AIDS and the death receptors

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Activation-induced cell death (AICD) of T cells involves the CD95 receptor/ligand system. T cell activation through the T cell receptor results in expression of the CD95 ligand (CD95L) that acts on CD95+ cells by direct binding and in a paracrine or autocrine fashion. In AIDS, upregulation of CD95L in T cells is accelerated by two viral gene products, HIV-1 Tat and gp120. The CD95 signaling pathway is, therefore, likely to represent an important road to cell death of the CD4+ T cells in AIDS.

Recently, the early events in the CD95 signaling pathway have been identified. A key role hereby plays a receptor-interacting member of the interleukin 1β-converting enzymes (ICE), FLICE, that could be a target for therapeutic intervention. In addition to CD95, the role of other members of the TNF receptor superfamily in AIDS is discussed.

In the immune system, apoptosis plays an important role in development and maturation of both normal T and B lymphocytes and in lymphoid malignancies. In the normal lymphoid system, apoptosis occurs in primary lymphoid organs such as the bone marrow, liver and thymus, and it is used to eliminate useless precursor cells with non rearranged or aberrantly rearranged, nonfunctional receptors (for review, see Krammer et al).

B and T lymphocytes contact antigen at different stages of maturation and in a different cellular context. Depending on these circumstances, their response can be entirely different. Immature double positive (CD4+CD8+) thymocytes can be positively selected and exit the thymus to populate the peripheral immune system. Alternatively, autoreactive thymocytes are negatively selected and die within the thymus by apoptosis. A similar process, however less well defined, might be operative for immature B cells in bone marrow and in germinal centers. In previously activated lymphocytes, triggering of the antigen receptor may lead to proliferation and memory or to death by apoptosis. The molecular switches leading to these different responses are partially unknown. Apoptosis in the peripheral lymphoid system may serve several functions. It may be a second safeguard to assure self-tolerance and eliminate autoreactive cells that have escaped negative selection in the central lymphoid organs. In addition, apoptosis may prevent uncontrolled expansion of specific, antigen reactive lymphocytes and may be one mechanism of immunosuppression.
Whether lymphocytes die by apoptosis or survive is controlled by various genes and their products. In principle, genes relevant for apoptosis might code for death or survival ligands and their receptors, or a cascade of signaling molecules. Such signaling molecules might be connected to second messengers and bridge membrane events to transcription factors and gene expression. Gene products might then either stimulate or block apoptosis. Genes directly implicated in lymphoid apoptosis comprise p53, c-myc, nur-77, several members of the bcl-2 family, the members of the ICE-like proteases, and the gene for CD95 (APO-1/Fas).

The death receptors

All members of the NGF/TNF receptor superfamily are type I transmembrane receptors characterized by extracellular cysteine-rich domains. They include NGF-R, TNFR-1, TNFR-2, CD27, CD30, CD40, 4-1BB and OX40 (for review, see Peter et al\textsuperscript{2}). Five of the members of this family have been reported to transduce an apoptotic signal under certain circumstances, the TNF receptors\textsuperscript{3}, CD30\textsuperscript{4}, CD40\textsuperscript{5}, the low affinity NGF receptor\textsuperscript{6} and the CD95 (APO-1/Fas)\textsuperscript{7,8}. The receptor most specialized in induction of apoptosis is CD95. Apoptosis via the CD95 receptor can be triggered by agonistic antibodies, such as anti-APO-1, or by the natural ligand of the receptor (CD95L). CD95L is a TNF-related type II transmembrane molecule (members of the ligand family include TNF, LT, LTβ, CD27L, CD30L, CD40L and TRAIL/APO-2L). From the crystal structure of TNFα, it was deduced that members of this family need to be trimerized in order to induce a signal via their cognate receptors. Indeed, dimerization of CD95, e.g. by F(ab')2 anti-APO-1, was found to be insufficient to induce an apoptotic signal. Multimerization of CD95, however, induced apoptosis\textsuperscript{9,10}. This finding suggests that, by analogy with TNF which triggers cytotoxicity as a trimer via TNFR-1, CD95L may also act as a trimer to induce apoptosis via the CD95 receptor.

Peripheral T cell deletion

We have previously shown that T cell receptor (TCR)-induced apoptosis of human T cell lines, T cell clones or activated peripheral CD4+ T cells could be blocked by reagents [F(ab')2 anti-APO-1 and CD95-Fc] that prevented CD95L/CD95 interaction\textsuperscript{11}. Dexamethasone-induced apoptosis, however, was not effected by these reagents. These data indicated
that the CD95/CD95L system is involved in activation induced cell death (AICD) of peripheral T cells. T cell apoptosis may occur as 'fratricide' by interaction of the membrane bound receptor with the membrane bound ligand on neighboring T cells that kill each other or by a mechanism that involves paracrine death or autocrine soluble CD95 ligand mediated suicide. PCR analysis also demonstrated that TCR stimulation resulted in expression of CD95L. These data were confirmed by others with murine TT hybridoma cell lines. Shortly after, it was also demonstrated that activated T cells produce TNF in addition to CD95L and that TNF was also involved in AICD of human peripheral T cells. The kinetics of the TNF release were delayed in comparison to the kinetics of CD95L production. It seems as if both ligands cooperate to kill activated T cells.

The role of apoptosis in AIDS

During the late phase of HIV-1 infection, a dramatic decrease of CD4+ T cells leads to the development of AIDS. The early stages of the infection are characterized by a severe dysregulation of the immune system affecting both the number and the function of CD4+ and CD8+ T cells. In spite of intensive investigation, the cause of T cell depletion and immune dysfunction is still unclear. Direct cytolytic effects of the virus were originally believed to be the main cause of T cell loss. However, direct cytolysis by the virus does not explain the extent of cell loss in various cell types. HIV-1-infected individuals show enhanced cell death of CD8+ T cells and NK cells and a decline of neurons, although these cells are not susceptible to infection. Viral cytolysis does not even explain the loss of CD4+ T cells since only approximately 0.1% of these cells in the peripheral blood are directly infected by the virus. Furthermore, infection of macrophages does not lead to enhanced macrophage death or to a decline of macrophage numbers.

Recent work by two groups supported the idea that viral load is the main reason for the development of AIDS. They have examined the viral load in plasma and extrapolated their findings using a mathematical model. They could show that up to $10^9$ virions were cleared every day and, therefore, assumed an equivalent HIV production. The authors argued that the synthesis of a billion virions per day is responsible for the daily loss of $20-200 \times 10^6$ CD4+ T cells. However, Levy has recently shown that, for the production of approximately a billion virus particles in the plasma of HIV positive individuals, only as little as approximately ten million CD4+ cells (T cells and macrophages) are required. Therefore, it is unlikely that the virus load is the only cause for CD4+
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T cell depletion and the development of AIDS. How can one then explain the loss of T cells during the progression of the disease?

In HIV-1-infection, the immune system is hyperactivated as seen, for example by increased expression of activation markers like HLA-DR, CD38 and CD57 on T cells21,22. In addition, CD4+ T cells of HIV-1-positive asymptomatic individuals show defects in response to recall antigen, superantigen SEB and pokewheat mitogen23. Furthermore, an increased level of cytokines including TNFα, IFN-γ, IL-10, IL-6 is found in sera of HIV-1-positive individuals (for review, see Oyaizu and Pahwa16). Thus, overexpression of the death-inducing cytokines IL-10, TNFα and IFN-γ and the depression of the death-preventing cytokine IL-1216 might render T cells more susceptible to apoptosis. Finally, T cell proliferation and clonal expansion requires co-stimulatory signals. In AIDS, such signals might be decreased and the T cells activated without proper co-stimulation. Thus, absence of a suitable co-stimulatory signal may render the T cells susceptible to apoptosis.

Finkel et al have shown that predominantly noninfected bystander cells and not the infected cells in lymph nodes of HIV-1-infected children and SIV-infected macaques are eliminated by apoptosis24. In addition, CD4+ and CD8+ T cells of HIV-1-infected individuals show enhanced spontaneous apoptosis and AICD25,26. This is not found in chimpanzees in which productive HIV-1-infection does not lead to AIDS27,28. These and other findings suggest that apoptosis is one of the main mechanisms for the loss of T cells in AIDS.

AIDS and the CD95 receptor/ligand system

There is striking evidence for a crucial role of the CD95/CD95L system in CD4+ T cell depletion in AIDS. HIV-1-infected individuals show enhanced expression of CD95 and the CD95L on CD4+ and CD8+ T cells29,30, as well as elevated levels of anti-CD95 autoantibodies31. Such autoantibodies might facilitate CD95-mediated apoptosis. Furthermore, since TNFα and IFN-γ were found to be upregulated in AIDS, these cytokines which also have been shown to lead to upregulation of CD95 may be essential for apoptosis and disease progression16. Furthermore, enhanced expression of CD95L on HIV-1-infected macrophages was found. CD95L+ macrophages that kill T cells might, therefore, be one cause of depletion of uninfected T cells32. In addition, crosslinking of CD4 increases expression of the death-inducing cytokines TNFα and IFN-γ which then leads to upregulation of CD9516. Crosslinking of CD4 might be induced by anti-CD4 autoantibodies present in patients’ sera. Such autoantibodies might arise from an immune response against
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Fig. 1 Enhancement of activation-induced cell death of T cells by two HIV-1 gene products TCR, T cell receptor; MP, metalloprotease, ROI, reactive oxygen intermediates, MnSOD, manganese superoxide dismutase, GSH, reduced glutathione. For details see text.

crossreactive sites of the viral envelope glycoprotein gp120 and CD4. CD4 crosslinking could also be induced by gp120 and anti-gp120 antibodies that are generated as the host’s immune response against HIV-1. gp120 is expressed in the outer membrane of infected cells and is present in patients' sera derived from free virus or shedded from infected cells.

Crosslinking of CD4 leads to apoptosis in vitro and in vivo. Apoptosis induced in this fashion can be partially inhibited by blocking antibodies against CD95 or by CD95 receptor decoys. Therefore, the CD95/CD95L system seems to be directly involved in CD4-mediated T cell death. In addition, preliminary evidence from our laboratory would indicate the existence of a CD4-induced cell death pathway that is independent of the CD95/CD95L or TNFR-1/TNF-system (unpublished data).
In addition to gp120, the HIV-1 transactivating protein Tat is involved in CD4+ T cell depletion. Tat protein is secreted by infected cells and is taken up by uninfected cells. We found that Tat enhances AICD triggered through the TCR most probably by the following mechanism: intracellular Tat causes downregulation of the manganese superoxide dismutase (MnSOD) and of glutathione (GSH) levels thereby leading to oxidative stress by accumulation of reactive oxygen species (Fig. 1). Oxidative stress causes enhanced CD95L expression after TCR triggering and, thus, an increased rate of apoptosis. This effect can be blocked by the reducing agents N-acetylcysteine, β-mercaptoethanol and the specific Tat inhibitor RO24-7429.

Tat-induced oxidative stress might operate at the level of transcription factors. A transcription factor reported to be sensitive to oxidative stress is NF-κB. We have previously shown that the Tat-enhanced activity of the IL-2 promoter involves NF-κB activation. This activation of NF-κB is caused by a Tat-enhanced production of hydroxyl radicals (unpublished data). In addition, other transcription factors are also involved in Tat-enhanced IL-2 promoter activity (unpublished data). In a similar fashion, therefore, expression of the CD95L gene might be influenced.

Absence of Tat-effects on AICD in HIV-1-infected chimpanzees

The organism closest related to man is the bonobo (pygmy chimpanzee). At the level of the genome, bonobo and man are 99.8% identical, while the common chimpanzee still shows 98% sequence identity with man. From the beginning of the AIDS epidemic, chimpanzees have, therefore, been used as an animal model to study this disease. As expected, chimpanzees could be infected with HIV-1 and viral replication in chimpanzees resembles the early infection in man. However, neither T cell depletion nor progression to AIDS occurs in these animals. The cause for the difference in disease susceptibility between man and ape is unknown. A number of cellular responses to the virus found in man cannot be observed in chimpanzees and bonobos. Most notably, no signs of oxidative stress induced by Tat can be observed in these animals. Thus, we have shown that, unlike in humans, Tat-treated chimpanzee T cells do not downregulate MnSOD and do not have reduced GSH levels. Therefore, chimpanzees do not upregulate CD95L and, in addition, chimpanzee and bonobo T cells are resistant to Tat-enhanced apoptosis and AICD. These data provide an explanation why enhanced apoptosis and progressive T cell depletion are not observed in HIV-1-infected chimpanzees.
infected chimpanzees. These findings might stimulate new therapeutic approaches of HIV-1 infection in man.

**Signal transduction of the death domain receptors**

AIDS is characterized by an increased rate of apoptosis in peripheral T cells. It is becoming clear that members of the TNF receptor superfamily and their ligands play an important role in the induction of apoptosis in AIDS. Learning more about the signal transduction pathways of these receptors might, therefore, provide the basis for development of new drugs that interfere with this process.

The cytoplasmic domain of members of the TNF receptor superfamily do not contain any known consensus motifs for enzymatic activities, such as kinases or phosphatases. However, four of the death-inducing members of the TNF receptor superfamily contain a homology region of about 65 amino acids in their intracellular parts, CD95, TNFR-1, the low affinity NGF receptor and CD40. This structure was called the death domain (DD). Its deletion results in a complete loss of apoptotic signaling. The DDs of CD95 and TNFR-1 are characterized best. A growing number of signaling proteins are identified that bind to these domains (for review, see Peter et al). Using the yeast two-hybrid system, two proteins were identified that bind to the CD95 death domain: FADD/MORT1 and RIP. Both proteins contain a DD themselves at their C-terminus. For FADD, direct binding to CD95 was demonstrated. We used a biochemical approach focusing on proteins that bind in vivo to the activated CD95 receptor. Four proteins were identified on high resolution IEF/SDS-PAGE 2D gels that associated only with activated CD95. These proteins were therefore termed CAP1-4 (for cytotoxicity-dependent APO-1-associated proteins). Together with the receptor, CAP proteins form the death-inducing signaling complex (DISC). CAP1 and CAP2 were identified as two differently serine-phosphorylated forms of FADD. Ectopically expressed, the N-terminal half of FADD induced cell death in various cells (unpublished data). It was, therefore, termed the death effector domain (DED). Deletion of this region resulted in a protein (FADD-DN) that functioned as a dominant negative. Biochemical analysis revealed that FADD-DN still bound to CD95 in a ligand dependent fashion. However, CAP3 and CAP4 did not bind to the DISC any more, suggesting that they required the FADD N-terminus for binding. CAP3 and CAP4 were, therefore, candidates for apoptosis effector molecules. Using the advanced nano electrospray tandem mass spectrometry method, CAP4 was sequenced from 200 fmol of silver-stained material. The sequence of four peptides was obtained
that was used to screen human genome data bases resulting in the identification of a full length cDNA. The predicted protein contained all of the sequenced amino acids. This protein contains two DEDs at its N-terminus and a domain highly homologues to the structure of a class of proteases that was recently identified to play a key role in apoptotic signaling, the IL-1β converting enzyme (ICE) proteases. CAP4 was, therefore, termed FLICE (for FADD-like ICE)\textsuperscript{49}. CAP3 was also sequenced. It contained the two most N-terminal peptides that were also found in FLICE. Therefore, it is likely to represent the N-terminus of FLICE. Recent data, however, indicate that the CAP3 C-terminus contains sequences not found in FLICE (unpublished data).

FLICE was also found by two other groups. It was called MACH\textalpha 1 by Boldin \textit{et al}\textsuperscript{50} and Mch5 by Fernandez-Alnemri \textit{et al}\textsuperscript{51}. Both reports indicated that FLICE exists in different isoforms most likely as the result of different splicing events. That might explain the existence of CAP3. No function could yet be assigned to CAP3. However, CAP3 is present in the DISC in all tested cells and tissues, so far. By replacing the cysteine residue in the active center of the FLICE ICE protease domain, Boldin \textit{et al}\textsuperscript{50} have generated a inactive FLICE molecule (FLICE-DN). Overexpression of this protein completely blocked CD95 signaling confirming that FLICE is essential for CD95-induced cytotoxicity.

While the main activity of CD95 is to trigger cell death, TNFR-1 can signal a diverse range of activities including fibroblast proliferation, resistance to intracellular pathogens including chlamidiae, and synthesis of prostaglandin E\textsubscript{2}\textsuperscript{52}. Upon triggering, TNFR-1 recruits a multivalent adaptor molecule termed TRADD which, like FADD, contains a death domain required for receptor association\textsuperscript{53}. TRADD has been shown to bind a number of signaling molecules including FADD, TRAF2 and RIP\textsuperscript{54,55}. FADD-DN blocks TNF killing\textsuperscript{48,54}, while dominant negative versions of TRAF2 and RIP block TNF-induced NF-κB activation\textsuperscript{54,55}. This indicates the existence of a signaling branch point dictated by the nature of the TNFR-associated adaptor molecules. Overexpression of FLICE-DN also blocked TNFR cytotoxicity. It is, therefore, likely that both receptors, CD95 and TNFR-1, actually use the same pathway for induction of apoptosis. TNFR-1 might indirectly use FADD to engage the death protease FLICE and, thereby, unite the death pathways that emanate from CD95 and TNFR-1. This is in contrast to some reports that demonstrate that CD95 and TNFR-1 use different signaling pathways to signal cell death (for review, see Peter \textit{et al}\textsuperscript{1}). However, the cloning of FLICE now favors the model that both receptors use one united death pathway. Conflicting data could be explained by additional signals generated by both receptors depending on the cell type that might modulate the apoptosis signal.
The elucidation of the early pathways used by CD95 and TNFR-1 to signal apoptosis might help to understand some of the pathological features of AIDS. As mentioned above, both the CD95 and the TNF receptor/ligand systems are involved in AICD of T cells. Upregulation of this form of apoptosis might be the main cause for the loss of CD4+ T cells during AIDS. The apoptosis pathway that contributes substantially to the elimination of T cells in AIDS can be described as follows (Fig. 1): restimulation of activated T cells through the antigen receptor results in the expression of CD95L. CD95L is secreted and can now kill cells by autocrine suicide or by fratricide. At a later time of T cell activation, secretion of TNFα also contributes to AICD. The mechanism of CD95-mediated apoptosis requires the following steps: CD95L binds to CD95 resulting in the formation of the DISC. The DISC contains and activates the receptor bound ICE-like protease FLICE and FLICE starts a cascade of events that involves activation of other intracellular ICE-like proteases and cleavage of various protein substrates. Further downstream, kinases, e.g. SAP kinases, are activated followed by the activation of endonucleases and eventually cell death. The whole process is accelerated by the action of HIV-Tat that reduces the intracellular MnSOD and GSH levels and by gp120 and anti-gp120 Abs present in sera of AIDS patients likely by inducing CD95L expression. Since FLICE also seems to be involved in TNFR-1-mediated apoptosis, this pathway might account for the early events in apoptosis signaling that contribute to the AICD of T cells. Apoptosis induced by CD95 and TNFR-1 can both be blocked by specific ICE protease peptide inhibitors. Recent data indicate that FLICE can be inhibited by zVAD-fmk a broad spectrum ICE protease inhibitor (unpublished data).

The death receptors without DD

In addition to the death domain containing members of the TNF receptor superfamily, some receptors without a death domain also seem to induce apoptosis. CD30 was shown to be involved in the deletion of T cells in the thymus. It is not known whether CD30 plays a role in the developments of AIDS. In addition to CD95L, TNF is secreted by activated T cells during the late phase of AICD. Zheng et al have demonstrated that TNF primarily affects CD8+ T cells by binding to TNFR-2. This is in line with reports that showed that both TNF receptors can generate a cell death signal.

Both the CD30 and the TNFR-2 do not contain a death domain. How do they induce apoptosis? Most signaling molecules that bind to members of the TNF receptor superfamily fall into two classes: the death
domain containing proteins that bind to the DD containing receptors; and the TRAF proteins. Candidate signal transducers first identified were TRAF1 and TRAF2 that bind to TNFR-2\textsuperscript{57}. They both contain a C-terminal TRAF domain. TRAF1 and TRAF2 both form homo- and heterodimers and TRAF2 mediates binding of TRAF1 to the C-terminal half of TNFR-2.

Soon after cloning of TRAF1 and TRAF2, a related molecule, TRAF3 was identified and shown to interact with CD40 (for review, see Peter et al\textsuperscript{5}). TRAF2 and TRAF3 contain an N-terminal RING finger motif. It might promote regulation of cellular proliferation. Both CD30 and TNFR-2 bind TRAF proteins. So far, direct involvement of these proteins in apoptosis induction has not been demonstrated.

Recently, a new member of the TNF family, TRAIL/APO-2L was cloned that killed certain cells in a similar fashion to CD95L\textsuperscript{58,59}. These similarities suggest that the TRAIL/APO-2 receptor also belongs to the TNF receptor family and it was argued that it also contains a DD. A very recent report indicated that FADD was not involved in apoptosis induction by the TRAIL/APO-2 receptor making the presence of a DD unlikely\textsuperscript{60}. This type of cell death, however, also involved ICE-like proteases since it could be inhibited by the cow pox virus serpin crmA. Whether the TRAIL/APO-2 receptor/ligand system is involved in AICD and, therefore, plays a role in AIDS development is not know at the present time.

In summary, apoptosis induced by the death receptors generally involves activation of ICE-like proteases. This includes TNFR-2, since apoptosis induced by this receptor can be inhibited by the broad spectrum ICE inhibitor zVAD-fmk (M. Grell, personal communication). Therefore, all death pathways important for AIDS development converge on the level of the ICE proteases. ICE proteases represent a suitable target to intervene with apoptosis signaling and to counteract the dysregulation of the activity of the death receptors in AIDS.

**Conclusion**

The cause for apoptosis of uninfected cells in AIDS was long unknown. It is a complex process involving T cell activation and induction of apoptosis through one of the death receptors of the TNF receptor superfamily. HIV-1 gene products contribute to the enhanced rate of cell death observed in AIDS patients. Together with recent advances in the delineation of the intracellular death pathways, these recent findings now provide the means to design drugs and reagents that interfere with the induction or the execution of apoptosis. These reagents might
include substances that block the effects of HIV-1 Tat or gp120, drugs that increase the intracellular glutathione level or reduce the activity of the ICE proteases that carry out the death signal received by one of the death receptors.

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