Activation of the CD95 (APO-1/Fas) System in T Cells From Human Immunodeficiency Virus Type-1–Infected Children

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Increased apoptosis of CD4+ T cells is considered to be involved in CD4+ T-cell depletion in human immunodeficiency virus type-1 (HIV-1)–infected individuals progressing toward acquired immunodeficiency syndrome (AIDS). We have recently shown that CD95 (APO-1/Fas) expression is strongly increased in T cells of HIV-1–infected children. In this report we provide further evidence for a deregulated CD95 system in AIDS. CD95 expression in HIV-1–infected children is not restricted to previously activated CD45RO+ T cells but is also increased on freshly isolated naive CD45RA+ T cells. In addition, specific CD95–mediated apoptosis is enhanced in both CD4+ and CD8+ T cells. Furthermore, levels of CD95 ligand mRNA are profoundly increased. Specific T-cell receptor/CD3–triggered apoptosis in HIV-1–infected children is more enhanced in CD8+ than in CD4+ T cells. Accelerated activation induced cell death of T cells could partially be inhibited by blocking anti-CD95 antibody fragments. These data suggest an involvement of the CD95 receptor/ligand system in T-cell depletion and apoptosis in AIDS and may open new avenues of rational intervention strategies.

DISEASE PROGRESSION in human immunodeficiency virus type-1 (HIV-1)–infected individuals toward acquired immunodeficiency syndrome (AIDS) is characterized by progressive CD4+ T-helper cell depletion. The mechanism of T-cell depletion may involve direct or indirect mechanisms caused by the virus. Increased apoptosis in infected as well as noninfected cells has previously been invoked in this process. It has been suggested that indirect mechanisms may sensitize for apoptosis because the number of HIV-1–infected cells in blood and in the peripheral lymphoid system may be too small to account for the massive loss of T cells in AIDS. Mechanisms that induce apoptosis in bystander T cells may involve effector molecules, communicated from HIV-1–infected to noninfected cells.

The CD95 receptor/ligand system is a key regulator of apoptosis in normal and malignant T cells. We and others have recently shown that the mechanism of T-cell receptor (TCR)–triggered activation–induced cell death (AICD) in peripheral T cells is mediated via CD95 receptor/ligand interaction. Upon activation T cells express the CD95 cell-surface receptor, a member of the TNF-R/NGF-R superfamily, that induces apoptosis upon oligomerization. Concomitantly, activated T cells produce the CD95 ligand (L), a member of the corresponding family of tumor necrosis factor (TNF)–related cytokines. CD95L can occur in a membrane-bound or in a soluble form proteolytically cleaved by a metalloprotease. CD95L may induce autocrine suicide in sensitive CD95+ T cells and cause fratricide by direct contact or paracrine death in neighboring T cells.

We hypothesized that during HIV-1 infection a constant upregulation of the CD95/CD95L system might contribute to disease progression. In support of this hypothesis we recently demonstrated a strong increase in CD95 expression on T cells from HIV-1–infected children. In addition, we showed that HIV-1 Tat increases CD95–mediated AICD by increasing CD95L expression. We also showed that HIV-1 gp120 and anti-gp120 antibodies confer CD4+ T-helper cell subset preferance to this effect by crosslinking the CD4 receptor. Furthermore, in sera of HIV-1–infected children we detected HIV-1 Tat in concentrations comparable to levels of soluble Tat in cultures with a low percentage of HIV-1–infected T cells.

To further elucidate the deregulation of the CD95 system we investigated the sensitivity of T cells in HIV-1–infected children toward induction of apoptosis. In addition, we studied CD95L–mRNA expression and CD95L activity in peripheral T cells from HIV-1–infected children and investigated the possibility to inhibit CD95–mediated autocrine suicide by disrupting CD95 receptor/ligand interaction.

MATERIALS AND METHODS

Patients. Heparinized venous blood was obtained from 24 HIV-1–infected children during routine blood sampling. All patients were seen at monthly intervals at the University Children’s Hospitals in Heidelberg and Frankfurt, Germany, as part of the “German Multicenter Study on HIV Infection in Children,” supported by the Federal Ministry of Health and approved by the ethical committee of the Children’s Hospital of Heidelberg. Patients were classified according to Centers for Disease Control (CDC) criteria as asymptomatic (n = 6), mildly symptomatic (n = 12), or severely symptomatic (n = 5). Twenty HIV-1–infected children received monthly Ig infusions, and 9 children were on azidovudine (AZT) nontherapy at the time of the study. Four HIV-1+ mothers were also tested for CD95L–mRNA expression in T cells.

Control blood samples were obtained from 15 age-matched healthy children of HIV-1–infected mothers who were followed up in our outpatient clinic and had repeatedly been shown to be HIV-1–negative as defined by negative results of HIV-1 Western blot, HIV-p24 antigen assay, and viral culture. Adults controls were recruited from HIV-1–healthy laboratory personnel (n = 11). All patients or their relatives and the controls gave informed consent before venipuncture.

Cytolourimetric analysis. Peripheral blood mononuclear cells (PBMC) obtained from HIV-1–infected patients and healthy donors were isolated by Ficoll (Seromed, Berlin, Germany) gradient centrifugation as described. T cells and their subsets were identified by...
fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies (anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, and anti-CD45RO; Dianova, Hamburg, Germany). CD95 expression was detected using biotinylated anti-AP0-1 antibodies (IgG3) and Streptavidin-PE. Immunofluorescence analysis was performed on a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

**PBMC culture and induction of apoptosis.** Freshly isolated PBMC from HIV-1- and HIV-1+ children were distributed in 96-well flat-bottom plates at a concentration of 5 x 10^6 cells per well with medium, coated IgG3, anti-CD95 (10 µg/mL-1) or anti-CD3 (100 µg/mL-1) antibodies as described.°° Cells were collected at different time points and cell death was determined on gated CD4+ and CD8+ T cells as described.°° Briefly, apoptotic cells were characterized by a decrease in forward and an increase in side scatter, reflecting the characteristic morphology of "shrinking death" of apoptotic cells.

Inhibition of anti-CD3-induced apoptosis was investigated in 10 HIV-1- children by using anti-CD95 antibody fragments (see below) that efficiently inhibit the interaction between CD95 and CD95L.°° Experiments were performed with PBMC on anti-CD3-coated plates in the presence of F(ab'), anti-CD95 (1 µg/mL-1) or F(ab'), control antibody fragments (1 µg/mL-1) derived from the isotype-matched antibody FI123.°° Percentage of specific AICD was calculated according to the formula: 100 x [experimental cell death (%) - spontaneous cell death (%)].

**Quantitative PCR for CD95L mRNA detection.** Peripheral T cells were purified from PBMC by use of anti-CD19 and anti-CD14 antibody-coated magnetic beads (Dynal, Hamburg, Germany). Over 90% pure T cells were obtained as described by FACScan analysis of the purified cells (data not shown). Total RNA was obtained by the procedure of Chomczynski and Sacchi,°° and converted to cDNA by avian myeloblastosis virus (AMV)-reverse transcriptase and oligo-dT(12) primers. For polymerase chain reaction (PCR) quantification of human CD95L, 250 ng of total RNA was coamplified with variable amounts of competitor DNA in a 50 µL PCR reaction.°° After amplification, the cDNAs were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. The ratio of competitor DNA/wild-type DNA reflects the amount of both cDNA species at the beginning of PCR.

**Statistical analysis.** Data are reported by their median values together with 95% Monte Carlo confidence intervals (CI95) of the median (created via bootstrapping on 1,000 samples). Differences between HIV-1- children and HIV-1+ controls were evaluated using the two-sided Wilcoxon rank-sum test. P values <.05 indicate statistical significance. To describe the differences in the changes spontaneous apoptosis of T-cell subsets (the mean response profile) over time between HIV-1+ children and HIV-1- controls, pointwise 95% confidence intervals were computed on the basis of the loess procedure (nonparametric curve fitting local regression).°° Numerical and graphical computations were done using the statistical software package S.PPLUS, Version 3.3 (Statistical Sciences, MathSoft Inc, Seattle, WA, 1995) on a Sun SPARCstation (Sun Microsystems, Mountain View, CA).

**RESULTS**

In previous experiments in a large cohort of children exposed to HIV-1 perinatally we could show that CD95 expression on T cells was increased in HIV-1+ children when compared with HIV-1- children.°° In this study we provide evidence that CD95 expression during HIV-1 infection is not restricted to CD45RO+ memory T cells because naive CD45RA+ T cells of HIV-1+ children also showed increased levels of CD95 expression (Fig 1). To assess the susceptibility of T lymphocytes for apoptosis, short-term cultures of PBMC were performed in the presence or absence of agonistic anti-CD95 antibodies (IgG3) or anti-CD3 antibodies coupled to the culture plates. T cells from HIV-1- children showed less spontaneous apoptosis when compared with T cells from HIV-1+ children (Fig 2, P < .001 for both CD4+ and CD8+ T cells). An increased spontaneous apoptosis was seen in both CD4+ and CD8+ T cells from HIV-1+ children. Spontaneous apoptosis was higher in CD8+ than in CD4+ T cells from HIV-1+ children (Fig 2, P < .001). Addition of interleukin-2 (IL-2) lead to a partial reduction of spontaneous apoptosis predominantly in CD8+ T cells (data not shown). Specific anti-CD95 triggered cell death (ie, apoptosis over the spontaneous background) was higher in T cells from HIV-1+ than from HIV-1- children (Fig 3, P < .005 for both CD4+ and CD8+ T cells). Addition of IL-2 did not influence CD95-triggered apoptosis (data not shown). In 22 of 24 HIV-1+ children TRC/CD3 triggered apoptosis exceeded spontaneous apoptosis in both CD4+ and CD8+ T cells. In HIV-1- children, specific TRC/CD3-triggered apoptosis of CD8+ T cells at 48 hours was significantly higher than in HIV-1+ children (Fig 3, P < .05). In the overall group mean specific TRC/CD3 triggered apoptosis of CD4+ T cells at 48 hours was not significantly different when comparing HIV-1+ children to HIV-1- children (Fig 3, P > .05). However, a subgroup of patients showed increased AICD in the CD4- T-cell subset (Fig 3). These patients are characterized by a CD4 count > 100 µL. Repeated analysis showed a constant increase of TRC/CD3-triggered specific apoptosis in CD4+ T cells from these children (data not shown). In two HIV-1+ children specific TRC/CD3-triggered apoptosis of CD4+ T cells could not be detected because of insufficient recovery of CD4+ T cells.

Because TRC/CD3-triggered early AICD in lymphocytes is primarily mediated via the CD95L/CD95L-system,°° we assessed CD95L mRNA expression in T cells freshly isolated from blood samples of 17 HIV-1+ patients (13 children, 4 adults) and 12 HIV-1- controls (3 children, 9 adults; see Fig 4). HIV-1+ patients showed two- to four-times higher levels of CD95L mRNA (median 1,000 fg/250 µg RNA, CI95 500 to 1,600) than HIV-1- controls (median 225 fg/250 µg RNA, CI95 100 to 500, P < .001). CD95L mRNA expression was increased in CD4+ as well as in CD8+ T cells (data not shown). In patients with normal immune function according to CDC criteria°° (CDC class 1), CD95L mRNA levels were only slightly elevated. However, in most patients with moderate or severe immunodepression (CDC class 2 and 3) CD95L mRNA levels were profoundly elevated (Fig 4).

To assess the influence of increased expression of CD95L on accelerated AICD of peripheral blood T cells, we measured TRC/CD3-triggered specific apoptosis after 48 hours in 10 HIV-1+ patients in the presence or absence of F(ab')2 anti-CD95 antibody fragments that inhibit CD95 receptor/ligand interaction (Fig 5). Only patients with a specific TRC/CD3-triggered apoptosis in CD4+ T cells of more than 10% were selected for this analysis. Spontaneous apoptosis of CD4+ and CD8+ T cells was not changed in the presence of CD95L.
CD95 SYSTEM IN HIV INFECTION

CD45RO

CD45RA

Fig 1. Increased expression of CD95 (APO-1/Fas) on freshly isolated CD45RO+ memory T cells and naive CD45RA+ T cells from 24 HIV-1+ and 10 HIV-1- children. Horizontal bars indicate median values for each group of patients and the respective T-cell subpopulation.

Fig 2. Increased spontaneous apoptosis in T cells from 24 HIV-1+ children (solid lines) and 15 HIV-1- children (dotted lines). The percentage of apoptotic CD4+ or CD8+ T cells was measured at 24-hour intervals during short-term cultures of freshly isolated PBMC. The plots show the loess-fits, vertical bars indicate the corresponding 95% confidence intervals over time. Nonoverlapping intervals indicate a difference at a 5% significance level.

DISCUSSION

The data in this report show that in HIV-1-infected children the CD95 receptor/ligand system is profoundly deregulated. In normal T cells this system is tightly controlled to maintain peripheral T-cell homeostasis. We and others previously described an increase in CD95 expression in T cells from HIV-1-infected children and adults. Here we show that increased CD95 expression is not confined to activated CD45RO+ T cells but also present on naive CD45RA+ T cells. In addition, T cells from HIV-1-infected children undergo increased spontaneous as well as CD95-triggered apoptosis ex vivo. CD3/TCR triggered apoptosis of CD8+ T cells over the entire culture period was found to be significantly higher in HIV-1-infected children than in healthy controls. A subgroup of patients also shows a significant increase of CD3/TCR-triggered apoptosis of CD4+ T cells after 48 hours of culture when compared with healthy controls (Fig 3). Furthermore, T cells from the majority of HIV-1-infected children show a substantial increase in constitutive expression of CD95L mRNA. This increase may be more pronounced in advanced stages of the disease (see Fig 4) and may contribute to the high levels of spontaneous apoptosis seen in these patients. Upon activation, eg, by agonistic anti-CD3 antibodies, T cells from HIV-1-infected children further upregulate CD95L mRNA (Herr I, Debatin K-M, unpublished results). Anti-CD3-induced, CD95-mediated AICD in these patients could partially be inhibited in both CD4+ and CD8+ T cells by interfering with CD95 receptor/ligand interaction using blocking anti-CD95 antibody fragments. Several mechanisms may account for the
inability to totally inhibit anti-CD3–induced, CD95–mediated AICD in these experiments. First, it has recently been shown that TNF contributed to AICD of T cells in prolonged cultures. The effect of TNF has not been addressed in our experiments and its contribution to apoptotic death of T cells from HIV-1–infected individuals remains to be shown.

Second, most circulating T lymphocytes from HIV-1–infected individuals already show mitochondrial dysfunction (decreased mitochondrial transmembrane potential and increased generation of superoxide anions) which correlate

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**Fig 3.** Increased anti-CD95–mediated apoptosis and anti-CD3–mediated apoptosis in T cells from 24 HIV-1+ children and 15 HIV-1–children. The percentage of apoptotic CD4+ or CD8+ T cells was measured after 48 hours of short-term culture of freshly isolated PBMC. Specific apoptosis was calculated as described in Materials and Methods. Horizontal bars indicate median values for each group of patients and the respective T-cell subpopulation.

**Fig 4.** CD95L mRNA levels in T cells of 17 HIV-1+ patients and 12 HIV-1–controls. Patients are grouped according to the CDC 1994 classification criteria: normal immune function (class 1) or immunosuppression (class 2 and 3). Horizontal bars indicate the arithmetic mean for each group of patients and the controls.

**Fig 5.** Inhibition of AICD by blocking anti-CD95 antibody fragments in T cells of 10 HIV-1+ children. PBMC were incubated for 48 hours with plate-bound anti-CD3 antibodies in the presence of F(ab')2 anti-CD95 or F(ab')2 control antibody fragments. Specific TCR/CD3–mediated apoptosis was in the range of 10% to 30% for CD4+ and 15% to 35% for CD8+ T cells and was calculated as described in Materials and Methods. Horizontal bars indicate the median percent inhibition of AICD of CD4+ and CD8+ T cells, respectively. No blocking was obtained in T cells undergoing spontaneous apoptosis in medium.
with the extent of spontaneous apoptosis ex vivo and are thought to represent early changes in T-cell apoptosis. Given the high constitutive expression of CD95L mRNA in these patients, it is probable that binding of CD95L to its receptor has already occurred before in vitro culture of the T cells. Thus, it may be difficult to inhibit spontaneous cell death by adding blocking anti-CD95 antibody fragments ex vivo to the cultures of cells already primed for apoptosis in vivo.

The data presented here suggest that HIV-1 infection is associated with upregulation and acceleration of CD95-mediated elimination of peripheral T cells at various levels. Increased expression of CD95 on naive and previously activated T cells may be a consequence of continuous antigen stimulation or, alternatively, may be induced by viral gene products such as HIV-1 Tat and gp120. Increased constitutive expression of CD95L mRNA may be caused by similar mechanisms. Thus, CD4+ and CD8+ T cells in HIV-1-infected individuals might be presensitized in vivo for CD95-mediated apoptosis by uptake of HIV-1 Tat. Preference to CD4+ T-cell depletion in vivo might be given through the presence of gp120 binding to the CD4 receptor and crosslinking anti-gp120 antibodies present in patient sera. In vivo, these interactions may direct the apoptotic signals predominantly toward the CD4+ T-cell population. The fact that spontaneous apoptosis ex vivo is more pronounced in CD8+ T cells compared with CD4+ T cells may point to a deficiency in costimulatory cytokines. Thus, addition of IL-2 to the cultures could partially abrogate spontaneous apoptosis, in particular in the CD8+ T-cell population. However, addition of IL-2 could not inhibit CD95-mediated apoptosis. In vivo, in addition to mechanisms that selectively target apoptosis into the CD4+ T-cell population, kinetics of cell turnover may determine T-cell depletion. Thus, replenishment of CD8+ T cells may be faster than that of CD4+ T cells, at least at certain stages of the disease. However, at late stages of the disease, progressive depletion of both subpopulations is observed. This corresponds to increased apoptosis sensitivity in both T-cell subpopulations.

In addition to the effects of viral gene products, accelerated T-cell loss might be triggered by constant activation of the immune system, eg, through frequent opportunistic infections. Deregulated activation of the CD95 receptor/ligand system might shift the balance of immune homeostasis toward apoptosis and cause progressive depletion of T cells that cannot be compensated by replenishment. Thus, during the course of HIV-1 infection various mechanisms may constantly lower the threshold for apoptosis in peripheral T cells.

Experiments in this report show that increased AICD in T cells from HIV-1+ individuals can be inhibited by interfering with binding of CD95L to its receptor. These experiments were done with unseparated PBMC to mimic the in vivo situation. Under these experimental conditions a variable degree of inhibition was observed. However, inhibition of AICD was more pronounced in CD4+ compared with CD8+ T cells and complete inhibition of AICD was observed in some cases. These data suggest that an upregulated CD95 system is involved in the pathogenesis of T-cell depletion during HIV-1 infection. This molecular concept may facilitate the development of rational therapeutic strategies that could be used to stabilize the pool of CD4+ T-helper cells. Thus, reducing CD95-mediated apoptosis in peripheral T cells, eg, by disrupting CD95 receptor/ligand interaction or inhibition of viral gene products such as HIV-1 Tat, may provide therapeutic means to maintain T-cell homeostasis, even in the presence of HIV-1.

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