Significance of T cell apoptosis for macrophages in HIV infection

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Abstract: Apoptosis is a major form of cell death in HIV infection. This review presents current ideas on the role of apoptosis in the development of AIDS. HIV may cause apoptosis either directly in individual CD4+ T cells through cellular infection and through the release of gp120 envelope protein, or indirectly by initiating systemic disturbances in the immune system. Furthermore, although apoptosis is often assumed to be a biological dead end, linear, unintegrated retroviral DNA survives apoptosis in avian leukosis virus systems. Macrophages avidly phagocytose apoptosing cells, and the viral DNA in apoptotic debris might spontaneously transfect macrophages and lead to the production of new virions. Such a hypothetical accessory infection pathway may explain why anti-HIV cytotoxic cells are unable to clear this virus from the body. Strategies directed against the “recycling” of the retroviral genomes present in apoptotic debris may ultimately have a role in the treatment of HIV infection. J. Leukoc. Biol. 56: 247-256; 1994.

Key Words: macrophage • preintegration complex • endonuclease • phagocytosis • transfection

INTRODUCTION

Tissue homeostasis occurs when cell death is balanced by the removal of dead cells and their replacement through cellular proliferation. In HIV infection this homeostasis is destroyed, which results in the marked depletion of CD4+ T lymphocytes (and eventually all lymphocytes) that is the hallmark of the progression from asymptomatic HIV infection to AIDS [1]. The acute fall in CD4+ T cells seen in primary infection at a time of very high viral replication and the rebound in this cellular population that follows seroconversion with the appearance of temporary antiviral immunity [2] both indicate that HIV itself can cause the destruction of CD4+ T cells. Our own data indicate that HIV can kill cells by inducing the process of cell death called apoptosis, an endogenous sequence of events also known as programmed cell death [3]. This review surveys current theories of the triggering stimuli for T cell apoptosis in HIV infection and presents a hypothetical accessory infection pathway through the recycling of the HIV DNA genome present in apoptotic debris after it is taken up by phagocytosing macrophages.

KEY FEATURES OF APOPTOTIC CELL DEATH

Apoptosis is usually described by comparing it to its opposite form of cell death, necrosis. More recently, various incomplete forms of apoptosis called atypical apoptosis have complicated the understanding of this form of cell death. However, one constant feature in all forms of apoptosis is the prolonged phase of plasma membrane integrity, which serves to contain the contents of the dying cell, often for a day or more after the cell has actually begun to die. Although the plasma membrane remains intact, subtle changes occur on its external face that permit macrophages to recognize and remove the dying cells. Meanwhile, within the apoptosing cell and undiscernible from the outside, a series of stepwise changes take place: (1) compaction of nuclear chromatin into dense masses that move to the edge of the intact nuclear envelope, usually associated with extensive degradation of DNA through the activation of an endogenous calcium-dependent endonuclease; (2) fragmentation of these chromatin masses and condensation of the cytoplasm with shrinkage of the cell; and (3) fragmentation of the cell into pieces called apoptotic bodies, which are still enclosed by intact cell membrane. During this process, cellular organelles such as mitochondria and lysosomes remain relatively normal. Because there is no spillage of cellular contents, there is no inflammatory reaction to apoptosis in vivo and scarring does not usually occur. In sharp contrast, necrosis is characterized by early swelling of mitochondria, disintegration of the cell membrane, and leakage of lysosomal enzymes. Not surprisingly, necrosis in vivo is usually accompanied by inflammatory exudation and often leads to a scar [4].

The concept of apoptosis was originated by Kerr et al. in 1972 [5]. Perhaps the major reason it was not recognized earlier is the fact that the apoptosing cells in a tissue environment are removed by phagocytosing macrophages (or occasionally epithelial cells) within a few hours after apoptosis begins [6]. However, after certain physical or chemical insults, large numbers of cells can be induced to undergo apoptosis in synchrony, which overwhelms the clearance capacities of the local macrophages and makes the apoptotic process more evident. For example, at any point in time, 95% of the cells in the thymus are destined to die by apoptosis, yet it is difficult to identify apoptosing cells in the thymus. However, when Bonyhadi et al. [7] and Stanley et al. [8] injected HIV into fragments of human fetal thymuses in SCID mice, a synchronous wave of apoptosing thymocytes could be identified in the areas where there was active viral

Abbreviations: ADCG, antibody-dependent cellular cytotoxicity; ALV, avian leukemia virus; APC, antigen-presenting cell; AZT, zidovudine; CEF, chicken embryo fibroblast; CTL, cytotoxic T lymphocyte; HBV, hepatitis B virus; HPETE, hydroperoxycicosatetraenoic acid; IL-1, interleukin-1; MAIDS, murine AIDS; PBMC, peripheral blood mononuclear cell; SCID, severe combined immunodeficiency disease; SIV, simian immunodeficiency virus; TCR, T cell receptor; TNF-α, tumor necrosis factor α.

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replication. In this study, even though the high flux of dying cells exceeded the rate of clearance by local macrophages, thymic macrophages were still seen engulfing HIV-infected thymocytes as they underwent the earliest stages of apoptosis (M. Bonyhadi, personal communication). Aside from this unusual situation, HIV-related cell death occurs stochastically, so that local macrophages can keep up with the apoptosing cells and prevent the accumulation of these dead cells in tissues. Thus, under steady-state conditions, histological examinations of tissues provide only a snapshot of the dynamic process of cell death and macrophage-mediated cell removal, and an underestimate of the frequency of apoptotic cell death is almost unavoidable.

**APOPTOSIS IN HIV-INFECTED INDIVIDUALS IN VIVO**

Despite these complexities, it is now becoming apparent that apoptosis is a major cause of lymphocyte depletion in HIV infection in vivo. Recently, Muro-Cacho et al. [9] described the results of a search for apoptosing cells in lymph nodes from HIV-infected volunteers. They used the TUNEL technique to detect cells containing highly fragmented DNA. This method utilizes terminal deoxynucleotidyl transferase to add biotinylated dUTP onto the 3' ends of cut DNA, followed by streptavidin-alkaline phosphatase histochemistry as a detection step. These investigators found that apoptotic cells were commonplace in the lymph nodes of HIV-infected individuals. The prevalence of apoptotic cells increased as CD4+ T cell numbers declined and disease progressed, and the apoptotic cells appeared to collect especially in the medullary sinuses of the lymph nodes, which is where macrophages ("sinus histiocytes") are concentrated [9].

**IN VITRO MODELS OF HIV-RELATED APOPTOSIS**

Preceding the demonstration that apoptosis occurs in HIV-infected individuals in vivo, numerous studies demonstrated HIV-related apoptosis in T lymphocytes in vitro. In fact, there are now at least 11 models for HIV-related T cell apoptosis in vitro. These models are listed in descending order of their relationship to cellular HIV replication or the products of HIV itself:

**HIV-induced apoptosis associated with an accumulation of unintegrated viral DNA**

Soon after HIV-1 was discovered, it was found to cause death of CD4+ T cells in culture [10]. In 1991, Laurent-Crawford et al. [11] and our group [3] reported that apoptosis was the mechanism of this cell death. This phenomenon is restricted to CD4+ T cells, because CD8+ T cells are not usually susceptible to HIV-1 in vitro and in vivo. Also, macrophages do not acutely apoptosis after HIV-1 infection and generally survive productive infection for several weeks in culture.

The mechanism of HIV-induced apoptosis in CD4+ T cells is currently an area of active investigation. Our experiments were modeled after a study by Keshet and Temin [12] on the induction of cell death in chicken embryo fibroblasts by spleen necrosis virus (SNV), a lytic form of avian leukosis virus (ALV) that causes apoptosis (see below). They found that cell death could be prevented if neutralizing antibodies were added a few hours after infection. Such a protocol limited cellular infection to a single virus replication cycle (virus entry and reverse transcription, followed by the production of progeny virions). Without the addition of neutralizing antibodies, multiple cycles of reinfection occurred, leading to an accumulation of unintegrated viral DNA in association with ALV-induced cell death [12, 13]. This led to the hypothesis that an accumulation of unintegrated retroviral DNA is somehow toxic to a cell.

In parallel with these studies on ALV, we found that HIV-1 caused apoptosis in productively infected CD4+ T cells and CD4+ T cell lines. The HIV-infected cells exhibited both the morphological changes of apoptosis and the degradation of their DNA into the classic "apoptotic ladder" pattern caused by internucleosomal cleavage. However, when CD4+ T cells were exposed to HIV-1 followed by the addition of a neutralizing monoclonal antibody, the infection was limited to a single virus replication cycle, high levels of virus were found in the medium, yet apoptosis did not occur [3]. In the presence of the neutralizing monoclonal antibody, it is likely that each cell contained only a few molecules of viral DNA [14], which again is consistent with the hypothesis that an accumulation of unintegrated retroviral DNA is a trigger for apoptosis.

However, there are exceptions to this apparent association between HIV infection in vitro with an accumulation of unintegrated viral DNA and apoptotic cell death. HIV-induced apoptosis is dependent on both the viral strain and the host cell. For example, sublines of CEM and WIL-2 cells differ widely in their susceptibility to HIV-1LAI (IIIb strain)—induced death [15]. In our experience, H9 cells, which are often used to produce HIV stocks, do not undergo apoptosis after infection by HIV-1LAI (LAV-1 strain) (data not shown). Such differences between cell lines may explain the data reported by Bergeron and Sodroski [16]. These investigators found that the HXBc2 strain of HIV-1 killed C8166 and Jurkat T cells and that this cell death could not be prevented by either neutralizing monoclonal antibody or zidovudine (AZT) added after the first cycle of virus infection. Since both of these treatments reduced the accumulation of unintegrated viral DNA, their data indicated that HIV-induced cell death can occur without this viral DNA accumulation, but they also noted that an apoptotic ladder of degraded DNA could not be detected in the dead cells, suggesting that they were not observing apoptosis. Perhaps more relevant to apoptosis, Laurent-Crawford and Hovanessian [17] performed experiments similar to ours using CEM T cells exposed to the HIV-1LAI (LAV-1 strain). They found that AZT added after infection could prevent the accumulation of unintegrated viral DNA but did not affect the subsequent development of apoptosis as measured by the release of histones from chromatin.

From these data, it appears that an accumulation of unintegrated viral DNA might not be causally related to apoptotic cell death. Nevertheless, it remains true that cell cultures infected by lytic retroviruses regularly contain high levels of unintegrated viral DNA (usually in the linear form) at the time that apoptosis is occurring. This association may also occur in vivo, because there is evidence that unintegrated lentiviral DNA accumulates in tissue. In macaques infected with simian immunodeficiency virus (SIV), over 85% of the detectable viral DNA was unintegrated in the five tissues examined (lymph node, liver, ileum, brain, and spleen), with circular genomes more prevalent than the linear form [18]. Similarly, in HIV-infected individuals, unintegrated circular viral DNA was detected by polymerase chain reaction study of brain extracts [19], but predominantly linear unintegrated viral DNA was detected by Southern blotting an extract of a single lymph node [20]. There are also reports of a high prevalence of unintegrated HIV DNA in peripheral blood mononuclear cells (PBMCs) and T cells [21, 22].
Apoptosis due to activation of the double-stranded, RNA-dependent kinase

In some in vitro systems, transcription initiated by the HIV long terminal repeat in the absence of Tat protein leads to preferential formation of prematurely terminated "short transcripts," which build up in the cytoplasm of the cell [23]. In some [24, 25] but not all studies [26], these short TAR loops of double-stranded RNA activate the double-stranded RNA-dependent kinase of the interferon system. It has been reported that excessive expression and activation of this kinase lead to apoptosis [27]. If these short transcripts were formed in vivo, they might contribute to T cell apoptosis. Conversely, Tat protein might reduce apoptosis by permitting the full elongation of HIV transcripts. Tat protein alone has been reported to inhibit apoptosis induced in cells by serum starvation in vitro by maintaining the expression of Bcl-2 protein, an antiapoptosis gene product [28].

HIV-induced apoptosis caused by envelope interaction with CD4

Cohen et al. found that Jurkat T cells that were engineered to express envelope (both gp120 and gp41) can induce cell death when mixed with standard Jurkat CD4+ T cells. This cell death occurs in association with the hyperphosphorylation of the p34<sup>cdc2</sup> cell cycle protein and has several features of apoptosis [29]. Similarly, CEM T cells infected with vaccinia recombinant viruses expressing the HIV-1 envelope form syncytia and induce apoptosis in uninfected CD4<sup>+</sup> CEM T cells [30]. This effect may require gp120 to be cell-associated, since gp120-expressing HeLa cells, but not free gp120, were found to induce apoptosis in a CD4<sup>+</sup> T cell clone [31]. In another study, however, free gp120 enhanced antigen-induced apoptosis in T cell clones as measured by the appearance of tissue transglutaminase in the dying cells [31a].

HIV-induced apoptosis caused by gp120-anti-gp120 antibodies plus T cell activation

Apoptosis can be induced in murine CD4<sup>+</sup> T cells using anti-CD4<sup>+</sup> antibodies and separate stimulation of the T cell receptor (TCR) [32]. Based on the fact that HIV gp120 binds to CD4, Banda et al. [33] reported that gp120 cross-linked with mouse anti-gp120 antibody and goat anti-mouse immunoglobulin G followed by TCR signaling induced apoptosis in human CD4<sup>+</sup> T cells. Cellular infection was not required for this to occur. One group [34], but not two other groups [35, 36], has been able to reproduce these data, and it is likely that technical factors are critical in this in vitro system. Because HIV-infected individuals have measurable quantities of gp120 and anti-gp120 antibodies in their blood [37, 38], there is the potential that if further cross-linking occurred (for example, on the surface of a cell with Fc receptors, such as a macrophage), this form of apoptosis might occur in vivo.

Apoptosis caused by cytotoxic cells

Apoptosis also occurs following the "lethal hit" of cytotoxic immune cells [39]. Cytotoxic T lymphocytes (CTLs) directed against HIV antigens are readily isolated from HIV-infected individuals [40]. Virus infection is not always necessary for cytotoxic cell attack, however. CD4<sup>+</sup> T cells exposed to gp120 can bind this antigen and present it to major histocompatibility complex class II-restricted CD4<sup>+</sup> CTLs [41]. Similarly, CD4<sup>+</sup> T cells carrying bound gp120 can be recognized and killed by antibody-dependent cellular cytotoxicity (ADCC) [42], which also induces apoptosis [43]. All of these cytotoxic cells would be expected to induce an apoptotic death in CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells. However, the presence of antilymphocytic autoantibodies may widen the spectrum of lymphocytes that are killed by ADCC.

Fas-related apoptosis

Fas protein (also called apo-1 or CD95) is a member of the tumor necrosis factor (TNF) receptor family. On certain cell lines, cross-linking Fas protein with anti-Fas antibodies can induce apoptosis. In PBMCS, Fas was found on certain CD4<sup>+</sup> and CD8<sup>+</sup> T cells (especially the CD45RO<sup>+</sup> subset of memory cells), γδ T cells, and B cells. On these normal cells, however, cross-linking Fas with anti-Fas antibodies alone does not induce apoptosis [44]. Fas is also a marker of T cell activation, which may be why it is found on a higher than normal percentage of T cells in the blood of HIV-infected individuals [45]. There is a short region of sequence homology between Fas and HIV gp120 [46], and it is possible that anti-gp120 antibodies might cross-react with Fas to transmit an apoptosis-inducing signal. However, normal lymphocytes must be activated for several days before apoptosis can be induced by anti-Fas antibodies [47]. In the future, it will be interesting to learn about the effects of HIV infection on the expression of Fas ligand, a naturally occurring T cell membrane protein that transmits an apoptosis-inducing signal to susceptible, Fas-bearing cells [48].

Oxidant excess- and antioxidant deficiency-related apoptosis

Systemic levels of glutathione, cysteine, and cystine (the disulfide-linked dimer of two cysteines) decline dramatically during the early stages of HIV infection [49, 50]. A similar decline occurs within 1 week after SIV infection in macaques (which eventually develop simian AIDS), but does not occur in SIV<sub>AGM</sub>-infected African green monkeys and HIV-infected chimpanzees (which do not develop AIDS) [51]. In humans, the subset of CD4<sup>+</sup> and CD8<sup>+</sup> T cells containing high levels of glutathione disappears soon after HIV infection [52]. For lymphocytes, depletion of glutathione or catalase could lead to a susceptibility to oxidant-induced DNA strand breaks [53].

Macrophages may have a Janus-like involvement in oxidant-induced apoptosis. First, they normally provide a trophic function for T cells by bathing them in cysteine, which macrophages, but not T cells, can produce from cystine [54]. Second, activated macrophages produce TNF-α, which in turn induces the production of reactive oxygen intermediates in susceptible cells. Malorni et al. [55] reported that TNF-α caused chronically HIV-infected U-937 cells (but not uninfected U-937 cells) to undergo apoptosis and that this was antagonized by the addition of N-acetylcysteine, an agent that increased intracellular glutathione levels. This is an example of how decreased levels of an antioxidant can sensitize cells to certain apoptosis-inducing stimuli.

Sandstrom and Buttké [56] have reported that CEM T cells secrete catalase into their medium, which permits them to resist H<sub>2</sub>O<sub>2</sub>-mediated damage. At low plating density in serum-free medium where catalase was limiting, the CEM cells spontaneously apoptosed. A chronically HIV-infected subline of CEM, 8E5, secreted much less catalase than its CEM-derived parent, A3.01, and readily underwent apoptosis after H<sub>2</sub>O<sub>2</sub> exposure [57]. It is believed that H<sub>2</sub>O<sub>2</sub> oxidizes membrane lipids into lipid hydroperoxides, converting, for example, arachidonic acid into hydroperoxyeicosatetraenoic acid (HETE).
acids (HPETEs). Cells protect themselves by using reduced glutathione and glutathione peroxidase to convert the lipid hydroperoxides into less reactive lipids. BE5 cells readily apoptosed after exposure to HPETE, which was traced to a marked deficiency of glutathione peroxidase in the HIV-infected cells [58]. These authors have suggested that similar defects in catalase production or intracellular deficiencies in the glutathione-glutathione peroxidase system could contribute to the culture-induced ex vivo apoptosis that occurs with lymphocytes taken from HIV-infected individuals [58].

Ceramide-related apoptosis

TNF-α and certain other apoptosis inducers activate sphingomyelinase, which leads to the production of ceramide from cell membrane sphingomyelin. Under some circumstances, ceramide alone can induce apoptosis [59, 60]. Consequently, it is interesting that as much as 60% of the sphingomyelin in CEM T cells infected by HIV-1LAI (HIB strain) is degraded into ceramide around the time the cells are apoptosing. In the HIV-infected cells, the ceramide levels were 2.8- to 4.1-fold higher than in the uninfected control cells [61]. This type of membrane lipid change may be related to the marked hydrolysis and release of free fatty acids that accompanies CTL-mediated target cell lysis, a form of apoptosis [62]. The membrane changes that result from these membrane lipid events may participate in the movement of phosphatidylserine to the outer leaflet of the plasma membrane, where it marks the apoptosing cell for recognition by phagocytosing macrophages.

Apoptosis facilitated by zinc deficiency

In many culture systems, apoptosis can be prevented by adding ZnSO₄ to the medium, and there is a report that the chelation of zinc is sufficient to induce apoptosis in T cells [63]. Consequently, it may be important that zinc levels are lower in the sera of HIV-infected individuals who progress to AIDS versus those who are nonprogressors [64]. This difference could not be explained by differences in dietary intake or malabsorption, suggesting that oral zinc supplements would be unlikely to restore serum zinc to its normal level [65].

Steroid-related apoptosis

The induction of apoptosis in thymocytes by corticosteroids is a now classical model of T cell apoptosis [66, 67]. Serum cortisol levels during all stages of HIV infection are about 35% higher than normal [68]. Since the normal morning (peak) levels of cortisol approach the lowest concentration needed to induce apoptosis in thymocytes, the further elevation that occurs in HIV infection may enhance T cell apoptosis. Recent work, however, suggests that cortisol can antagonize activation-induced thymocyte apoptosis under certain circumstances [47]. Other changes, such as the decline in dehydroepiandrosterone in AIDS [68] may have additional effects on T cells [69].

Culture-induced, ex vivo apoptosis

In 1991, Gougeon et al. [70] reported that lymphocytes taken from HIV-infected individuals have a markedly decreased survival in culture and undergo an apoptotic death in vitro. In the same year, Groux et al. [71] reported a similar form of ex vivo lymphocyte apoptosis, which occurred only after the cells had been activated in culture by mitogens or superantigens [72]. Similarly, Meyda et al. [73] found a statistically significant increase in apoptosis after 72 h in culture in PBMCs from HIV-infected individuals compared with HIV-seronegative controls, and the difference was even greater after TCR stimulation.

It has been proposed that this ex vivo phenomenon represents the result of in vivo priming for activation-induced T cell death in HIV-infected individuals [74-76]. Because CD8 T cells are even more susceptible to apoptosis ex vivo than CD4 T cells [77], it is likely that this phenomenon results from some generalized change that occurs after HIV infection. The changes proposed include decreased cytokine support for T cells, either in the form of interleukin-1 (IL-1) plus IL-2 [78] or a factor(s) present in fibroblast-conditioned medium [79]; insufficient costimulatory signaling through T cell receptors such as CD28 [72]; and inadequate antigen-presenting cell (APC) function due to HIV-induced alterations in macrophages and other APCs [80]. Alternatively, an excess of a positive, apoptosis-inducing factor such as prostaglandin E₂ [81] could be involved. Of course, some of the abnormalities already described (i.e., envelope interaction with CD4, complexes of cell-associated gp120 with anti-gp120 antibodies, dysregulation of Fas, excessive oxidants and/or deficient antioxidants, zinc deficiency, and abnormal steroid levels) might also contribute to this phenomenon.

Several considerations support a role for this ex vivo phenomenon in the development of AIDS. First, the phenomenon could explain part of the T cell loss that occurs as HIV infection progresses, and it discloses some gross dysregulation or hyperactivation that requires explanation. Second, ex vivo apoptosis occurs in simian AIDS caused by SIV [78], in feline AIDS caused by FIV [82], and in the murine model of AIDS, MAIDS [83]. Significantly, it does not occur in HIV-infected chimpanzees, in which HIV infection does not cause progressive immunodeficiency or AIDS [78, 84]. Third, ex vivo apoptosis also occurs in CD4 hematopoietic stem cells taken from HIV-infected individuals [85], which suggests that it could also play a role in the bone marrow insufficiency that often complicates AIDS.

In contrast, several considerations confound any easy correlation between ex vivo apoptosis and progressive lymphocyte depletion. First, the phenomenon is greater in CD8 T cells than in CD4 T cells [73]. Second, in one study, the magnitude of ex vivo cell death had no correlation with CD4 T cell number, the rate of decline in CD4 T cells, viral load, the appearance of syncytium-inducing strains of HIV, the stage of HIV infection, or the progression of disease [77]. However, another group found that ex vivo apoptosis did correlate with CD4 T cell depletion and the rate of decline in CD4 T cells in HIV-infected individuals [86]. Third, this phenomenon is not unique to HIV infection and also occurs in Ebstein-Barr virus-induced infectious mononucleosis, cytomegalovirus infection, and murine lymphocytic choriomeningitis virus infection [77].

Based on the models for HIV-related apoptosis just described, there is increasing interest in blocking apoptosis as a means of preventing the decline in CD4 T cells that occurs in HIV infection. This approach may be very beneficial, but it has the potential drawback that HIV-infected cells may survive longer and therefore release even more virus over time. For example, we found that when neutralizing monoclonal antibodies were added soon after HIV infection of CD4 T cells in vitro, apoptosis was prevented but there was a disconcerting increase in the net amount of viral p24 antigen accumulation over time [3]. Consequently, potential antiapoptosis therapies may need to be given in combination with effective antiretroviral agents in order to be maximally effective.
UNINTEGRATED RETROVIRAL DNA SURVIVES APOPTOSIS

The unintegrated, linear viral DNA of lytic avian leukemia viruses (ALV) survives apoptotic cell death

In 1981, Weller and Temin [87] reported that certain strains of ALV (also known as Rous-associated viruses) kill chicken embryo fibroblasts (CEF') within a week after infection. They analyzed the DNA within these dead cells by agarose gel electrophoresis and found what in retrospect is a classic apoptotic ladder of intranucleosomal DNA cleavage (Fig. 2 of ref. 87). Remarkably, by Southern blotting, they further showed that linear, unintegrated viral DNA survived this cleavage and they speculated that it might be protected somehow from degradation [87]. As already noted, they also found an association between the accumulation of unintegrated viral DNA, apoptotic cell death, and the survival of the unintegrated viral DNA in this system.

The relationship of the retroviral DNA that survives apoptosis to the "infectious DNA" concept

Historically, after the discovery of reverse transcriptase, it became important to show that the DNA copy of the retroviral RNA genome contained all of the genetic information needed to generate new viirions. This was accomplished primarily by Hill and Hillova [88]; by Cooper, Fritsch, and others in Temin's laboratory [89, 90]; and by Weinberg's group [91]. Each of these laboratories found that the transfection of retroviral DNA resulted in the production of new virions. This DNA came to be known as infectious DNA. Consequently, in the ALV experiment described above, Weller and Temin transfected fresh CEF cells with unintegrated, protein-depleted ALV DNA isolated from the apoptotic CEF cells by the Hirt procedure. Consistent with prior studies [89], they found that about one in a million molecules of unintegrated viral DNA would generate a new infection [87]. (Presumably, the introduction of an authentic preintegration complex into a cell would be a more efficient way to initiate infection, since this complex contains integrase and other viral proteins.) Evidently, no one at this time considered what might be the fate in vivo of the retroviral DNA that survived the apoptotic death of its host cell and whether there might be some naturally occurring form of DNA transfection that could "recycle" the retroviral genome.

UPTAKE OF APOPTOTIC DEBRIS BY PHAGOCYTOSING MACROPHAGES

Macrophages avidly phagocytose apoptotic debris

Apoptotic cells are very rapidly phagocytosed by macrophages [4, 92]. Macrophages recognize apoptotic debris by the changes that occur in their plasma membranes, probably without regard to the internal changes taking place in the apoptosing cells. Three ligands have been proposed as the molecules that macrophages recognize on the surface of apoptosing cells: (1) N-acetylglucosamine, recognized by the macrophage receptor for mannose, may appear on the surface of apoptotic cells as a result of fusion of intracellular membranes with the cell membrane [93]; (2) phosphatidylserine, recognized by an uncharacterized macrophage receptor, an anionic phospholipid that is normally only on the inner leaflet of the cell membrane, but rapidly flows onto the outside of the cell membrane during apoptosis [94]; (3) a thrombospondin-binding ligand, recognized by a combination of the αβ1 "vitronectin receptor" integrin and CD36, appears on the surface of certain apoptosing cells [95]. In most experimental systems, macrophage recognition of apoptotic cells cannot be 100% blocked by an excess of any one putative ligand, which suggests that multiple receptors are used. All studies indicate that a major function of macrophages is to remove apoptotic cells.

A HYPOTHESIS FOR THE REEXPRESSION OF THE HIV GENOME IN MACROPHAGES INGESTING APOPTOTIC DEBRIS

Efficient transfection can occur after the introduction of DNA into cells via membrane receptors

Given that macrophages phagocytose apoptotic cells, it has been proposed that the endonuclease digestion of DNA is important for host defense because it will destroy any viral DNA genomes present in the dying cells [96-99]. It was specifically proposed that unless viral DNA is degraded within the apoptosing cell, it might somehow retain its infectious potential after phagocytosis by a macrophage [100]. This theoretical possibility has been substantiated by the demonstration that the introduction of DNA into cells via a membrane receptor is one of the most efficient ways to transfect cells. For example, hepatocytes can be transfected by hepatitis B virus (HBV) DNA bound to a poly-L-lysine/asialo-galactosomucoid conjugate resulting in the production of HBV particles [101]. An even more efficient receptor-mediated transfection process that can be applied to a variety of cells utilizes DNA bound to a poly-L-lysine/transferrin conjugate ("transferrinfection"). This process is enhanced more than 1000-fold if an inactive adenovirus is incorporated in the carrier. The adenovirus envelope disrupts the endosomal membrane at low pH and provides a nuclear localization signal for the transport of the DNA-protein complex into the nucleus [102]. Therefore, Weller and Temin's finding that retroviral DNA is not destroyed by apoptosis coupled with the avid uptake of apoptotic debris by macrophages raises the possibility that macrophages may undergo a naturally occurring form of receptor-mediated transfection. We term such an infection pathway DNA-mediated phagoinfection.

"DNA-mediated phagoinfection": a hypothetical pathway for the recycling of HIV genetic information after the death of its initial host cell

In summary, we propose an alternative infection pathway based on the phagocytosis by macrophages of apoptotic debris containing intact HIV DNA (Fig. 1). The steps in this pathway are the following:

HIV infection of a susceptible cell

Although any infectible cell type is a candidate for this event, we conceptualize CD4+ T cells as the prototypic cell in this pathway.

Apopotosis of the HIV-infected cell with preservation of linear, unintegrated viral DNA

Once the cell is infected by HIV, we hypothesize that any process that triggers apoptosis may permit the survival of linear, unintegrated viral DNA. Our experiments have shown that, like the ALV system of Weller and Temin described earlier, linear, unintegrated HIV-1 DNA survives infection-induced apoptosis in vitro (data not shown). It will be interesting to determine whether linear, unintegrated
HIV DNA also survives apoptosis initiated by cytotoxic cells and other stimuli (discussed above).

We envision two reasons why the unintegrated viral DNA is spared degradation: (1) The DNA may be contained within a "preintegration complex," which is a 300-Å particle in the infected cell that is protected from digestion by HIV p17 matrix protein, integrase, reverse transcriptase, and other proteins [103-105]. (2) Preintegration complexes are most prevalent in the cytoplasm of infected cells [103], where the endonuclease of apoptosis is absent [47].

Recognition and phagocytosis of the apoptotic HIV-infected cell

As already described, macrophages avidly phagocytose apoptotic cells. In the studies on human thymuses transplanted into SCID mice [7, 8], thymocytes in areas of active HIV replication were phagocytosed by thymic macrophages while they were still undergoing the apoptotic process (M. Bonyhadi, personal communication).

Escape from lysosomal degradation in the phagosome

There are now several examples of receptor-mediated transfection in nonphagocytic cells. However, macrophages have an extensive lysosomal system and they are able to degrade most of the substances that they ingest, including DNA [106]. Consequently, it is important to postulate some mechanism for the escape of the infectious material from the phagosome—a common theme in intracellular infections [107].

The DNA in the apoptotic cell debris moves to the nucleus

It is likely that the linear, unintegrated DNA that survives in apoptotic cells is in the form of a preintegration complex. It has been shown that the HIV p17 gag protein that is part of this complex contains a nuclear localization signal that allows the complex to be transported into the nucleus of non-dividing cells [108], including macrophages [109].

The DNA is transcribed to produce new virions

Once inside the nucleus, the integrase present in the preintegration complex may mediate integration into macrophage DNA. With or without integration, there appears to be no intramacrophage block to HIV expression, because the electroporation into macrophages of plasmids containing lym-photropic proviral HIV DNA (including HIV-1LAI, IIIB strain) results in the production of infectious progeny virions [110, 111]. Thus, the delivery of HIV infectious DNA into macrophages may be all that is needed to complete the cycle by initiating the production of new virions and new cellular infections.
Clinical significance of the DNA-mediated phagoinfection

Following initial infection by HIV, a massive amount of viral replication occurs and it is not unusual to find millions of viruses per milliliter of blood. Within weeks, however, there is a 2 to 4 log reduction in the amount of virus found in blood, which precedes the appearance of antibody and seroconversion. This reduction in virus is more impressive than the effects of AZT therapy and it correlates with the development of CTLs directed against HIV-infected cells [112]. After this stage, anti-HIV CTLs are easily detected at relatively high levels until the very late stages of disease [40]. However, HIV infection is not like influenza infection in which immune CTLs clear the virus from the body by killing infected cells prior to virion assembly. Instead, we propose that CTLs are only partly successful in limiting HIV infection. Although they may dramatically reduce the number of virus-producing, infected cells that occur during acute infection, CTLs may be unable to eliminate the DNA form of the HIV genome that survives apoptosis. Because this form of the HIV genome undergoes a "life after death" experience in macrophages, the virus does not die along with its host cell and is instead resurrected via the DNA-mediated phagoinfection pathway. Conversely, if this pathway were blocked, then naturally occurring or vaccine-induced immune responses might be sufficient to eventually eliminate HIV from the body.

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REFERENCES


