

Original article

New knowledge about T-cell cytotoxicity

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Introduction

Ever since the initial reports that antigen-specific cytolytic T lymphocytes (CTLs) are generated during allograft rejection or viral infection [1], many attempts have been made to elucidate the precise mechanism(s) by which CTLs destroy target cells. Earlier *in vitro* studies demonstrated that target-cell lysis requires intimate contact between the CTL and its target and is highly specific, i.e., it does not affect antigenically irrelevant bystander target cells. The assessment of target-cell lysis *in vitro* was facilitated by the development of assays based on the extracellular release of radioactive markers from the cytoplasm (e.g., ⁵¹Cr) or the nucleus (e.g., ¹²⁵I-UdR) of labeled target cells. Moreover, it was clearly established that CTL-mediated lysis, in contrast to lysis induced by antibody and complement, is not simply caused by a loss of the integrity of the target cell plasma membrane (resulting in colloid-osmotic lysis), but involves an active process similar, if not identical, to apoptosis that includes nuclear events such as chromatin condensation and extensive DNA fragmentation.

Recent work indicates that two major mechanisms are responsible, at least *in vitro*, for CTL-mediated lysis [2-5]: (1) the exocytosis of cytolytic granules at the site of membrane contact between the CTL and its target, resulting in the local delivery of soluble lytic proteins such as perforin and granzymes, and (2) the expression on the CTL surface membrane of a molecule, the Fas ligand (FasL), which induces a death signal upon engagement with its receptor, Fas, on the target cell membrane.

The existence of two independent lytic pathways has been clearly established *in vitro* by using CTL generated in mice lacking perforin or with a nonfunctional Fas ligand as well as target cells derived from Fas-deficient mice. Moreover, such mice have been used to assess the relative role of the two pathways in T-cell-mediated effector mechanisms *in vivo*. Here, we summarize the recent advances made in the understanding of the molecular mechanisms operating in CTL-mediated lysis *in vitro*. The relevance of these findings to T-cell immunity is briefly discussed.

The perforin/granzyme-dependent pathway

CD8+ CTLs possess specialized lytic granules which, although they share distinctive features with regulated secretory granules (such as those found in neuroendocrine cells, for example), are unusual in their nature, i.e., they are 'secretory lysosomes'. Indeed, while in most secretory cells lysosomal and secretory granule components are packaged into separate organelles, the CTL lytic granules are organelles combining both secretory and lysosomal compartments. In addition to classical lysosomal proteins, the granules contain several other proteins, including perforin and a set of serine proteases termed granzymes. Following recognition of the target cell antigen (a complex made of an antigenic peptide bound to a major histocompatibility complex class I molecule) by the CTL antigen receptor (TCR), the granules within the CTL reorient toward the CTL target-cell contact site and then fuse with the plasma membrane resulting in the polarized delivery of their content.

It should be emphasized that CD8+ (naïve) T cells do not possess lytic granules before they are activated in a primary response to antigen. Once they are activated into CTLs, the presence of lytic granules with stored proteins precludes the need for protein synthesis in perforin-dependent CTL-mediated lysis. It appears, however, that CTLs not only secrete lytic granules when they interact with target cells, but may also be triggered to synthesize additional lytic proteins that are stored in newly formed granules or secreted directly from the cell via a constitutive secretory pathway [4]. While this phenomenon may explain the ability of a single CTL to kill *in vitro* several target cells sequentially, its significance remains to be defined *in vivo*.

Perforin, one of the soluble lytic proteins stored in CTL granules, is a glycoprotein with sequence homology to the complement component C9. It is generally agreed that, following granule exocytosis, perforin monomers can bind and insert into the target-cell plasma membrane and subsequently aggregate to form homopolymeric pore structures [3-6]. While pores visible by electron microscopy contain 10-20 aggregated monomers, there is evi-

dence that 3–4 monomers may already constitute a functional channel. While such pores may perturb the permeability of the target cell membrane, it is clear that this effect is not sufficient to explain the various apoptotic changes associated with CTL-mediated lysis. The search for additional soluble mediators stored in CTL granules has led to the identification of a family of serine proteinases termed granzymes [3, 7]. Granzyme B is by far the most abundant granzyme in CTL granules and cleaves proteins to the carboxyl site of acidic amino acids (e.g., aspartic acid). This substrate specificity is very unusual among serine proteinases. Another granzyme, granzyme A, exhibits a trypsinlike activity and thus cleaves proteins to the carboxyl side of basic residues (e.g., arginine or lysine). Following the demonstration that granzyme A or B can induce DNA fragmentation in target cells permeabilized with a sublytic dose of perforin, it has been proposed that granzymes are involved in target-cell apoptosis. As these soluble mediators have to enter the target cell in order to activate the apoptotic program, perforin may promote their entry, either directly through the poly-perforin pores, or indirectly by stimulation of endocytosis.

The absolute requirement for perforin and granzyme B in rapid CTL-mediated lysis has been confirmed unequivocally by studies using CTL generated in mice in which the perforin gene or the granzyme B gene has been destroyed by homologous recombination [7, 8]. Perforin- or granzyme B-deficient mice develop normally and produce activated CD8⁺ cells upon appropriate antigenic stimulation. However, activated perforin-deficient CD8⁺ cells are unable to lyse *in vitro* antigenically relevant target cells (provided these target cells do not express Fas; see below). Activated granzyme B-deficient CD8⁺ cells, although they induce ⁵¹Cr release from target cells, are unable to induce rapid target cell DNA fragmentation. While these findings do not rule out that other CTL granule proteins may play a supplementary role, they point to the essential role of both perforin and granzyme B in the triggering of target cell apoptosis resulting from CTL granule exocytosis.

DNA damage induced by the perforin/granzyme-dependent pathway does not depend on new protein synthesis in the target cell. Thus, all the molecules necessary for this pathway are already present, in contrast to some other pathways leading to apoptosis. Although DNA fragmentation is a biochemical hallmark of apoptosis, a central role for DNA cleavage in the initiation of apoptosis has not been convincingly demonstrated. Indeed, it appears that intracellular proteases, and not endonucleases, are the primary initiators of apoptosis [9–11]. Accordingly, recent attention has focused on the identification of intracellular proteases that may serve as substrate(s) for granzyme B. While interleukin 1 β converting enzyme (ICE) has been proposed as a putative substrate, proteases related to ICE may be more relevant than ICE itself. For example, a recently identified member of ICE-related proteases involved in programmed cell death has been shown to be activated by granzyme B. As this protease, termed apopain/Yama/ CPP32, is also involved in

Fas-dependent apoptosis (see below), it may represent a target cell component essential for CTL-mediated lysis.

The Fas ligand/Fas-dependent pathway

In addition to the regulated and polarized exocytosis of lytic granules, another mechanism of CTL-mediated lysis involves the interaction of membrane-bound Fas ligand (FasL) on the CTL with its cognate receptor Fas on the target-cell surface membrane, resulting in a signal leading to apoptotic cell death [12–14].

FasL is a type II membrane protein belonging to the tumor-necrosis-factor family. Interestingly, FasL is hardly detectable on the surface of CTLs before they interact with target cells. It is therefore likely that engagement of the CTL TCR with its ligand on the target cell triggers FasL expression at the CTL surface, in addition to exocytosis of granules. In contrast to the latter phenomenon, however, inhibition of RNA or protein synthesis in a CTL prevents its ability to mediate target-cell lysis via the FasL/Fas-dependent pathway, thus suggesting the need for continuous FasL synthesis (or the production of a putative FasL-activating protein).

Fas (CD95) is a type I membrane protein belonging to the tumor-necrosis-factor-receptor family. Upon proper engagement (including its cross-linking), Fas can transduce an apoptotic signal. A 70-amino-acid-long segment of the Fas cytoplasmic domain is necessary and sufficient for transduction of the apoptotic signal and, hence, is called a death domain. There is considerable variability in the expression patterns of Fas in various tissues. Abundant expression is found in liver, heart, kidney, and ovary. Lymphocytes transformed with human T-cell leukemia virus, human immunodeficiency virus, or Epstein-Barr virus, as well as activated lymphocytes, express a relatively large amount of Fas on the cell surface.

The biochemical mechanisms involved in the Fas-dependent cytotoxic pathway are still poorly understood. As mentioned earlier, there is increasing evidence that activation of ICE-related cytoplasmic proteases such as CPP32 and/or ICE-LAP3 [15] is an important step in the mediation of apoptotic death. Recent studies suggest that such an activation could be triggered by the association of the Fas cytoplasmic domain with an intracellular adaptor molecule termed FADD, leading to the assembly of other proteins that participate in a cascade of enzymatic reactions resulting in the cleavage of several cytoplasmic and nuclear substrates. It is not yet clear whether a single or several of the substrates of ICE-related proteases may play a critical role in Fas-dependent cytotoxicity.

The relative role of the FasL/Fas-dependent pathway in CTL-mediated lysis has been clarified by the use of CTL or target cells derived from mice with loss-of-function mutations in FasL or Fas, respectively [12]. In mice carrying the *gld* (for generalized lymphoproliferative disease) mutation, there is a replacement of a phenylalanine by a leucine in the extracellular C-terminus of FasL. This

substitution abolishes the ability of FasL to bind to Fas. In mice with the *lpr* (for lymphoproliferative) mutation, transcription of the Fas gene is impaired by the insertion of a transportable element in an intron of the gene. Another allelic mutation (*lpr^{cg}*) in the *lpr* locus results in the replacement of an isoleucine by an asparagine in the Fas death domain, which affects the ability of Fas to transduce the apoptotic signal.

With the availability of such mice, it is now feasible to perform comparative *in vitro* assays in which normal CTL, perforin-deficient CTL, and FasL-deficient CTL are tested for lysis of Fas-positive or -negative target cells [16]. Studies along this line indicate that there is little cross-talk between the two mechanisms of CTL-mediated lysis: perforin-deficient CTL can perfectly lyse Fas-positive target cells by means of the FasL/Fas-dependent pathway, whereas FasL-deficient CTL and Fas-negative target cells can lyse or be lysed, respectively, by means of the perforin-dependent pathway. Moreover, while there is direct evidence that the two pathways are operative in CD8+ CTLs, it appears that CD4+ CTLs utilize primarily, although not exclusively, the FasL/Fas-dependent pathway, in contrast to NK cells, which utilize mainly the perforin/granzyme-dependent pathway, although they express FasL after activation [17, 18].

Significance for T-cell immunity

It is generally assumed that T-cell-mediated cytotoxicity is one of the major effector mechanisms responsible for immunity to viruses, intracellular bacteria, and tumor cells. With few exceptions, this assumption is mainly based on circumstantial evidence, i.e., lack or loss of specific immunity after depletion of CD8+ cells *in vivo*, or indirect detection (i.e., after expansion *in vitro*) of specific CTLs in immune mice. Since most CTLs also secrete lymphokines, including interferon- γ , upon target-cell recognition, it is evident that effector mechanisms other than cell-mediated cytotoxicity may be responsible for CD8+ cell-dependent immunity.

The availability of perforin-deficient mice now allows the direct assessment of the role played by perforin-dependent cytotoxicity in immune reactions *in vivo* [8]. The results reported so far indicate that the perforin-dependent cytotoxic pathway plays a crucial role in the immunological control of some viral infections, but not of others (in spite of similar CTL responses). Similarly, perforin-dependent cytotoxicity has been shown to play a major role in immune protection against *Listeria monocytogenes*, a model system for immunity against intracellular bacteria. Impaired immune rejection of tumor cells in perforin-deficient mice has been reported, but further work is needed to ascertain the role of perforin-dependent cytotoxicity in various model systems of tumor immunity. An important role of perforin-dependent cytotoxicity has also been established in model systems of autoimmune tissue damage [19]. As far as rejection of allogeneic grafts is concerned, the role of perforin-

dependent cytotoxicity may be greatly influenced by the tissue or organ involved as well as by the participation, or lack thereof, of the graft vascular bed in the rejection process.

In contrast to perforin-deficient mice, lymphocyte development is abnormal in mice with *lpr* or *gld* mutations. Indeed, these mice develop lymphadenopathy and splenomegaly characterized by the accumulation of T lymphocytes which do not express the CD4 or CD8 surface markers. It is likely that these lymphocytes correspond to mature T cells which have not been eliminated after antigen-induced activation because of the lack of a Fas-based mechanism normally involved in the regulation of T-cell homeostasis. Because of such abnormality, the use of *lpr* or *gld* mice to determine the role of FasL/Fas-dependent cytotoxicity in T-cell immunity is relatively limited. As shown recently, however, short term studies based on the adoptive transfer of T cells from *gld* mice may bring direct information as to the participation of Fas-based immune mechanisms *in vivo*. In a model system of lethal graft-versus-host disease (GVHD), spleen cells from perforin-deficient mice or from FasL-deficient mice were able to induce the disease, although mortality was delayed compared to that observed after transfer of normal T cells. In contrast, GVHD was abrogated when spleen cells lacking both molecules were used as the source of effector cells [20].

Conclusions

It is now clear that two independent pathways are involved in T-cell-mediated cytotoxicity. The first one is based on the local delivery of lytic proteins, including perforin and granzymes, normally stored in CTL granules, whereas the second one depends on the expression of both FasL on the CTL plasma membrane and Fas on the target-cell membrane. Both pathways are triggered by the engagement of the CTL TCR with its antigenic ligand and result in target cell apoptosis. While the biochemical mechanisms involved in the apoptotic processes mediated by either pathway are still poorly understood, activation of distinct target-cell cytoplasmic proteases belonging to the ICE-like family may be a crucial step common to both pathways. The relative role of the two pathways in T-cell-mediated immunological phenomena *in vivo* is under intensive investigation thanks to the availability of mice deficient in one or the other molecule identified *in vitro* as a key component of T-cell-mediated cytotoxicity. Elucidation of the mechanisms leading to target-cell apoptosis *in vivo* will further our understanding of the role of CTL in immunity and autoimmunity, and may provide new tools for manipulating their activity, positively or negatively, in a variety of immune processes.

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