Minireview

How Do Lymphocytes Kill Tumor Cells?[1]

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Introduction

The combined modalities of chemotherapy, radiotherapy, and surgery have had a significant impact on cancer mortality. Still, nearly 50% of the adult victims of cancer die of their disease as a result of primary or acquired resistance (1). The enormously diverse immune system, able to generate specific soluble and cell-mediated responses to as many as 10^{10} ligands, has had strong intuitive appeal for investigators hoping to find more effective cancer therapies.

There is good evidence that the immune system is active against tumors. Immunocompromised hosts (*e.g.,* patients infected with HIV, receiving immunosuppressive medications, or recovering from solid organ or bone marrow transplants) are at increased risk of developing oligoclonal or monoclonal proliferations that regress occasionally if immunocompetence is restored (2). The occasional spontaneous regression of tumors (*e.g.,* melanoma) is also taken as evidence of immune surveillance (3).

All arms of the immune system appear to be involved in tumor surveillance. The generation of antibodies to tumor-specific antigens or to host antigens that are expressed aberrantly are the basis of the tumor vaccine effort, which has reached clinical trials. Soluble mediators such as TNF[3], IFN-γ, lymphotoxin, and other cytokines clearly have potent antitumor activity. These subjects have been reviewed recently elsewhere and will not be discussed further (4, 5). Coverage of immunotherapy trials, including strategies using gene-transfer techniques, is likewise beyond the scope of this review (6, 7). Here, we will focus on the molecular mechanisms of contact-dependent, cell-mediated cytotoxicity and the evidence that suggests a role for this pathway in tumor cell killing.

Cell-mediated Response to Tumors

There is abundant evidence suggesting that lymphocytes play a central role in the host response to tumors. Nonspecific phagocytes (neutrophils and macrophages) may also be involved but will not be discussed further here. Participation by all lymphocyte compartments in cell-mediated cytotoxicity against tumor cells has been implicated using a variety of experimental approaches. $CD8^+$ T cells from immunized animals can provide immunity against syngeneic tumors when adoptively transferred into naive hosts (8). This property is specific and long-lived. Furthermore, $CD8^+$ and $CD4^+$ T cell clones can be propagated *in vitro* and have been used to identify tumor-specific peptides. Although disputed initially, it is now clear that $CD4^+$ T cells (of the Th1 subclass) can also be cytolytic (9). Circumstantial evidence suggests that NK cells may also participate in tumor rejection (10, 11). NK cells are able to lyse targets without previous sensitization. The prototypical tumor cell lines sensitive to NK-mediated lysis *in vitro* are K562 (human chronic myelogenous leukemia-derived erythroleukemia) cells and YAC-1 (murine T-cell lymphoma) cells. LAK cells are derived from a precursor common to NK cells but acquire potent antitumor activity only upon exposure to IL-2. Tumor-infiltrating lymphocytes are enriched for specificity to the tumor from which they were explanted but may assume the nonspecific LAK phenotype when incubated with IL-2 *in vitro.*

Lymphocytes kill targets (including tumor cells) by inducing them to undergo programmed cell death (apoptosis). This is a process with unique biochemical and morphological features that distinguish it from necrotic cell death (12). The process of CTL-induced apoptosis has been characterized extensively at the molecular level through genetic experiments performed during the past several years, the results of which are summarized below.

Two Effector Pathways Account for Cell-mediated Cytotoxicity

The Granule Exocytosis Pathway. Two molecular mechanisms of cellular cytotoxicity appear to have evolved independently in lymphocytes. The first is the granule exocytosis pathway. In this pathway, the recognition and tight binding of a susceptible target cell by a CTL or NK cell causes vectorial alignment of their secretory apparatus, promoting delivery of electron-dense cytoplasmic granules to the target cell membrane at areas of close intercellular contact (13). These secretory granules contain material sufficient to initiate an apoptotic program in the target cell. One granule constituent is perforin [cytolysin/pore-forming protein (PFP)], a protein with significant homology to the terminal components of complement (14). The Ca^{2+}-dependent polymerization of perforin on the target cell membrane forms a channel through which other granule constituents probably gain access to the target cell cytoplasm to deliver the lethal hit (Ref. 15; Fig. 1). The gzms are a family of neutral serine proteases that are critical for this process. In one experimental test of this model, purified perforin induced membrane damage causing leakage of cytoplasmic contents from target cells, but it was not sufficient to trigger DNA fragmentation, the *sine qua non* of apoptosis (16–18). Purified gzm A or gzm B alone was ineffective. Perforin and gzm A or gzm B together resulted in the apoptotic death of the target cell. In a similar experiment, the nonlytic RBL (rat basophilic leukemia) cell line became weakly cytotoxic when transfected with the

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[3] The abbreviations used are: TNF, tumor necrosis factor; NK, natural killer; LAK, lymphokine-activated killer; gzm, granzyme; FasR, Fas receptor; FasL, Fas ligand; ICE, interleukin-1 converting enzyme; crmA, cytokine response modifier A.

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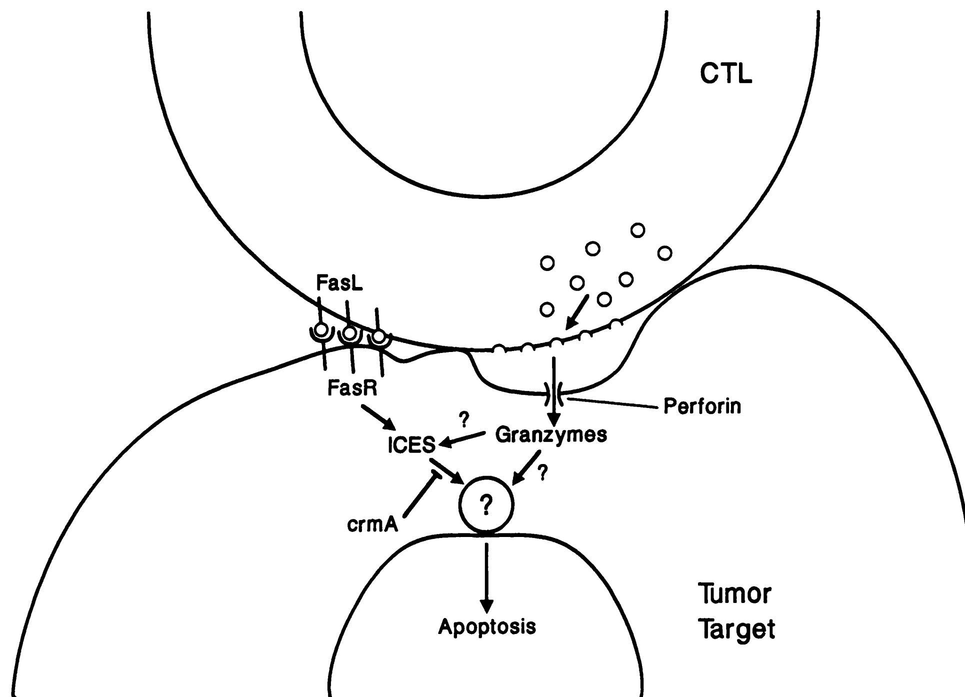

Fig. 1 Perforin/gzm and Fas pathways of cell-mediated cytotoxicity. At sites of tight intercellular conjugation, cytotoxic lymphocytes release secretory granules containing perforin, a protein that permeabilizes tumor target cell membranes, probably allowing gzms to enter the cytoplasm, where they activate substrates (possibly including members of the ICE family) by proteolytic cleavage. Subsequent events (not yet defined) culminate in tumor cell DNA fragmentation and apoptotic cell death. Alternatively, apoptosis may be induced when the FasR, present on the surface of most tumor targets, is engaged by the FasL, present on cytotoxic lymphocytes. The transduction of this death signal may converge on substrates in the perforin/gzm pathway and is inhibitable by a gene product of the cowpox virus, crmA.

cDNA for perforin (19). Transfectants expressing both perforin and gzm A or gzm B generated all of the hallmarks of apoptosis in target cells. In triple transfectants, gzm A and B synergized (in the presence of perforin) to yield even higher levels of cytotoxicity.

Effector cells differ in their expression of these molecules. The activation of a T cell upon recognition of antigen by its surface receptor in the context of self-MHC and the appropriate costimulatory signals results in transcriptional up-regulation of the genes encoding perforin, gzm A, gzm B, and other granule proteins (20–22). The CTL becomes fully armed within 3–5 days. NK cells contain preformed toxic granules (generated presumably at a precursor stage of development) and consequently do not require sensitization to trigger the formation of granules (23).

Through the use of gene-targeting technology, mice have been generated with null mutations at several critical loci encoding granule exocytosis pathway proteins. These loss-of-function mutants have validated the model presented above. *In vitro* analysis of perforin-deficient $CD8^+$ lymphocytes using standard lytic assays demonstrated a severe defect in cytotoxicity against allogeneic target cells (24). Perforin-deficient NK cells were compromised similarly when directed against the NK-sensitive YAC-1 tumor cells. Perforin-deficient mice also exhibit profound *in vivo* defects in viral clearance (lymphocytic choriomeningitis virus) as well as impaired cardiac allograft rejection responses (24, 25).

gzm B-deficient $CD8^+$ CTLs and LAK cells exhibit a severe defect in their ability to induce apoptosis rapidly in susceptible target cells (26), but cytotoxicity recovers partially with prolonged incubation of effectors with target cells (as is true for perforin-deficient CTLs; Ref. 24). In contrast, gzm B-deficient NK cells cannot induce apoptosis in susceptible target cells, despite high E:T ratios or prolonged incubation (27). In an acute graft-*versus*-host disease model used in our laboratory, gzm B-deficient $CD8^+$ T cells demonstrate a reduction in cytotoxic activity, but $CD4^+$ T cells do not (28).

A human model for the complete disruption of the granule exocytosis pathway is provided by patients with Chédiak-Higashi syndrome. These patients have impaired cell-mediated immunity, resulting in increased susceptibility to infection, as well as a lymphoproliferative disorder in the terminal phase of the illness (29). CTLs, NK cells, and neutrophils from these patients have a severe defect in the granule exocytosis pathway. Although the azurophil granules are synthesized and loaded properly, they fuse aberrantly with secondary granules and are rendered completely nonfunctional (30, 31). Although Chédiak-Higashi CTLs still exhibit some cytotoxicity, Chédiak-Higashi NK cells are virtually devoid of cytotoxicity. These observations suggest that $CD8^+$ CTLs have a second killing pathway but that NK cells do not.

The Fas Pathway. CTLs with an ineffective granule exocytosis pathway (*i.e.,* from perforin-deficient mice) exhibit residual cytotoxicity only against target cells that express the Fas antigen, a cell surface receptor related structurally to the TNF receptor family. The purification of the FasR led to the cloning and characterization of FasL, a membrane-bound protein that is induced during T-cell activation (32). The Ca^{2+}-independent interaction of FasR and FasL triggers target cell apoptosis through a "death domain" on the cytoplasmic tail of the FasR via a series of docking proteins that transmit the death signal through unknown mechanisms (Refs. 33–35; Fig. 1). Although it is argued that the Fas pathway serves primarily an immunoregulatory role (by deleting activated T cells), Fas-dependent

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cytotoxicity against nonlymphoid targets can be demonstrated readily (36–38).

Naturally occurring mutations that provide loss of function models for the Fas pathway have been identified recently. Mice with the *gld* mutation have a single nucleotide substitution in a region of FasL that is critical for transmission of the apoptotic signal (39). Conversely, *lpr* mice have a mutation causing truncation of FasR that results in a similar phenotype (40). These animals accumulate $CD4^-$ $CD8^-$ T cells as a consequence of the failure of Fas-dependent deletion of activated T cells in the periphery (41, 42). *In vitro* cytotoxicity assays using FasL-deficient effectors demonstrate defects primarily in the $CD4^+$ compartment (43). In an acute graft-*versus*-host disease model, FasL-deficient $CD4^+$ CTLs have a profound reduction in cytotoxicity.[4] Cytoxicity is also reduced in the $CD8^+$ compartment, but this is only apparent when superimposed on a gzm B-deficient background.

Human patients with mutations of FasR have also been identified recently (44, 45). The failure of peripheral clonal T-cell deletion seen in FasR- or FasL-deficient mice is shared by these patients. Clinically, affected individuals present with lymphadenopathy and autoimmunity; increased susceptibility to infections and malignancy have not yet been noted in these patients (46).

There is strong experimental evidence to suggest that these two pathways account for nearly all contact-dependent, cell-mediated cytotoxicity. For example, when perforin-deficient CTLs are used to kill FasR-deficient targets, no cytoxicity is detectable (47, 48). $CD8^+$ CTLs and LAK cells depend on both pathways for cell-mediated cytotoxicity, but $CD4^+$ CTLs appear to utilize primarily the Fas pathway. NK cytotoxicity, in contrast, seems to depend entirely on the granule exocytosis pathway as noted above.

The intracellular intermediates that link the signals delivered by perforin/gzms or Fas to nuclear events resulting in apoptosis have not yet been identified. An important clue was provided by the cloning of ICE, a human homologue of *CED-3*, a death-inducing gene critical for development in the nematode *Caenorhabditis elegans* (49). ICE is the second protease identified that cleaves protein substrates after aspartic acid residues; the first was gzm B. This unusual coincidence of substrate specificity suggested that the granule exocytosis pathway may initiate apoptosis through a proteolytic cascade reminiscent of the coagulation system. An additional clue came with the recognition that crmA, a gene product of the cowpox virus, is a cross-class inhibitor, inhibiting specifically both a serine protease (gzm B) and cysteine protease family members (*e.g.,* ICE; Ref. 50). The demonstration that crmA blocks apoptosis mediated by Fas, TNF, or gzm B (51)[5] reinforces further the analogy to the coagulation system, with two independent initiation arms converging on a final common pathway (Fig. 1). It is tempting to speculate that a system of protease inhibitors may have evolved to regulate this cascade, and that exploitation of this system by viral gene products may represent a novel road to neoplasia.

Evidence That the Fas and Granule Exocytosis Pathways Mediate Tumor Cell Killing *in Vivo*

The null mutations of FasR/FasL and perforin/gzm B provide critical reagents to test the importance of these pathways for tumor cell killing. The *in vitro* defects in allogeneic tumor cell killing were described above. To date, only a few *in vivo* experiments have been performed.

Perforin-deficient mice were able to lyse allogeneic P815 tumor cells injected i.p. as effectively as their wild-type littermates (24). Nonsyngeneic tumor models such as this, however, raise the formal possibility that tumor clearance represents allogeneic tissue rejection rather than tumor antigen-specific immunity. However, in an additional experiment, the footpad swelling response to the intradermal injection of syngeneic MC57G fibrosarcoma tumor cells was significantly greater in perforin-deficient mice, indicating compromise of $CD8^+$-dependent tumor rejection in these animals (24).

In an *in vivo* tumor surveillance model, mice overexpressing the oncogenic *lck*^{pr}-*HOX11* transgene were bred into a gzm B-deficient background. After 1 year of observation, there was no significant difference in the incidence or latency of tumors in the gzm B-deficient cohort, compared with wild-type controls,[6] suggesting that gzm B is not critical for immune surveillance in this model.

The composition of the lymphocyte compartments is quantitatively normal in perforin and gzm B-deficient animals. This simplifies greatly the interpretation of these experiments. Data from the following experiments is not as easy to interpret for a number of reasons, as outlined below.

Killing of syngeneic tumors has not been studied in either FasR- or FasL-deficient mice. Abnormalities in thymocyte development and peripheral T cell clearance caused by these mutations leads to lymphoproliferation and autoimmune disease in these animals, however, making interpretation of such experiments potentially difficult. One recent report does demonstrate indirectly a death effector role for this pathway against tumors *in vivo*; i.p. administration of supernatants from a cell line transfected with FasL cDNA caused dose-dependent apoptotic death of encapsulated YAC-1 cells implanted in the peritoneal cavities of syngeneic mice (52). Within the constraints of this experimental model, this finding demonstrates that soluble FasL is sufficient to cause apoptosis of tumor cells *in vivo*.

Class I- and class II-deficient animals have been generated and are developmentally normal apart from a profound reduction in $CD8^+$ and $CD4^+$ T cells, respectively. Their phenotype in syngeneic tumor surveillance or clearance models has not yet been analyzed. These animals will potentially yield important information about the role of individual lymphocyte compartments in the cell-mediated response to tumors.

[4] T. Graubert, J. Russell, and T. J. Ley, unpublished observations.
[5] L. Shi and A. Greenberg, personal communication.
[6] T. Graubert, S. Korsmeyer, and T. J. Ley, unpublished observations.

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Conclusions

The experiments described above suggest that the granule exocytosis and Fas pathways may fully account for cytotoxicity mediated by CTLs and NK cells against tumor targets. Powerful *in vitro* observations, confirmed to some extent *in vivo*, demonstrate that disruption of these pathways by targeted or naturally occurring mutations compromise significantly tumor immunity. Although the effector molecules are segregated differentially in lymphocyte compartments (Fas for $CD4^+$ CTLs, perforin/gzm for NK cells, and both pathways in $CD8^+$ CTLs), once the death signal is delivered to a target cell, a common mechanism, possibly involving a proteolytic cascade, triggers apoptosis. It is this latter phase of the signal transduction pathway that is least understood.

Spontaneous tumors have not been observed to occur at an increased rate in any of these animals with null mutations of death effector molecules. Rather than refuting the importance of these pathways in immune surveillance, this observation likely reflects the significant redundancy among the effectors. It would not be surprising if a tumor phenotype emerges if both pathways are disabled, either by breeding mutant lines together or by transgenic overexpression of inhibitors like crmA that act late in the apoptotic cascade, below the convergence of the pathways.

As the molecular description of tumor immunity is defined, new insights into the means by which tumor cells circumvent host defenses should follow. Hopefully, this will lead in turn to novel therapeutic strategies that capitalize on this information.

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