Granzymes at a glance

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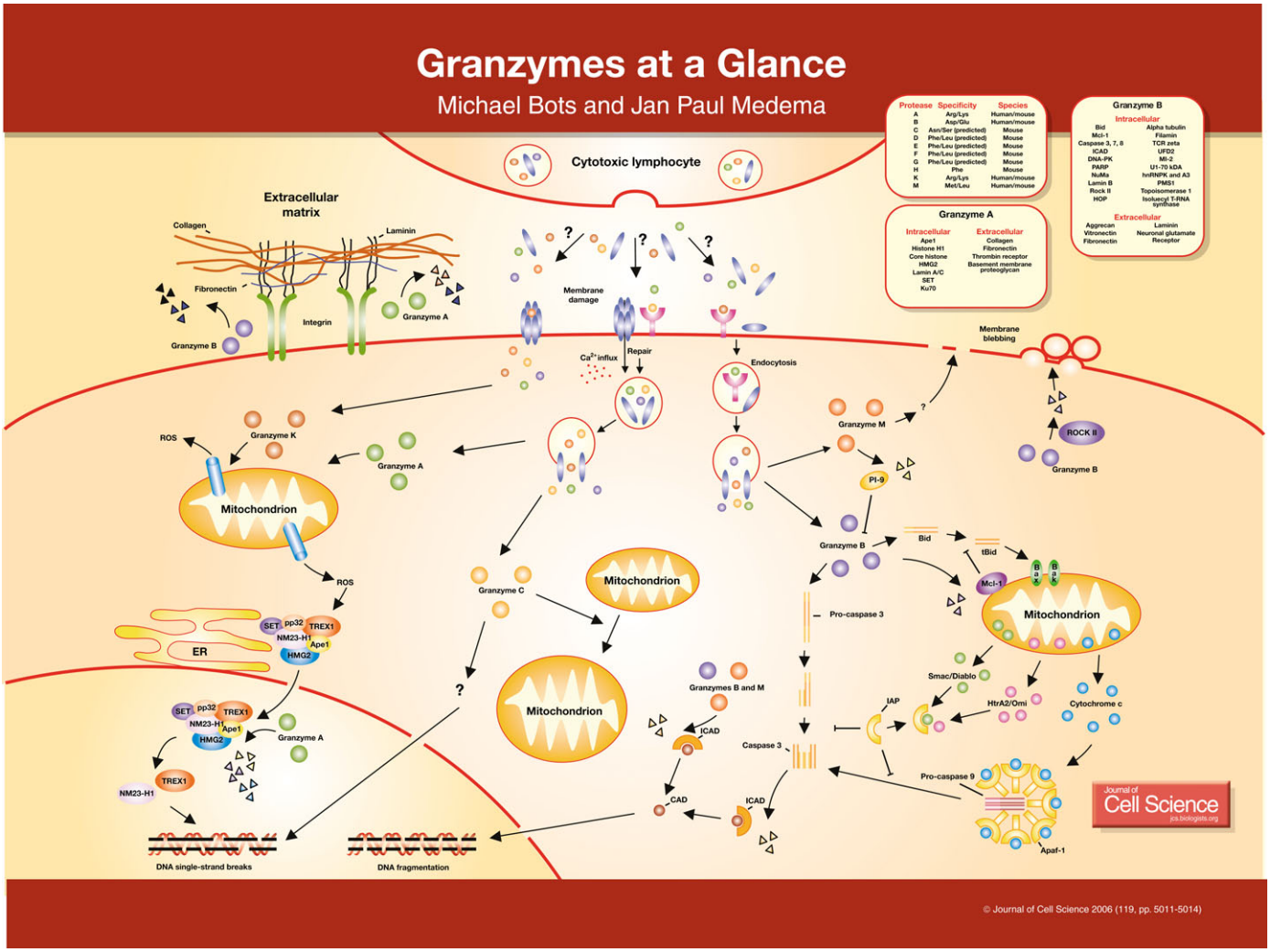
Defence against virally infected and malignant cells depends on the action of cytotoxic T lymphocytes and natural killer cells (Barry and Bleackley, 2002; Russell and Ley, 2002). Although these use several mechanisms to eliminate target cells, the principal event is secretion of cytotoxic granules. These granules contain the pore-forming protein perforin together with a variety of granule-associated proteases, which include the granzymes. Target cell recognition by cytotoxic lymphocytes induces secretion of the cytotoxic granular content towards the target and the induction of death. Here, we briefly provide a generalized view of granzyme signalling and the substrates involved.

Expression of granzymes

Granzymes are structurally related serine proteases that differ in their substrate specificity. To date, five different granzymes have been described in humans: granzymes A, B, H, K and M (Grossman et al., 2003). In mice, clear orthologues of four of these granzymes (A, B, K and M) can be found, and granzyme C seems the most probable murine orthologue of granzyme H. The murine genome encodes several additional granzymes (D, E, F, G, L and N), of which D, E, F and G are expressed by cytotoxic lymphocytes; L appears to be a pseudogene and N is expressed in the testis. Except for their proposed specificities (see inset), relatively little is known about these additional granzymes. Nevertheless, the ubiquitous expression in cytotoxic lymphocytes suggests a role for these proteases in host protection, but more information on the timing and levels of their expression, as well as their substrates, is required. Here we focus on the granzymes for which a function in cytotoxicity has been reported.

Extracellular substrates

Upon secretion by cytotoxic lymphocytes, granzymes cleave both extracellular and intracellular proteins. The extracellular events are still ill



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defined but clearly involve remodelling of the matrix. In vitro, granzyme B can mediate detachment of cells and induces cell death by anoikis (death due to lack of extracellular contact) (Buzza et al., 2005; Waterhouse et al., 2006), but the physiological relevance of this is unknown. Initial studies also indicated that granzyme A acts extracellularly: it induces cytokine production (interleukin 6/8), cleaves matrix proteins (fibronectin and collagen type IV) and processes the thrombin receptor (Barry and Bleackley, 2002). Although these events are likely to be important, the intracellular granzyme targets have attracted most attention over the years.

Granzyme delivery into the cytoplasm

The entry of granzymes into target cells is a pivotal step in cell death. Granzymes can enter the endosomal compartment in a receptor-dependent fashion, which probably involves electrostatic interactions with heparan sulphate proteoglycans and possibly the mannose-6-phosphate receptor. However, their entry into the cytoplasm and subsequent cell killing entirely depend on the protein perforin (Catalfamo and Henkart, 2003). How perforin facilitates cytoplasmic entry is a debated issue. Perforin itself is a pore-forming molecule that is capable of membrane permeabilization. Initially these perforin pores were suggested to be the gates for granzyme entry, but such pores are probably too small and too transient for optimal passage. A more realistic view depicts these pores as transient ion channels that destabilize the plasma membrane. Repair of the plasma membrane would then result in internalization of membrane-bound cytolytic molecules, including perforin and granzymes, into endosomes and subsequent release from this compartment (Keefe et al., 2005). An alternative hypothesis suggests that perforin does not affect the plasma membrane but is co-internalized with granzymes into the endosomal compartment and destabilizes these vesicles to allow granzymes to enter the cytoplasm (Metkar et al., 2002). However, interpretation of these models calls for caution, as all evidence has been gathered in experiments using purified perforin at concentration that may not reflect the physiological situation at the immunological synapse.

Intracellular substrates

Granzyme B

Granzyme B is the most extensively studied granzyme and is responsible for the rapid induction of caspase-dependent apoptosis (Trapani and Sutton, 2003; Waterhouse et al., 2006). Human granzyme-B-mediated apoptosis is in part mediated by mitochondria. To induce mitochondrial changes, granzyme B cleaves the BH3-only pro-apoptotic protein Bid. Upon cleavage, truncated BID translocates to the mitochondria and together with Bax and/or Bak results in release of pro-apoptotic proteins and mitochondrial outer membrane permeabilization. Cytochrome c release is crucial in apoptosome formation and subsequent caspase-9 activation, which in turn cleaves downstream effector caspases. In addition to Bid, granzyme B can induce cytochrome c release by cleavage and inactivation of the anti-apoptotic Bcl-2 family member Mcl-1.

Besides its Bcl-2-family-directed actions, granzyme B can process several caspases, including the effector caspase 3 and initiator caspase 8. The granzyme-B-mediated activation of caspase 3 is especially important in mice, in which Bid appears to be a weak substrate (Adrain et al., 2005). Activation of caspase-3 is controlled by inhibitor of apoptosis protein (IAP) family members, which are often over-expressed in tumour cells. For caspase 3 to be unleashed, IAPs need to be sequestered by Smac/Diablo or HtrA2/Omi, which are released from disrupted mitochondria (Trapani and Sutton, 2003; Waterhouse et al., 2006). Mitochondrial disruption may therefore be needed for this direct pathway as well; this notion is supported by the absence of active caspase 3 in granzyme-B/perforin-treated Bcl-2-overexpressing cells.

Granzyme B has also been reported to process several known caspase substrates directly, such as poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), ICAD, the nuclear mitotic apparatus protein (NuMa) and lamin B. Although most research has focused on the caspase-related pathways, granzyme B also induces caspase-independent events. For instance, membrane blebbing can be induced directly by cleavage of the Rho-regulated kinase Rock II, which is involved in actomyosin contraction, and this subsequently regulates bleb protrusion. Several other substrates unrelated to caspase-dependent death were recently identified in proteomic screening and these studies should help to complete the picture of granzyme-B-mediated killing (Adrain et al., 2005; Bredemeyer et al., 2004). A list of reported granzyme B substrates is given on the poster, but caution is warranted, because the vast majority have not been rigorously tested in physiologically relevant settings.

Granzyme A

Granzyme-A-induced cell death is mainly characterized by generation of single-stranded DNA nicks, rather than the oligonucleosomal DNA fragments typical of granzyme-B-induced apoptosis. Moreover, granzyme-A-induced death does not result in activation of caspases. Despite these differences, mitochondria are thought to fulfil a critical role in granzyme-A-mediated cell death. However, in contrast to granzyme B, granzyme A induces loss of mitochondrial inner membrane potential and the release of reactive oxygen species (ROS). Because the outer membrane stays intact, pro-apoptotic proteins such as cytochrome c, HtrA2/Omi or Smac/Diablo are not released. In response to ROS, the ER-associated SET complex, which includes SET, Ape1, pp32, HMG2, NM23-H1 and TREX1, translocates to the nucleus (Chowdhury et al., 2006; Martinvalet et al., 2005), where granzyme A cleaves three members of the SET complex that are involved in DNA repair: HMG2, Ape1 and SET (Lieberman and Fan, 2003). More importantly, cleavage of SET releases the DNase NM23-H1 from its inhibitor and allows NM23-H1, together with TREX1, to nick DNA (Chowdhury et al., 2006; Lieberman and Fan, 2003).

Granzyme A also targets the nuclear lamins and histone H1 (Lieberman and Fan, 2003). Because these proteins are important for stabilizing the nuclear envelope and maintaining chromatin structure, cleavage of these substrates probably facilitates the activity of DNases.

Granzyme C/H and granzyme K

Granzyme C/H and granzyme K are so-called orphan enzymes because their substrates have not been identified. Granzyme-C-induced cell death is independent of caspase activation (Johnson et al., 2003) and the main feature of this pathway is thought to be rapid mitochondrial swelling and loss of mitochondrial membrane potential. Similarly to granzyme A, granzyme C induces single-stranded DNA nicks, but the DNase responsible for this effect has not yet been identified.

Granzyme K induces caspase-independent cell death (MacDonald et al., 1999). Experiments performed with recombinant granzyme K also show ROS production, hinting at the possibility that the mechanism of action is similar to that of granzyme A.

Granzyme M

Granzyme-M-induced cell death occurs rapidly and in a caspase- and mitochondria-independent fashion (Kelly et al., 2004). Cells treated with granzyme M display large cytoplasmic vacuoles, which may be indicative of autophagy, and show rapid plasma membrane permeabilization via an unknown mechanism. However, this view was recently contested following the identification of ICAD and PARP as potential substrates for granzyme M and clear signs of apoptosis when granzyme M was 'protein-transfected' into target cells (Lu et al., 2006). Moreover, granzyme M was suggested to regulate granzyme B activity by cleavage of the endogenous inhibitor PI-9 (see below). Which mechanism will hold up awaits further investigation, but it is clear that this granzyme uses alternative cleavage sites to cause cell death.

Granzymes in disease

The importance of granule-mediated death for host protection can be deduced from several genetically determined clinical syndromes (Menasche et al., 2005). For instance, patients with familial haemophagocytic lymphohistiocytosis (FHL), which in most cases results from mutations in the perforin, *MUNC13-4* or *STX11* gene, show impaired granzyme delivery and cytotoxicity and, as a consequence, cannot clear infectious pathogens and display uncontrolled activation and expansion of T cells. Similarly, patients with Grisceli type 2, Cediak-Higashi and X-linked lymphoproliferative syndromes are all characterized by impaired granule secretion and thus impaired cytotoxicity (Menasche et al., 2005). In perforin-deficient mice, similar symptoms can be observed when animals are infected with lymphocytic choriomeningitis virus (LCMV). Importantly, clearance of LCMV or murine orthopox virus is also greatly affected in mice lacking granzymes, which suggests a major role for granule exocytosis in viral control.

Besides impaired responses to viruses, mice lacking components of the granule-induced death mechanism are more prone to tumours. For instance, perforin-deficient mice develop spontaneous B-cell lymphomas and cannot control tumour growth in experimental settings (Catalfamo and Henkart, 2003). The role of granzymes in tumour clearance is controversial and conflicting results have been reported that do or do not attribute a role of granzyme A and B in tumour clearance (Davis et al., 2001; Pardo et al., 2002; Smyth et al., 2003).

Granule-mediated death thus forms an important defence mechanism against viruses and tumours, but escape mechanisms have been described. Several intracellular inhibitors of granzymes have been reported and some of these are encoded by the viruses or can be overexpressed in tumour cells (Silverman et al., 2004; Trapani and Sutton, 2003). The serpin class of serine protease inhibitors can render tumour and virus-infected cells immune to the actions of at least granzyme B and M in vitro (Bots et al., 2005; Medema et al., 2001), but inhibitors for the other granzymes are likely to exist. Whether this inhibition is effective in vivo is debated (Bots et al., 2006), but it is clear that the plethora of cell death pathways induced by the different granzymes will in most cases act as 'fail-safe' mechanisms that makes granule-induced death hard to beat.

We sincerely apologize to those colleagues whose original articles we have not cited. Owing to space limitations we have mainly cited reviews discussing the various topics and encourage everyone to read these. Authors are supported by the Dutch Cancer Society and the AICR.

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