 μM) for 12 h (data not shown). We next examined the effects of activation states of LINE-IIIB cells on the magnitude of apoptosis induction. When stimulated CTLs were further treated with peptide I-10 after 1, 4, 7, or 10 days after stimulation with 15-12 cells, maximum induction of apoptosis was observed on day 1 after stimulation, and the level of apoptosis decreased with time (Fig. 2*A*). Moreover, when the CTLs were stimulated with various numbers of target cells, DNA fragmentation was decreased upon stimulation with decreasing target cell numbers (Fig. 2*B*).

Free antigenic peptide-induced apoptosis is mediated by signaling through TCR

We next addressed the mechanism of apoptosis induction with free antigenic peptide in stimulated CTLs. Two major pathways might be employed in the induction of this kind of apoptosis: through the MHC class I molecules on the T cells or via their own TCR, or through some combination of these possible pathways. To eliminate the possibility that antigenic peptide induced a signal on the

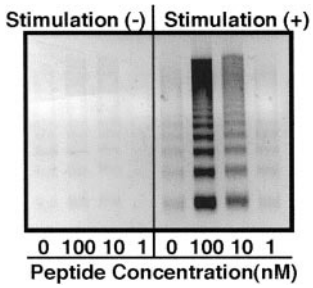

FIGURE 1. Free antigenic peptides induce a rapid onset of apoptosis in stimulated CTLs. LINE-IIIB cells were stimulated with or without target cells (MMC-treated 15-12 cells). After 1-day incubation the CTLs were treated with various concentrations of peptide I-10 for 30 min, followed by washing to remove free peptide and further incubation. At 2.5 h after addition of each peptide, cells were harvested, and DNA fragmentation was estimated as described in *Materials and Methods*.

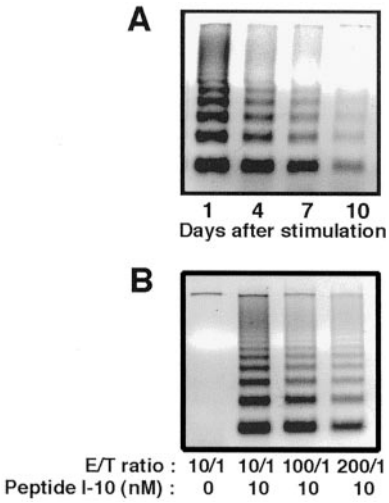

FIGURE 2. Kinetics of apoptosis induction in stimulated CTLs by free antigenic peptide. *A*, LINE-IIIB cells were stimulated with target cells at an E:T cell ratio of 10:1 and further incubation for indicated period. After incubation, the CTLs were treated with 10 nM peptide I-10 for 30 min, followed by washing to remove free peptide and further incubation. *B*, LINE-IIIB cells were stimulated with target cells at the indicated E:T cell ratio. After 1-day incubation, the CTLs were treated with 10 nM peptide I-10 for 30 min, followed by washing to remove free peptide and further incubation. At 2.5 h after addition of peptide I-10, cells were harvested, and DNA fragmentation was estimated as described in *Materials and Methods*.

T cell, we prepared a multivalent H-2D^d/P18-I10 tetramer that would be expected not to bind to T cell class I molecules, but only to the TCR of the specific T cells. As shown in Fig. 3*A*, treatment of the stimulated CTLs with the D^d/P18-I10 tetramer, but not with the D^d/motif peptide tetramer control, potently induced apoptosis. This is consistent with the view that apoptosis in the recently re-stimulated CTL is due to further signals conveyed via the TCR.

Another way to confirm the importance of the TCR in serving as a receptor for this apoptotic signal is to evaluate the efficiency of the signal when single amino acid-substituted peptide variants of P18-I10 were studied. As shown in Fig. 3*B* substitutions of 322(R-A), 324(F-A), and 325(V-Y) resulted in a significant reduction of apoptosis induction. We previously reported the importance of amino acid position 325 for TCR recognition and of positions 322 and 324 for binding to the MHC-I molecule (19, 20). The effect of residue 324 substitution on MHC binding may be indirect, since crystalographically the side chain at this position is exposed to solvent and is not in the binding cleft (21, 22). These results are consistent with those findings and strongly indicate that the free antigenic peptide must bind to the H-2D^d MHC class I molecule and interact with the TCR on the same or possibly a neighboring cell to induce the observed apoptosis.

One of the first intracellular biochemical events that occurs after TCR recognition of peptide/MHC is phosphorylation of the CD3 ζ-chain. Therefore, we next measured tyrosine phosphorylation of the CD3 ζ-chain in stimulated or peptide I-10-restimulated CTLs by Western blotting. Anti-phosphotyrosine immunoblot analysis showed that treatment of stimulated CTLs with peptide I-10 induced a higher level of phosphorylated proteins corresponding to the CD3 ζ-chain (Fig. 3*C*).

Identification of molecules related to apoptosis in stimulated CTLs

The TCR-mediated apoptosis that has previously been described was mainly Fas dependent and resulted in rapid up-regulation of

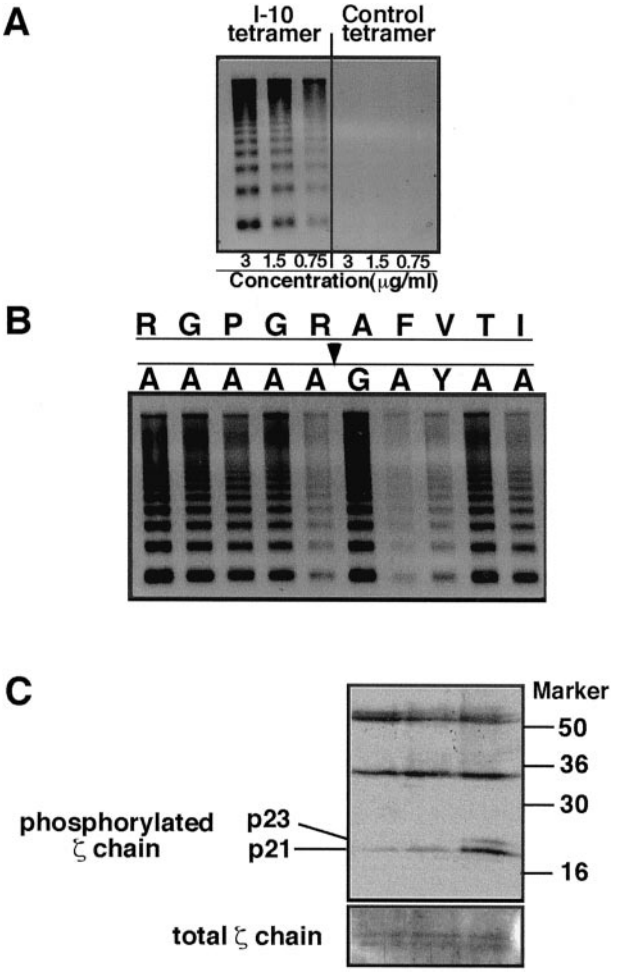

FIGURE 3. Free antigenic peptide-induced apoptosis is mediated by signaling through TCR. The CTLs were stimulated with target cells at an E:T cell ratio of 10:1. After 1-day incubation, the CTLs were treated with various concentrations of H-2D^d/P18-I10 tetramer (*A*), or 10 nM analogs of peptide I-10 with single amino acid substitutions (*B*) for 30 min. At 2.5 h after addition of peptide, cells were harvested, and DNA fragmentation was estimated as described in *Materials and Methods*. For detection of phosphorylated CD3 ζ-chain, cells were lysed with lysis buffer, and proteins in lysates were separated in a 13% polyacrylamide gel and blotted on nylon membrane. The blot was probed with peroxidase-conjugated mouse anti-phosphotyrosine mAb (*C*, *upper column*) or hamster anti-mouse CD3ζ, followed by peroxidase-conjugated goat anti-hamster Ab (*C*, *lower column*), and developed using a tetramethylbenzidine substrate kit. *Left lane*, Untreated CTLs; *middle lane*, stimulated CTLs; *right lane*, stimulated and further peptide-treated CTLs.

FasL on the cell surface following TCR ligation (23). Therefore, we examined the expression of molecules related to apoptosis in these restimulated CTLs by FACS analysis. As demonstrated in Fig. 4, stimulation of the CTLs with target cells or further treatment with peptide I-10 slightly up-regulated Fas, but had no effect on FasL expression. Bcl-2 expression was almost unchanged with any treatment of the CTLs. The CTLs showed a slight sign of activation, up-regulation of IL-2Rα in both stimulated and further peptide treated-CTLs, but TCR and CD8 expressions were unchanged with any treatment of the CTLs (data not shown).

Determination of the death pathway in apoptotic cells induced by antigenic peptide

Next, to determine whether TNF-α, Fas, or CD8 molecules were involved in the induction apoptosis observed in our system, we

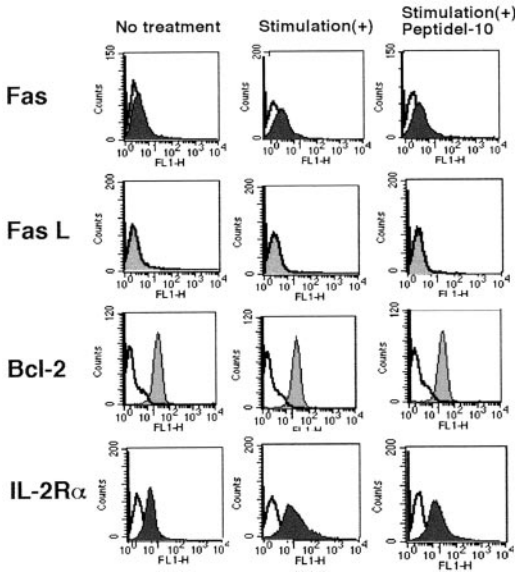

FIGURE 4. Expression of apoptosis-related molecules in stimulated CTLs. The CTLs were stimulated with or without target cells at an E:T cell ratio of 10:1. After 1-day incubation, the CTLs were treated with or without 10 nM peptide I-10 for 30 min. Then treated or untreated CTLs were strained with FITC-labeled rat anti-mouse Fas mAb, FITC-labeled rat anti-mouse IL-2Rα, or hamster anti-mouse FasL mAb, followed by treatment with FITC-labeled goat anti-hamster Ab. To detect intracellular Bcl-2 expression, cells were fixed and permeabilized before staining with FITC-labeled hamster anti-mouse Bcl-2. The filled curve represents the staining with each tested-Ab, whereas the open curve shows the staining with class-matched control Ab.

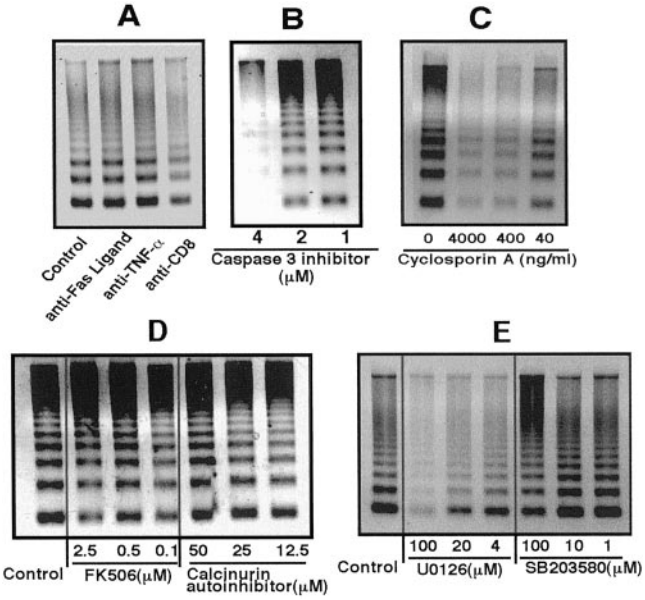

FIGURE 5. Determination of the death pathway in apoptotic cells induced by free antigenic peptide. The CTLs were stimulated with target cells at an E:T cell ratio of 10:1. After 1-day incubation, the CTLs were treated with 10 μg/ml anti-FasL, TNF-α, or CD8 mAb (*A*); caspase 3 inhibitor (*B*); cyclosporin A (*C*); FK506 or calcinurin autoinhibitor (*D*); or MAPK kinase inhibitor (U0126) or p38 MAPK-specific inhibitor (SB203580; *E*) for 1 h at 37°C before treatment with 10 nM peptide I-10 for 30 min. At 2.5 h after addition of peptide I-10, cells were harvested, and DNA fragmentation was estimated as described in *Materials and Methods*. To confirm the activity of the Abs used in this study, we tested the ability of anti-FasL Ab (10 μg/ml) to inhibit AICD of DO.11.10 T hybridoma (94% inhibition) as reported previously (36), the ability of anti-TNF-α Ab (10 μg/ml) to inhibit the sensitivity of L929 cells to 50 ng/ml of recombinant mouse TNF-α (74% inhibition) based on our previous observation (37), or the ability of anti-CD8 Ab (10 μg/ml) to inhibit the proliferation of LINE-IIIB cells 2 days after stimulation with target cells (69% inhibition) based on our unpublished observation.

added anti-TNF-α, anti-FasL, or anti-CD8 mAb to stimulated CTLs cultures before treatment with peptide I-10. As shown in Fig. 5*A*, anti-TNF-α and anti-FasL mAb did not inhibit, and anti-CD8 mAb slightly inhibited the induction of apoptosis in the restimulated CTLs. However, apoptosis induction was markedly inhibited by the addition of caspase 3 inhibitor in a dose-dependent manner (Fig. 5*B*). Moreover, pretreatment of cyclosporin A significantly inhibited apoptosis induction in stimulated CTLs (Fig. 5*C*). Cyclosporin A and FK506 are known to exert a selective inhibitory effect on T cells by binding with immunophilin, resulting in the inhibition of calcineurin catalytic activity. On the other hand, it has been reported that calcineurin induces apoptosis through a mechanism that suppresses the function of Bcl-2 (24). To test whether calcineurin is involved in the induction of apoptosis in our system, we examined the effect of FK506 or the calcineurin autoinhibitor, the 43 residues C-terminal of the calmodulin binding domain of calcineurin (25), on the induction of apoptosis. Unexpectedly, pretreatment of the CTLs with FK506 or the calcineurin autoinhibitor failed to inhibit apoptosis induction. Also, the level of Bcl-2 expression in stimulated or untreated CTLs was found to be the same (Fig. 4). These findings suggest that the apoptosis induction observed in our system is not dependent on the calcineurin-mediated pathway.

Recently, Matsuda et al. (26) have demonstrated that cyclosporin A inhibited activation of MAPK pathways. Three major types of MAPK cascades, ERK1/ERK2 MAPK, c-Jun kinase/stress-activated protein kinase, and p38 kinase, have been reported in mammalian cells that respond synergistically to different upstream signals. To extend our analysis of the pathway in cyclosporin A-sensitive apoptosis, we next examined whether the ERK1/ERK2 MAPK pathway inhibitor (U0126) or the p38 MAPK pathway inhibitor (SB203580) can block the apoptosis induction. As demonstrated in Fig. 5*E*, U0126 significantly inhibited apoptosis induction of stimulated CTLs in a dose-dependent manner, while SB203580 did not. Thus, the rapid induction of apoptosis in restimulated CTLs by their brief exposure to peptide I-10 may be mediated by ERK1/ERK2 MAPK and the caspase 3 cascade.

Discussion

The restimulation of recently activated $CD4^+$ T cells via their Ag-specific TCR triggers a fatal response, termed AICD. Recent studies have shown that AICD in $CD4^+$ T cells is mainly mediated by Fas and TNF-α and occurs ~12 h after restimulation. However, in our experiments we observed that stimulated CTLs treated 24 h later with antigenic peptide enter an apoptotic phase within 3 h in a reaction that was unaffected by either anti-FasL or anti-TNF-α, suggesting a unique pathway involved in AICD of $CD8^+$ CTLs.

In our system rapid onset of apoptosis by antigenic peptide seemed to be mediated by signaling through TCR. In general, TCR engagement with peptide/MHC initiates signal transduction through tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs of the cytoplasmic domain of the CD3 molecule. Our results clearly showed that treatment of recently stimulated CTLs with antigenic peptide induced an increased level of CD3ζ phosphorylation. Rodriguez-Tarduchyth et al. (27) reported that impairment of association of CD3ζ with TCRβ by TCRβ mutants resulted in the specific inhibition of apoptosis.

Since other T cell activation events were not affected by the mutation, they speculated the existence of an independent intracellular signaling pathway for apoptosis in which CD3ζ seems to be involved. Therefore, enhancement of CD3ζ phosphorylation by peptide I-10 appears to contribute to the rapid onset of apoptosis in the CTLs. In our experiments, CD3ζ phosphorylation was clearly involved in an apoptotic event that depended on TCR engagement.

Several reports have shown that MAPK family members might be involved in inducing apoptosis signals via regulating FasL expression in T cells (28, 29). We have also observed that the ERK1/ERK2 MAPK pathway inhibitor U0126 inhibited apoptosis in stimulated CTLs after exposure to free antigenic peptide. However, both the expression of FasL mRNA (data not shown) and surface FasL molecules of the CTLs were unchanged after stimulation with target cells or further treatment with peptide, and anti-FasL did not inhibit apoptosis, suggesting that the regulation of FasL expression by MAPK activation might not be involved in the induction of apoptosis, and that a Fas-independent pathway through MAPK activation may be considered. Thus, in addition to its role in T cell proliferation, MAPK signaling plays an important role in CTLs apoptosis, indicating that proliferation and AICD of the CTLs share a common activation pathway.

We performed additional experiments to address the question of whether the CTLs are killing each other (fratricide) by recognizing peptide I-10/MHC class I complexes on their surface. Since both ERK1/ERK2 MAPK inhibitor (U0126) and caspase 3 inhibitor significantly inhibited apoptosis induction of stimulated CTLs in a dose-dependent manner (Fig. 5, *B* and *E*), we examined the effects of these inhibitors on CTL activity. Treatment of the CTLs with either ERK1/ERK2 MAPK inhibitor (U0126) or caspase 3 inhibitor resulted in no or little inhibition of CTL activity (Fig. 6). These findings demonstrate that cell death is not due to the fratricide, but due to the induction of apoptosis.

We have reported previously that cytolytic activity of the CTLs was markedly inhibited by brief exposure to peptide I-10 when the CTLs were in a resting state (>day 7 after routine stimulation) (30). This inhibition was temporary and was not due to damage to the CTLs or their elimination by apoptosis, but to exhaustion, as evidenced by the reduction of granzyme B and perforin, key components of cytotoxic granules (31). In another series of studies we found that apoptosis was induced in high avidity CTLs by supraoptimal densities of peptide-MHC complexes on APCs even when the cells were rested and were exposed to the high density of Ag for only 2 h (7, 8, 32); that mechanism involves TNF and the TNF receptor. In the present study we describe yet a third, distinct mechanism in which recently stimulated CTLs are induced to enter apoptosis within 3 h following brief exposure to antigenic peptide P18-I10 at optimal, rather than supraoptimal, concentrations. The level of apoptosis was dependent on both the time after stimulation with target cells and the number of target cells for stimulation, and this mechanism does not seem to involve TNF or Fas. These findings suggest that the activation state of the CTLs before exposure to antigenic peptide is very important in determining the anergic or apoptotic fate of the cell; that is, recently stimulated CTLs might enter apoptosis, while resting CTLs might become anergic due to the brief exposure to antigenic peptide.

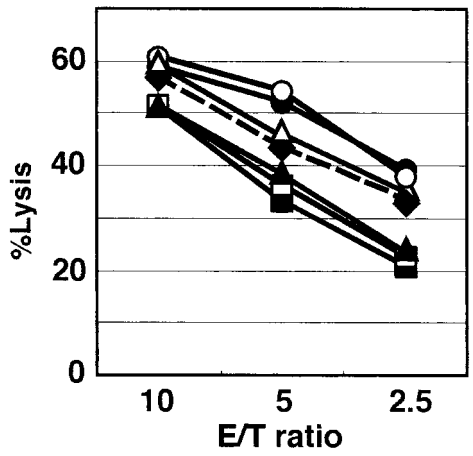

FIGURE 6. Effect of ERK1/ERK2 MAPK inhibitor (U0126) or caspase 3 inhibitor treatment on CTL activity. CTLs were incubated with ERK1/ERK2 MAPK inhibitor (U0126) or caspase 3 inhibitor for 1 h at 37°C. After the incubation, the treated cells were further cultured with ^{51}Cr-labeled 15-12 cells in the presence of inhibitor in 96-well, round-bottom microtiter plates for 4 h, followed by ^{51}Cr release assay. The symbol in the parentheses represents untreated control (♦) or cells treated with 100 μM U0126 (●), 20 μM U0126 (○), 4 μM U0126 (△), 4 μM caspase 3 inhibitor (■), 2 μM caspase 3 inhibitor (□), or 1 μM caspase 3 inhibitor (▲).

Our model may help to explain the disappearance/deletion of peripheral, virus-specific CTLs during an acute viral infection in vivo. For example, primary HIV infection is associated with overwhelming virus replication throughout the lymphoid system. Moreover, HIV-1-infected individuals are known to have a high frequency of HIV-1-specific CD8$^+$ T cells during primary infection. Under such circumstances, cells infected with high titers of virus are lysed by effector CTLs, and large amounts of intracellular viral protein may be released into the environment of the CTLs. These virus proteins may be digested by various types of proteases into peptides and recognized by the CTLs as free antigenic peptide. Therefore, the CTLs stimulated with the target cells might be further stimulated by exposure to free antigenic peptide, leading to the induction of AICD. Mice with transgenic TCR have allowed more direct approaches to be used to assess the fate of Ag-specific CD8$^+$ T cells in vivo. Koniaras et al. (33) and Wack et al. (34) have reported that peptide-specific CD8$^+$ T cells proliferated and subsequently underwent apoptosis in situ in lymphoid organs in response to antigenic peptide following injection of TCR transgenic mice with peptide. Using P18-I10-specific TCR transgenic mice (35) we are also currently in the process of examining whether our model system, in which free antigenic peptides induce apoptosis in stimulated CTLs, occurs in vivo.

The CTLs are thought to play a key role in preventing both virus spread and disease progression in viral infections such as AIDS and chronic hepatitis. Thus, to maintain this important arm of immunity, it is crucial to preserve such CTLs even in the presence of a high viral burden, particularly during the early phase of primary infection. Our study demonstrates that death pathways involving ERK and caspase activation may be critical in regulating this early CTL response, and that agents such as cyclosporin A might prove useful in preventing such CTLs from succumbing to an apoptotic pathway. Thus, a better understanding of the mechanisms by which virus-specific CTLs are damaged or killed in the early phases of viral infection should contribute to new approaches for immunotherapeutic intervention.

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