Susceptibility to Cytotoxic T Lymphocyte–induced Apoptosis Is a Function of the Proliferative Status of the Target

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Summary

Cytotoxic T lymphocytes (CTL) kill cells by perturbing the target's plasma membrane and by inducing the disintegration of the target cell's DNA into oligonucleosomal fragments, a process characteristic of apoptosis. We show that the DNA fragmentation event is distinct from the membrane lysis event and is dependent on the state of target cell activation or commitment into the mitotic cycle. Quiescent cells were refractory to DNA fragmentation, but not to membrane lysis. Log phase growth, transformation with c-myc, or infection of quiescent G0 targets with herpes simplex virus-1, which induces a competent state for DNA synthesis, all enhanced target cell susceptibility to CTL-induced DNA fragmentation without altering the membrane lysis. These results suggest that G0 cells are resistant to CTL-induced apoptosis, but that entry into G1 or a G1-like state by growth factors, cellular transformation, or DNA virus infection renders them competent to enter the apoptotic pathway(s).

Materials and Methods

Effectors.

In this investigation we used purified primary CD8+ spleen CTLs, induced by acute infection of adult C3H/HeSnJ × C57BL/6J)F1 mice for 8 d with lymphocytic choriomeningitis virus (LCMV), strain Armstrong (5). The use of these primary CTL (LCMV-CTL) precludes artifactual problems associated with CTL lines. These LCMV-CTL contain azurophilic granules and specifically induce the lysis and DNA fragmentation of LCMV-infected targets in an MHC class I-restricted fashion (7).

CTL Assays.

Mouse target cells (L-929, BALB/c NIH 3T3, P815, YAC-1, and A20) were labeled either with sodium S-chloro for a cytoplasmic label or with [3H]thymidine or [3H]iododeoxyuridine (3H-IUDR) for a DNA label and examined for the release of labels as specified in the figure legends. Where indicated, DNA was purified by guanidine HC1 denaturation in the absence of detergent and electrophoresed in 1.5% agarose gels, as described (5).

Results and Discussion

When fibroblasts are cultured in decreasing concentrations of serum, there is a concomitant decrease in the percentage of cells in log phase growth (8). Culture of L929 fibroblasts in 2, 5, 10, or 20% FBS led to greater cell proliferation with increasing FBS concentration. After 2 d of growth under these conditions, [3H]thymidine uptake was 12, 29, 45, and 87 × 103 cpm, respectively, after a 1-h pulse. When assayed as CTL targets in their respective culture conditions, there was...
no significant difference in the membrane lysis of these cells as assessed by release of the cytoplasmic radiolabel, "Cr (Fig. 1 A). However, the fragmentation of the target DNA, as assessed by liberation of a 125I-IUDR label, increased dramatically in cells grown in increasing serum concentrations (Fig. 1 B). These results suggested that the degree of DNA fragmentation was related to the proliferative status of the target cell. More importantly, however, this experiment established, without any drug-induced modulation of the target cell, that the process of target cell membrane lysis induced by the CTL lethal hit is distinct from the process of CTL-induced DNA fragmentation.

We next sought to determine the susceptibility of serum-starved quiescent cells to CTL-induced DNA fragmentation. These experiments required that the target cells be able to reach a state of quiescence upon serum deprivation or growth factor removal. Initial experiments with prototypic apoptotic CTL targets, i.e., the P815 mastocytoma, the A20 B cell lymphoma, and the T cell lymphomas YAC-1 and EL-4 (9) proved unsuitable, as these cells underwent serum deprivation-induced apoptosis during our attempts to render them quiescent. L929 cells have been reported to enter a Go-like state after prolonged serum deprivation (10), but in our hands the L929 cells displayed significant levels of DNA synthesis even after a 3-d serum starvation protocol. We, therefore, selected for these studies the NIH BALB/c 3T3 cell, clone A31 (3T3-A31), which would undergo quiescence upon serum deprivation (11). 3T3 cells are reported not to undergo high levels of DNA fragmentation when lethally hit by CTLs (9). We found, however, that, in 9-12-h assays, lethally hit 3T3-A31 cells did undergo apoptosis, which allowed for tenable DNA fragmentation comparisons. When quiescent 3T3-A31 cells were exposed to LCMV-CTLs, the level of membrane lysis was similar to 3T3-A31 cells in log phase growth (Fig. 2 A), but the level of DNA fragmentation was dramatically lower (Fig. 2 B). When quiescent 3T3-A31 cells were assayed in the presence of 10% FBS, added at the beginning of the cytotoxicity assay, the level of lysis (Fig. 2 A) and the level of DNA fragmentation increased only slightly (Fig. 2 B). These results support the concept that the DNA fragmentation of the target cell may be mediated by a mechanism(s) regulated by cell cycle controls.

c-myc is an immediate early gene that is expressed as cells gain competence to exit Go and progress into G1 (12-14). Cells expressing high levels of myc proliferate at a faster rate than their normal untransformed counterparts and bypass Go by entering G1 from mitosis (12-14). If the Go state protects cells from CTL-induced apoptosis, as it does in growth factor deprivation- and p53-induced apoptosis (15, 16), one would predict that c-myc-transformed cells would be more susceptible to apoptosis because they do not enter Go. We transfected the 3T3-A31 cells with a c-myc containing retrovirus (17) and selected a transformant in soft agar. In the presence of 10% FBS, the population doubling time of these cells (td) was 12 ± 1 h vs. 20 ± 2 h for the parental 3T3-A31 cells. The enforced expression of c-myc did not alter the membrane lysis between these targets (Fig. 3 A) but markedly enhanced their susceptibility to CTL-induced DNA fragmentation (Fig. 3 B). We were unable to compare the differences in quiescent A31-myc versus quiescent 3T3-A31 cells because the A31-myc cells would undergo apoptosis upon serum deprivation. L929 cells (td = 15 ± 1 h), which have a high to intermediate...
Figure 3. The susceptibility between different target cells to CTL-induced apoptosis may be reflected in their growth rates. (A and B) Comparison of CTL-induced membrane lysis (A) and DNA fragmentation (B) between 3T3-A31, A31-myc, and L929 cells. Target cells grown as monolayers (3T3-A31, A31-myc, L929) were cultured in DMEM + 5% FBS. Target cells were labeled with 

\[ ^{31} \text{Cr} \] before use (5) or with 1 \( \mu \)Ci/ml IUDR for 18 h. Cytotoxicity assays were assessed in Fig. 1 in RPMI 1640 + 10% FBS. Assay time was 10 h. SEM, for 

\[ ^{51} \text{Cr} \] release <8%; for IUDR release <14%. (C and D) Comparison of CTL-induced membrane lysis (C) and DNA fragmentation (D) of prototypic CTL-induced apoptotic targets P815 and A20 cells. Target cells grown in suspension (P815 and A20 cells) were cultured and assayed in RPMI 1640 + 10% FBS. Targets were labeled with 

\[ ^{51} \text{Cr} \] before use (3) and with 1 \( \mu \)Ci/ml IUDR for 12 h. Assay time was 6 h. SEM, for 

\[ ^{51} \text{Cr} \] release <7%; for IUDR release <10%.

Many DNA viruses induce quiescent cells to progress into a late G1/S-like phase to obtain cell-regulated components of the cell's DNA synthetic machinery (18-21). The mechanism of induction into the DNA synthetic state by DNA viruses differs, but the effects are the same, i.e., activation of the competence mechanism for cell cycle progression and the production of enzymes needed for DNA synthesis even if the DNA synthesized is preferentially viral (18-21). Numerous herpes viruses induce the activation of quiescent cells into the DNA synthetic phase and upregulate the activity of DNA topoisomerases I and II, which the viruses require for replication (20, 21). When quiescent 3T3-A31 cells were infected with HSV-1, these cells became more susceptible to CTL-induced DNA fragmentation than 3T3-A31 cells in log phase growth and markedly more susceptible than their quiescent counterparts (Fig. 5, A and B). The level of CTL-induced membrane lysis was not significantly different between the three groups (Fig. 5 A). A previous study (22) suggested that HSV-1-infected cells were highly sensitive to killing by TNF-α, but inclusion of anti-TNF antibodies in our assays did not block the DNA fragmentation nor the membrane lysis of the target cells. Pulse-labeling studies of the HSV-infected quiescent vs. uninfected quiescent targets showed, as expected, that DNA synthesis was initiated in the HSV-infected quiescent cells and remained high throughout the time of assay (data not shown). This DNA synthesis was probably viral, as HSV-1 infection inhibits cellular DNA synthesis while stimulating cellular DNA synthetic enzymes (23). Thus, HSV-1 infection of the target cell induced a state of competence for extreme susceptibility to CTL-induced apoptosis. This virus-induced competence was not due to serum con-
were processed and assayed as in Fig. 1 in AIM-V media without or with counting the number of cells in the infected flask, was 20. Target cells harvest of the assay was 20 h.

5 ml of HSV-1 with a titer of 10^7 PFU/ml for 2 h at 37~ with rocking to quiesce for 72 h. For HSV-1 inf~ion, quiescent cells were infected with moved, fresh RPMI + 5% CPSK-2 was added, and the cells were allowed (3). Assay time for the data presented was 14 h. Time = 0 when the LCMV-B). The possibility that HSV-1 directly induced fragmenta-

tion of the target cell DNA is unlikely, as the control virus-

infection of the virus inoculum, because FBS added back to quiescent targets or to HSV-1-infected quiescent targets, to a final concentration of 10%, did not significantly increase their susceptibility to CTL-induced apoptosis (Fig. 2, A and B). The possibility that HSV-1 directly induced fragmenta-
tion of the target cell DNA is unlikely, as the control virus-infected samples did not show oligonucleosomal ladder proteins, nor was the level of spontaneous DNA release different from uninfected target cells. Evaluation of whether HSV-1 infection could directly induce apoptosis revealed that the HSV-1 cytopathic effect kills cells via necrosis (data not shown), consistent with a recent report (24). In preliminary studies, various RNA viruses, i.e., the coronavirus mouse hepatitis virus, strain A-59, the picornavirus encephalomyocarditis virus, and LCMV, were unable to render the quiescent targets more susceptible to CTL-induced apoptosis, nor did they stimulate DNA synthesis in these cells. However, vaccinia virus, a DNA virus that encodes its own DNA topoisomerase I and other proteins that interact with cell cycle progression factors (25), increased the susceptibility of quiescent targets to CTL-induced apoptosis.

The data presented suggest that cells must be in cycle or in a state of competence to be susceptible to CTL-induced apoptosis. Transformation of cells with c-myc enhanced their growth rate and rendered them very susceptible to CTL-induced apoptosis, but the specific role of c-myc in apoptosis is unclear. Our analyses by immunofluorescence of c-myc protein levels between the targets shown in Figs. 3 and 4 did not reveal a corresponding relationship with their levels of DNA fragmentation. Since others have shown that antisense to c-myc can block antigen receptor–induced apoptosis in T cells (26), we asked whether similar treatment of target cells would block CTL-induced apoptosis. Although a 4–18-h treatment of P815 and L929 cells with antisense to c-myc (5'- CACGTGAGGGGCAT-3') significantly reduced myc protein levels and arrested the cells in the G1 (but not G0) phase of the cell cycle, as shown previously (27), it did not impair the level of CTL-induced DNA fragmentation versus untreated targets (data not shown). This suggests that c-myc, per se, is only indirectly involved in rendering targets sensitive to CTL-induced apoptosis.

HSV-1 can induce the up-regulation of the immediate early (IE) transcription factor c-jun/AP-1 (c-jun) (28). c-jun and c-fos are required for the serum-stimulated transition out of G0 into G1 (29). The c-jun–inducing activity was localized to the HSV-1 IE protein ICP0, which is required for the expression of IE proteins ICP4 and ICP27 (30–32). ICP4 and ICP27 have been defined as containing immunodominant CTL epitopes in BALB/c mice (33, 34). It is interesting to note that immunodominant MHC class I–restricted CTL epitopes have also been identified in the IE gene, pp89, of murine cytomegalovirus, which induces c-fos expression and cellular DNA synthesis in host cells (35). Thus, DNA viruses induce cellular activation and replication competence through protooncogene expression, and DNA viral proteins synthesized early after infection may both induce cellular activation through protooncogene expression and provide CTL epitopes to render these unwanted cells susceptible to the full CTL armament. The early recognition of the virus-infected cell by specific CTL could also allow for the degradation of viral DNA before viral packaging and release. Cell death by apoptosis is irreversible, but purified perforin has been shown to induce a transient membrane permeability that can be repaired (36). Thus, the membrane lysis mechanism of CTL may be less efficient than the apoptosis mechanism for irreversible killing of a target, and the induction of competence for CTL-induced apoptosis may therefore be of pathological significance.

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