Caspase Inhibition Activates HIV in Latently Infected Cells

ROLE OF TUMOR NECROSIS FACTOR RECEPTOR 1 AND CD95*

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Stimulation of tumor necrosis factor receptor 1 (TNF-R1) triggers both caspase-dependent and caspase-independent signaling activities. The caspase-dependent signaling pathway induces apoptotic cell death in susceptible cells, whereas the caspase-independent signaling cascade leads to activation of nuclear factor κB and induces antiapoptotic signaling activities. Stimulation of nuclear factor κB via TNF-R1 is known to activate human immunodeficiency virus (HIV) replication in infected cells. Here we show that the broad range caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (ZVAD) activates HIV replication in the chronically infected T-cell line ACH-2. Virus activation was caused by a sensitization of TNF-R1 toward endogenously produced tumor necrosis factor α (TNF-α). Neutralizing anti-TNF-α antibodies completely abolished the virus-inducing activity of ZVAD. Treatment of cells with TNF-α in the presence of ZVAD caused increased expression of TNF-α and induced enhanced virus replication. Activation of CD95, another member of the TNF receptor family, similarly triggered HIV replication, which was further enhanced in the presence of ZVAD. Our data show that caspase inhibitors sensitize both CD95 and TNF-R1 to mediate activation of HIV in latently infected cells. Activation of HIV replication in latent virus reservoirs is currently discussed as a therapeutic strategy to achieve eradication of HIV in patients treated with antiretroviral therapy. Our results point to a novel role for caspase inhibitors as activators of virus replication in vivo.

Stimulation of the tumor necrosis factor receptor 1 (TNF-R1) by TNF-α activates two different signaling pathways. One of them involves receptor-interacting protein and tumor necrosis factor receptor-associated factor 2 and activates NF-κB and activator protein 1, which in turn induce genes involved in acute and chronic inflammatory response. The other is mediated by caspase activity and induces apoptosis in susceptible cells (1–3). Another member of the TNF receptor family, CD95, has been recognized as a cell surface molecule mediating primarily apoptotic signaling (4). This molecule has been implicated in the control of cell homeostasis in the immune system and in cytotoxic T lymphocyte-induced cell killing (4). Activation of CD95 by its natural ligand or by cross-linking antibodies recruits a death-inducing signaling complex to CD95 that consists of the adaptor molecule Fas-associated death domain and caspase-8 (5, 6). Death-inducing signaling complex formation activates caspase-8, which in turn activates other downstream effector caspases that transmit and exercise the apoptotic process (7). Peptidase caspase inhibitors such as benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (ZVAD) block the proteolytic activation of caspases and therefore prevent induction of apoptotic cell death.

It has recently been demonstrated that CD95 not only triggers caspase-mediated apoptosis but also activates an alternative signaling cascade that induces activation of NF-κB (8). In our studies, we found that this alternative signaling cascade is independent of caspase activation. Moreover, under conditions of caspase inhibition, this signaling pathway induces expression of various proinflammatory cytokines in both T lymphoblasts and primary T lymphocytes.2

The human immunodeficiency virus (HIV) can infect activated and naïve CD4+ T cells. However, only activated T cells support subsequent viral gene expression and virus replication. In nonactivated T cells, the viral replication cycle ends after integration of the virus genome into the host DNA. Activation of these latently infected cells results in prosecution of the viral replication cycle, and viral genes become expressed. One important transcription factor involved in the expression of HIV is NF-κB. The 5′ long terminal repeat of HIV contains NF-κB binding elements that are functionally active (9–12), and activation of NF-κB in cells latently infected with HIV causes activation of virus replication. Two prominent activators for HIV replication are the cytokines TNF-α (9) and interleukin 2 (13). In recent clinical trials, a combination of highly active antiretroviral therapy (HAART) and interleukin 2 has been studied to achieve eradication of HIV from infected patients (14–18).

In this study, we report that caspase inhibitors activate HIV replication in chronically infected T cells. We show that caspase inhibitors not only block death receptor-induced apoptosis but also allow the execution of caspase-independent signaling pathways triggered by TNF-R1 or CD95 that lead to activation of NF-κB and HIV in chronically infected T cells.

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1 The abbreviations used are: TNF-R1, tumor necrosis factor receptor 1; ZVAD, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; HIV, human immunodeficiency virus; NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor α; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbet assay.

2 C. Scheller, S. Sopper, C. Ehrhard, E. Flory, P. Chen, E. Koutsilier, S. Ludwig, V. ter Meulen, and C. Jassoy, unpublished data.
**Fig. 1. Caspase inhibition activates HIV in ACH-2 cells.** ACH-2 T lymphoblasts were cultured in the absence (Medium) or presence (ZVAD) of the caspase inhibitor ZVAD (100 μM). A, HIV-p24 expression was monitored by intracellular staining and flow cytometry. B, the absolute cell number was determined by quantitative flow cytometry. C and D, dot plot analysis of HIV-p24 expression of two selected data points from A: untreated (C) and ZVAD-treated cells (D) cultured for 16 h. A and B, the presented data were obtained from the same experiment. All assays were adjusted to the same solvent (Me2SO) concentration. Values represent the means ± S.D. from triplicate analyses.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents**—The latently HIV-infected T lymphoblast line ACH-2 (1B) was cultured in RPMI 1640 and 10% fetal calf serum. The anti-CD95 mAb 7C11 (Coulter Immunotech, Hialeah, FL) was used at 200 ng/ml. Recombinant human TNF-α (BD PharMingen) was used at the indicated concentrations. The caspase inhibitor ZVAD (Bachem Biochemica, Heidelberg, Germany) was used at 100 μM and dissolved in Me2SO. All assays were adjusted to identical solvent concentrations.

**ELISA**—Cells were cultured in a 96-well flat-bottomed plate (10⁵ cells/well) in a total volume of 200 μl. Concentration of released TNF-α was determined by ELISA using 50 μl of the supernatants. The ELISA was performed according to the instructions of the manufacturer (OptEia; BD PharMingen).

**NF-κB Electrophoretic Mobility Shift Analysis**—To prepare nuclear fractions, cells were incubated in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride for 15 min. Swollen cells were ruptured by aspiration with a syringe, and nuclei were collected by centrifugation. The nuclei were dissolved in 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The solution was cleared by centrifugation, and the protein concentration was determined with the Bradford technique.

Double-stranded oligonucleotide probes were labeled in a reaction mixture containing 200 ng of double-stranded DNA probe, 50 μCi of [32P]dCTP, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 500 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, and 2 units of Klenow fragment. After a 30-min incubation at 37 °C, oligonucleotides were separated on a 0.25% Sephadex spin column (Roche Molecular Biochemicals) and finally resuspended in Tris-EDTA buffer (30,000 cpm/ml). 3 μg of nuclear proteins were preincubated on ice with 2 μg of poly(dI-dC) (Roche Molecular Biochemicals) and 1 μg of bovine serum albumin in bandshift buffer (20 mM HEPES, pH 7.9, 1 mM dithiothreitol, 1 mM EDTA, 10 mM KCl, and 4% Ficoll) for 5 min. 32P-labeled oligonucleotide (60,000 cpm) was added to a total volume of 20 μl, incubated at room temperature for 15 min, and loaded onto 5% native polyacrylamide gels in 0.5× Tris borate-EDTA buffer. Gels were dried and exposed for autoradiography.

**Flow Cytometry**—To determine intracellular HIV-p24 expression, ACH-2 cells were fixed with 4% formalin for 20 min. Cells were permeabilized with phosphate-buffered saline containing 5% bovine serum albumin and 0.5% saponin, stained with the mouse anti-HIV-p24 mAb 183-H12-5C, counterstained with a fluorescein isothiocyanate-labeled anti-mouse IgG antibody (DAKO), and analyzed by flow cytometry using a FACScan flow cytometer (BD PharMingen). Markers were set according to staining with an isotype-matched control antibody (DAKO). For quantitative flow cytometry, equal amounts of calibration beads (BD PharMingen) were added to all samples. Living cells were gated by propidium iodide-negative staining. Relative numbers of living cells were determined by comparison with the number of calibration beads.

**RESULTS**

**Caspase Inhibition Activates HIV in ACH-2 Cells**—TNF-α is known to induce HIV replication in ACH-2 cells, a T lymphoblast line that is latently infected with HIV (19). Virus activation by TNF-α is caused by the proinflammatory signaling cascade of TNF-R1 that activates NF-κB. The other signaling pathway triggered by TNF-R1 is mediated by caspases and induces apoptosis. To study any interfering effects between these two different signaling cascades on HIV replication, we blocked apoptotic TNF-R1 signaling with the peptidic broad range caspase inhibitor ZVAD. Cells were treated with ZVAD or with the solvent Me2SO alone (untreated cells), and HIV production was measured by flow cytometry detecting intracellular expression of the HIV Gag protein p24. ZVAD induced marked expression of HIV in ACH-2 cells (Fig. 1A). To exclude the possibility that the observed increase in p24 expression was mediated by a potential prolonged survival of HIV-expressing cells due to apoptosis inhibition by ZVAD, we determined the absolute cell number in the assays using quantitative flow cytometry. Treatment with the apoptosis inhibitor ZVAD did not result in increased cell numbers (Fig. 1B). Therefore, the observed increase in HIV-expressing cells was not mediated by a prolonged survival of spontaneously activated cells but was in fact caused by induction of HIV replication in previously non-activated cells.

**ZVAD-induced Activation of HIV Replication Is Dependent on TNF-α**—To elucidate the mechanism of the observed ZVAD-induced activation of HIV, we analyzed the potential role of TNF-α. We incubated ACH-2 cells with different concentrations of ZVAD for 24 h in the absence and presence of a neutralizing TNF-α antibody. ZVAD induced a dose-dependent activation of HIV (Fig. 2A). ZVAD-induced virus activation was inhibited in the presence of the anti-TNF-α antibody, suggest-
ZVAD induced a dose-dependent increase of TNF-α in the absence of ZVAD, which is endogenously produced by ACH-2 cells in low amounts. This conclusion is confirmed by measurement of TNF-α concentrations in the supernatants of the ACH-2 cells after stimulation with TNF-α in the absence and presence of ZVAD. TNF-α concentrations were markedly increased in the presence of ZVAD. Further analysis of the culture medium revealed augmented TNF-α concentrations in the assays containing ZVAD (Fig. 3A). Therefore, stimulation of TNF-R1 in the presence of ZVAD increases TNF-α expression in ACH-2 cells, which in turn contributes to activation of HIV.

Stimulation of CD95 Activates HIV—The previous results have demonstrated that caspase inhibition results in an enhancement of HIV expression in ACH-2 cells in the presence of ZVAD. CD95-stimulated HIV expression was markedly increased in the presence of ZVAD. Further analysis of the culture medium revealed augmented TNF-α concentrations in the assays containing ZVAD (Fig. 3B). Therefore, stimulation of TNF-R1 in the presence of ZVAD increases TNF-α expression in ACH-2 cells, which in turn contributes to activation of HIV.

Caspase Inhibition Stimulates TNF-α Production in CD95-activated Primary T Cells—Our results show that caspase inhibition activates HIV in the chronically infected T-cell line ACH-2. One pathway involved in this virus activation is a caspase-independent signaling pathway triggered by CD95. CD95 stimulation activates NF-κB, which in turn leads to HIV expression in ACH-2 cells.

Stimulation of CD95 Activates NF-κB in the Absence and Presence of ZVAD—To examine the involvement of NF-κB in CD95-induced virus activation, we performed electrophoretic mobility shift analysis with nuclear extracts of the cells using a 32P-labeled NF-κB probe as described previously (20). Activation of HIV replication by CD95 was accompanied by nuclear translocation of NF-κB in both the absence and presence of caspase inhibition (Fig. 6A). To test whether NF-κB controls HIV expression after stimulation of CD95, cells were treated with the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal, which prevents IκB degradation and subsequent activation of NF-κB (21). N-acetyl-leucyl-leucyl-norleucinal completely inhibited activation of HIV after CD95 stimulation in both the absence and presence of ZVAD (Fig. 6B). These data indicate that CD95 stimulation activates NF-κB, which in turn leads to HIV expression in ACH-2 cells.
Stimulation of the TNF-R1 activates both caspase-dependent and caspase-independent signaling pathways that either induce apoptotic cell death or activate NF-κB, respectively (1–3). It has been known for several years that stimulation of the NF-κB pathway by TNF-α activates HIV (19). This study now demonstrates that caspase inhibitors that block the apoptosis-inducing activity of TNF-R1 can enhance the HIV-stimulating effect of TNF-R1. Caspase inhibitor-mediated enhancement of virus was not due to a prolonged cell survival but was specifically caused by an up-regulation of NF-κB signaling activities of TNF-R1.

In contrast to TNF-R1, CD95 has mainly been viewed as an apoptosis receptor that is not involved in NF-κB signaling (1–3). Recent studies, however, have shown that CD95 signaling can lead to activation of NF-κB (8). Here we demonstrate that this alternative signaling activity results in activation of HIV upon CD95 stimulation in ACH-2 cells. Similar to the situation with TNF-R1, caspase inhibitors enhanced CD95-induced virus replication. Because we used intracellular staining of HIV Gag expression to detect virus replication, we were able to analyze virus activation at the level of single cells. Therefore, the observed increase in virus replication in ZVAD-treated cultures was not simply caused by a potential prolonged cell survival but was specifically caused by a potential prolonged cell survival but was specifically caused by an up-regulation of NF-κB signaling activities of CD95.

Because caspase inhibitors enhanced both CD95 and TNF-R1-induced HIV replication, caspase activation seems to counteract the NF-κB signaling activity of both receptors. It has been shown that caspses degrade several molecules that bind to TNF-R1 or CD95 and are involved in NF-κB activation, such as IKK (15, 16). The present data suggest that this is a general mechanism by which TNF and CD95 receptors inhibit HIV production and that caspase activity is required to overcome this inhibition. These data may be relevant to therapy since it is known that selective caspase inhibitors enhance HIV replication in vivo (4). Furthermore, our data suggest a mechanism by which HIV replication can be activated by caspase inhibitors that act on both TNF-R1 and CD95 receptors. This may be a general mechanism by which HIV replication is activated by caspase inhibitors that act on both TNF-R1 and CD95 receptors. This may be a general mechanism by which HIV replication is activated by caspase inhibitors that act on both TNF-R1 and CD95 receptors.

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as receptor-interacting protein, Raf-1, and tumor necrosis factor receptor-associate factor. 

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as receptor-interacting protein, Raf-1, and tumor necrosis factor receptor-associated factor 1 (22–25). Cleavage of tumor necrosis factor receptor-associated factor 1 inhibits tumor necrosis factor receptor-associated factor 2, which mediates TNF-R1-induced NF-κB activation (26). Based on these observations, we suggest that caspase inhibitors prevent degradation of these signaling molecules and therefore lead to an up-regulation of NF-κB signaling activities induced by CD95 and TNF-R1. However, further analysis of the signaling pathways involved in caspase inhibitor-induced activation of HIV is needed to confirm this assumption.

We performed our experiments in the T lymphoblast cell line ACH-2 derived from the A3.01 cell line (19, 27). There is evidence that our results may also reflect the situation in primary T cells latently infected with HIV. We have shown that stimulation of CD95 in the presence of caspase inhibitors induces production of TNF-α in primary T lymphocytes. In another study, we have further analyzed the effects of caspase inhibition on CD95 signaling in primary T cells. We have observed that CD95 stimulation in the presence of caspase inhibitors induces a switch from apoptotic to proinflammatory signaling in primary T lymphocytes.

This switch is accompanied by activation of NF-κB and expression of several proinflammatory cytokines, including TNF-α. Therefore, at the level of NF-κB activation and TNF-α expression, CD95-activated primary T cells show the same response to caspase inhibition as ACH-2 cells do. Because HIV expression is enhanced by activation of NF-κB, CD95 stimulation in the presence of caspase inhibitors may also result in virus replication in latently infected cells in vivo. It is noteworthy that caspase inhibitors have already been shown to enhance virus replication in stimulated peripheral blood mononuclear cells infected with HIV strains isolated from infected patients (28).

Activation of HIV replication in its cellular reservoirs under antiviral therapy has been suggested as a strategy to eradicate the virus from the infected body. This approach is currently being tested in several clinical trials (14–18). Based on the experience that activation of CD95 and TNF-R1 occur physiologically, enhancement of CD95- or TNF-R1-induced activation of HIV by caspase inhibitors should be explored as an alternative approach for activation therapy to achieve eradication of HIV under highly active antiretroviral therapy.

The authors thank Ingeborg Euler-Koenig for technical assistance. The chronically HIV-infected T lymphoblast ACH-2 cell line was obtained from T. Folks and the anti-HIVp24 hybridoma 183-H12-5 mAb from B. Chesebro. The ACH-2 T lymphoblasts were cultured for 7 h with medium alone, mAb 7C11 (200 ng/ml), ZVAD (100 μM), or both (7C11/ZVAD). Nuclear extracts were prepared, and activation of NF-κB was monitored by electrophoretic mobility shift analysis. A, ACH-2 T lymphoblasts were cultured for 7 h with medium alone, mAb 7C11 (200 ng/ml), ZVAD (100 μM), or both (7C11/ZVAD) in the absence (Medium) or presence (ALLN) of the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (100 μM). HIV-p24 expression was monitored by intracellular staining and flow cytometry. A and B, all assays were adjusted to the same solvent (Me2SO) concentration.
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