Brief Definitive Report

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 G_0 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 G_0 cells are resistant to CTL-induced apoptosis, but that entry into G_1 or a G_1-like state by growth factors, cellular transformation, or DNA virus infection renders them competent to enter the apoptotic pathway(s).

CTLs, which are principal mediators in the elimination of transformed and virus-infected cells from the host, kill their targets by the direct disruption of the plasma membrane and by the induction of the suicide program known as apoptosis (1–4). The CTL's arsenal includes the pore-forming protein perforin, a family of proteases called granzymes, and tumor necrosis factorlike molecules packaged together in cytoplasmic granules (3, 4). The granule exocytosis model of CTL killing proposes that, upon recognition of viral- or tumor-associated antigen(s) presented via the MHC class I molecule, the CTL is triggered to release its cytotoxic granule components onto the target cell's plasma membrane. Perforin inserts into the target's plasma membrane and forms transmembrane channels to cause the osmotic lysis of the target (3). The remaining cytotoxic granule components interact with as yet undefined target cell elements to trigger the apoptotic death of the target, characterized by cellular disintegration and fragmentation of the target's DNA into nucleosome particles (2–4).

In a previous investigation (5), we demonstrated that specific inhibitors for DNA topoisomerases I (camptothecin) and II (mAMSA and VM-26) prevent the CTL-induced DNA fragmentation of the target cell's DNA without impairing the disruption of the target cell's plasma membrane. DNA topoisomerases are cell cycle–regulated enzymes with low to undetectable activity in early G_1 and peak activity in the S and G_2/M phases of the cell cycle (6). We therefore, sought to determine whether the susceptibility of target cells to CTL-induced apoptosis was cell cycle–related and show here that it is.

Materials and Methods

Effector Cells. In this investigation we used purified primary $CD8^+$ spleen CTLs, induced by acute infection of adult C3H/HeSnJ × C57BL/6J)F_1 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). A Con A lectin–dependent killing assay was used to bypass the requirements for specific antigen, in order to compare the susceptibility of various target cells to CTL-induced apoptosis (5).

CTL Assays. Mouse target cells (L-929, BALB/c NIH 3T3, P815, YAC-1, and A20) were labeled either with sodium ^{51}chromate for a cytoplasmic label or with [^{3}H]thymidine or [^{125}I]iododeoxyuridine (^{125}I-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 HCl 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, [^{3}H]thymidine uptake was 12, 29, 45, and 87 × 10^3 cpm, respectively, after a 1-h pulse. When assayed as CTL targets in their respective culture conditions, there was

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no significant difference in the membrane lysis of these cells as assessed by release of the cytoplasmic radiolabel, ^{51}Cr (Fig. 1 *A*). However, the fragmentation of the target DNA, as assessed by liberation of a ^{125}I-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 G_0-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 G_0 and progress into G_1 (12–14). Cells expressing high levels of *myc* proliferate at a faster rate than their normal untransformed counterparts and bypass G_0 by entering G_1 from mitosis (12–14). If the G_0 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 G_0. We transinfected 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 (t_d) 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 (t_d = 15 ± 1 h), which have a high to intermediate

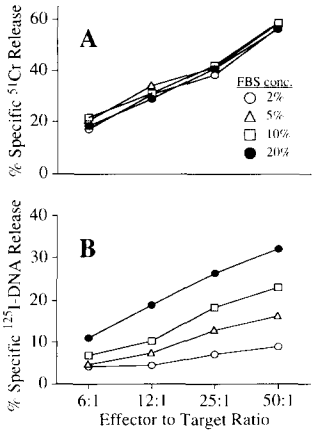

Figure 1. CTL-induced DNA fragmentation of L929 cells is enhanced in target cells grown in high serum concentration. (*A*) Membrane lysis of L929 target cells grown for 48 h in FBS as measured by the release of [^{51}Cr]chromium. 3 d before use (day −3) L929 fibroblasts were subcultured into T75 cm^2 flasks at ~50% confluency in MEM (GIBCO BRL, Gaithersburg, MD) with 5% FBS (Sigma Chemical Co., St. Louis, MO). On day −2, the medium was removed and replaced with RPMI 1640 (Sigma Chemical Co.) containing the desired concentration of FBS. The cultures were processed as described (3) and used as targets on day 0. Just before use, purified LCMV-CTL were resuspended in medium containing the desired concentration of FBS. Cytotoxicity assays were performed as described (5) with Con A (Sigma Chemical Co.) at 5 μg/ml. L929 cells were cultured identically for both the ^{51}Cr- and ^{125}I-IUDR-release portion of the assay. Assay time for *A* and *B*, performed in parallel, was 6 h. Standard error of mean (SEM) for ^{51}Cr release was <5%. (*B*) Influence of serum on target cell's susceptibility to CTL-induced DNA fragmentation. L929 target cells were cultured as above and labeled with [^{125}I]iododeoxyuridine (^{125}I-IUDR) at 1 μCi/ml on day −3 for 18 h as described (3). SEM: ^{125}I-DNA release <10%.

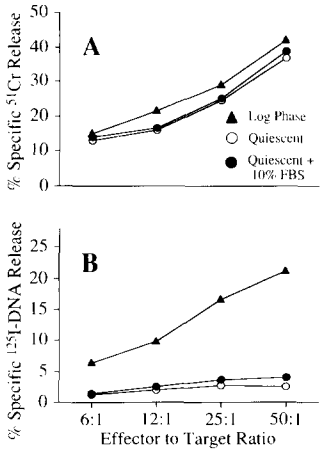

Figure 2. Quiescent NIH BALB/c 3T3, clone A31 cells are refractory to CTL-induced DNA fragmentation but not membrane lysis. (*A*) Membrane lysis of 3T3-A31 cells included in the assay medium. SEM, ^{51}Cr release <8% (*B*) Solubilized DNA (^{125}I-DNA) released from targets in log phase growth, quiescent targets, or quiescent targets with 10% FBS added during the assay. 3T3-A31 target cells were cultured in DMEM+10% calf serum (Hyclone Labs., Logan, UT). 5 d before use (day −5) 3T3-A31 cells were subcultured into T150 cm^2 flasks to obtain ~75% confluency. On day −4, they were labeled with ^{125}I-IUDR at 1 μCi/ml in 5 ml for 18 h. After labeling, the medium was removed, and fresh RPMI 1640+5% CPSR-2 (Sigma Chemical Co.) was added, and the cells were allowed to quiesce for a minimum of 72 h. CPSR-2 is a nonmitogenic serum substitute. For cells in log phase growth, 3T3-A31 cells were subcultured to ~50% confluency 2 d before use. The cells were labeled as above on day −1. Cytotoxicity assays were performed with LCMV-CTL in lectin-dependent assays in AIM-V media without or with 10% FBS. Assay time for parts *A* and *B* performed in parallel, was 10 h. SEM, ^{125}I-DNA release <15%.

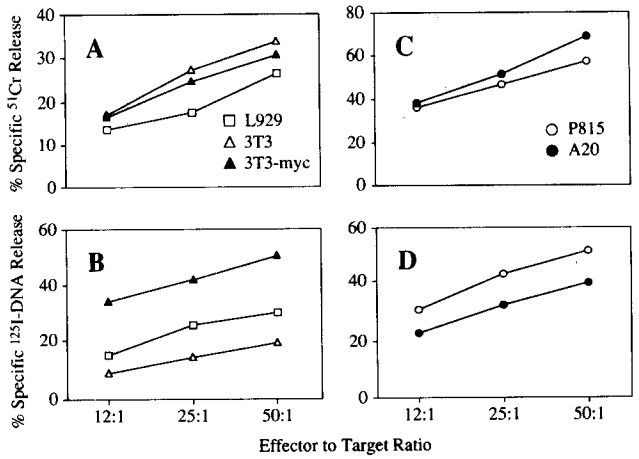

Figure 3. The susceptibility between different target cells to CTL-induced apoptosis may be reflected in their growth rates. (*A* and *B*) Comparison of CTL-induced membrane lysis (*A*) and DNA fragmentation (*B*) between 3T3-A31, A31-*myc*, and L929 cells. Target cells grown as monolayers (3T3-A31, A31-*myc*, L929 cells) were cultured in DMEM + 5% FBS. Target cells were labeled with ^{51}Cr before use (3) or with 1 μCi/ml ^{125}I-IUDR for 18 h (3). Cytotoxicity assays were as described in Fig. 1 in RPMI 1640 + 10% FBS. Assay time was 10 h. SEM, for ^{51}Cr release <8%; for ^{125}I-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}Cr before use (3) and with 1 μCi/ml ^{125}I-IUDR for 12 h. Assay time was 6 h. SEM, for ^{51}Cr release <7%; for ^{125}I-IUDR release <10%.

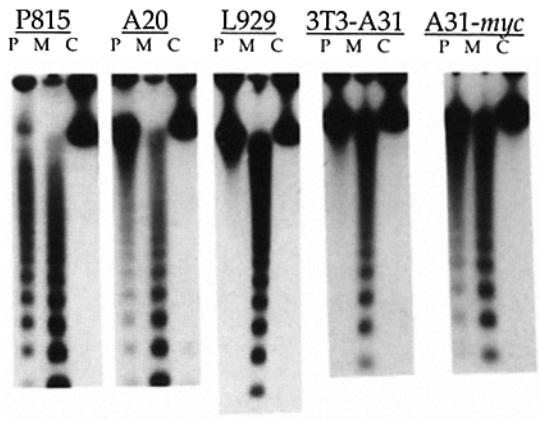

Figure 4. Target cell DNA is fragmented into the nucleosomal ladder pattern typical of apoptosis. Autoradiogram of target cell DNA purified as described from either the assay medium (*M*) or from the cell pellet (*P*) after the medium had been removed or from target cells, only, without CTL added (*C*) (5). Targets were exposed to CTL for either 6 (P815, A20) or 10 h (L929, 3T3-A31, A31-*myc*) assays. DNA fragmentation was assessed from samples at an E/T ratio of 50:1. Control samples were from targets without effector cells. 30,000 cpm ± 10% of each sample component was loaded onto 1.5% agarose gels. Gels were dried and exposed at −80°C with two screens (5).

growth rate under these conditions, had an intermediate level of DNA fragmentation even though they showed a lower level of membrane lysis than the 3T3-A31 cells (Fig. 3, *A* and *B*). To further dissect the relationship between the proliferative status of the target cell and CTL-induced DNA fragmentation, we analyzed CTL-induced apoptosis in the prototypic targets for apoptosis, P815 (t_d = 11 ± 2 h) and A20 (t_d = 14 ± 1 h) cells. These suspension-grown cells were extremely sensitive to apoptosis and consequently were analyzed in 4–6-h cytotoxicity assays. Although the level of membrane lysis was slightly greater in A20 cells than in P815 cells (Fig. 3 *C*), the level of DNA fragmentation was greater in the faster growing P815 cells (Fig. 3 *D*). Thus, P815 and A20 cells underwent apoptosis more rapidly than 3T3-A31, A31-*myc*, or L929 cells, but in each case the faster growing cell underwent apoptosis more readily (P815>A20; A31-*myc*>L-929>3T3-A31). Demonstration that each cell line underwent apoptosis associated with distinct oligonucleosomal ladders is presented in Fig. 4, which shows that the level of DNA fragmentation in cells not exposed to CTL (*C*), and in the cell pellets (*P*) and culture medium (*M*) from cells exposed to CTL in 6- (P815, A20) or 10- (L929, 3T3-A31, A31-*myc*) h assays. Note both the presence of the ladders and the disappearance of high molecular weight DNA, the latter being most noticeable with the P815 cells. Fig. 4 also shows that the DNA fragmentation in A31-*myc* cells is far more extensive than in the 3T3-A31 cells.

Many DNA viruses induce quiescent cells to progress into a late G_1/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 differ, 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/or 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 antisera 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-

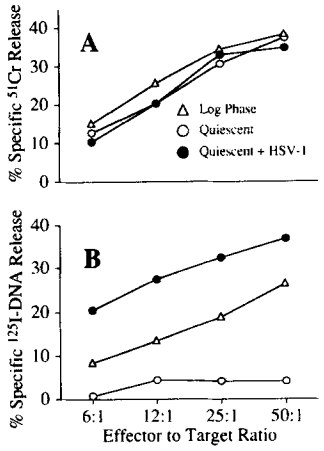

Figure 5. Infection of quiescent 3T3-A31 cells with HSV-1 renders these targets, otherwise refractory to apoptosis, highly susceptible to CTL-induced apoptosis. (*A*) Lysis of targets either in log phase growth, quiescent, or quiescent+HSV-1 infection. (*B*) Solubilized ^{125}I-DNA release from targets as in *A*. Quiescent targets and targets in log phase growth were prepared as in Fig. 2. 5 d before use (day −5), 3T3-A31 cells were subcultured into T150 cm^2 flasks to obtain ~75% confluency. On day −4, they were labeled with ^{125}I-IUDR at 1 μCi/ml in a total volume of 10 ml for 18 h. On day −3 the medium was removed, fresh RPMI + 5% CPSR-2 was added, and the cells were allowed to quiesce for 72 h. For HSV-1 infection, quiescent cells were infected with 5 ml of HSV-1 with a titer of 10^7 PFU/ml for 2 h at 37°C with rocking every 15 min. Multiplicity of infection, determined after infection by counting the number of cells in the infected flask, was 20. Target cells were processed and assayed as in Fig. 1 in AIM-V media without or with 10% FBS as indicated. Cytotoxicity assays were performed as described (3). Assay time for the data presented was 14 h. Time = 0 when the LCMV-CTLs were added to the target cells. Con A was at a final concentration of 5 μg/ml. Total time from the HSV-1 infection of quiescent targets to harvest of the assay was 20 h.

tamination 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 fragmentation 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 G_1 (but not G_0) 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 G_0 into G_1 (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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