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News and Commentary

A 'polarized' look at α-tubulin cleavage by granzyme B

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Granzyme B-induced apoptosis is one mechanism by which cytotoxic lymphocytes kill tumour or virus infected cells. As such, identifying critical substrates for granzyme B is of great interest with respect to mechanisms of cytotoxic lymphocyte (cytotoxic T lymphocytes (CTL) and natural killer (NK))-induced cell death and our general understanding of how cytototoxic lymphocytes (CL) protect the body from cancer and infection. The concerted effort of several labs has identified a number of granzyme B substrates that when cleaved promote apoptosis[1–7] (Figure 1). Granzyme B has also been shown to cleave proteins for which an effect on cell death has not been shown.[8–11] It is unclear if granzyme B preferentially targets one or a few critical substrates or if granzyme B exerts its full effect by cleaving several substrates. Adding to this complexity, human and mouse granzyme B have been shown to target different substrates *in vitro*[12] and are selectively blocked by different inhibitors. It is unclear if human and murine granzyme B preferentially target the same critical substrates or if these granzymes target diverse substrates, several of which overlap.

Of the almost 30 substrates reported for granzyme B (Figure 1), several have not yet been rigorously tested in cells suggesting that the number of bona-fide physiological substrates may be smaller. In contrast, recent proteomics studies have identified new potential targets for granzyme B, suggesting that the list may be incomplete. Adrain *et al.*[12,13] recently employed a proteomic approach to identify granzyme B substrates by comparing 2D-polyacrylamide gel electrophoresis (PAGE) gels of Jurkat cells treated with granzyme B in the presence of zVAD-fmk or depleted of executioner caspases (to prevent cleavage of substrates by caspases activated by granzyme B). Using this approach they have noted 15 potential substrates for granzyme B. A similar approach was employed by Goping *et al.*[14] in which protein fragments from granzyme B-treated lysates were resolved by conventional 1D-PAGE and identified by mass spectroscopy. Both groups identified α-tubulin as a substrate for human granzyme B, supporting a previous report which showed that α-tubulin was cleaved in lysates from YAC-1 (murine lymphoma) cells treated with murine granzyme B.[8]

CL deliver granzyme B to their targets by a process known as granule exocytosis. During this process, granules in the CL move to the CL:target interface and their contents are released into the immunological synapse. Granzyme B is then delivered to the target cell cytosol by a perforin-dependent mechanism. Adrain *et al.*,[13] showed that α-tubulin was cleaved in a caspase-independent manner in HeLa cells that were killed by the NK cell line YT, while Goping *et al.*[14] showed similar results using CTL stimulated with Epstein Barr virus transformed Rosewell's Park Memorial Institute media (RPMI)-8666 cells. In assays using intact NK (YT) or CTL (RPMI-8666) it is difficult to discount a potential role of other granzymes, delivered to the target cell along with granzyme B, in cleaving α-tubulin. Many papers have shown that purified granzyme B delivered to target cells by sublytic concentrations of perforin or replication-deficient adenovirus can induce apoptosis of the target cells by a mechanism that is regulated by the anti-apoptotic protein Bcl-2. These studies would be more compelling if the authors had shown that purified granzyme B-cleaved α-tubulin in whole target cells over-expressing Bcl-2 (to prevent mitochondrial damage) in the presence of zVAD-fmk (to prevent cleavage of proteins by caspases), rather than in cellular lysates.

While we suggest that more rigorous tests should have been carried out to verify that α-tubulin is a bona-fide physiological substrate for granzyme B, these studies leave us with little doubt that human and murine granzyme B have the ability to cleave α-tubulin in cell lysates[8,13,14] and that α-tubulin is cleaved in a caspase-independent manner in target cells killed by cytotoxic lymphocytes[13,14] strongly suggesting that α-tubulin is cleaved by granzyme B in cells. It is therefore intriguing to speculate why granzyme B cleaves α-tubulin and what consequences this has for the target cell.

The Dynamic Life of α-Tubulin

In cells, α-tubulin heterodimerizes with β-tubulin in an alternating pattern to form the main structural subunit of microtubules. α-Tubulin is a polarized protein containing an N-terminal GTP binding domain, an intermediate taxol binding domain and an acidic tail. The polar nature of the tubulin subunits and the distinct alternating binding pattern with β-tubulin results in microtubules that are also polarized. This polarity allows for a process known as 'dynamic instability' in which the positive end is extended by a net addition of subunits with a concurrent net loss of subunits at the negative end. Adrain *et al.*[13] and Goping *et al.*,[14] demonstrated that granzyme B-cleaved α-tubulin at Asp438, thereby removing a short sequence of acidic amino acids (SVEGEGEEEGEEEY) from the C-terminus. As the polarized nature of α-tubulin contributes to the dynamic nature of microtubules, removal of

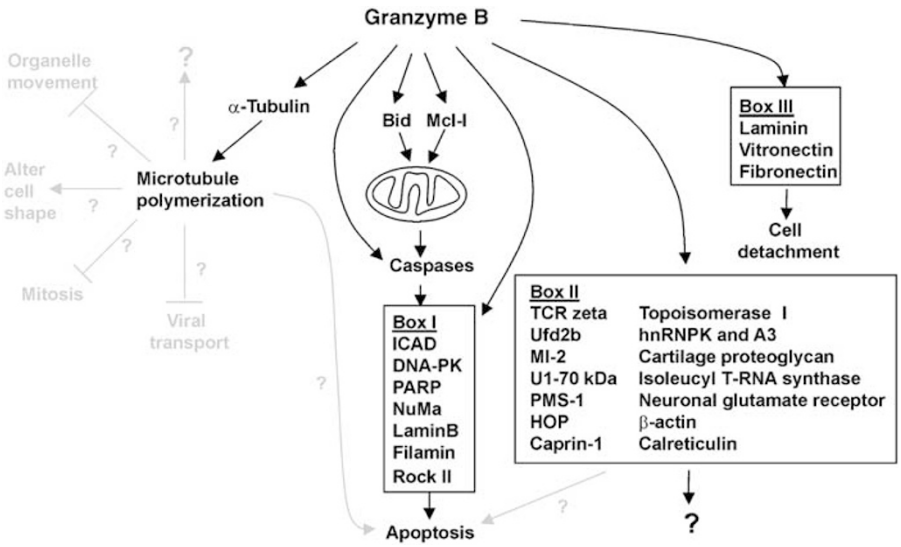

Figure 1 Substrates for granzyme B placed in pathways that indicate a potential physiologal role. Box I shows granzyme B substrates that are also substrates for caspases, Box II shows granzyme B substrates for which a defined role in cell death has not yet been shown and Box III shows granzyme B extracellular substrates that are involved in cell detachment. Grey lines indicate hypothetical pathways

these acidic (negative) residues is likely to impact on microtubule stability. Consistent with this, Adrain *et al.*[13] found that truncated α-tubulin polymerized faster than the full-length protein *in vitro* and microtubules of cells treated with granzyme B and streptolysin O (SLO) were more pronounced when stained with α-tubulin than in cells treated with SLO or granzyme B alone.

Cleavage of α-Tubulin and Cell Death

Granzyme B has been shown to induce apoptosis by cleaving proteins such as the proapoptotic Bcl-2 family member Bid[3,5,15] and caspase proteases.[2,4] Cleavage of Bid triggers mitochondrial outer membrane permeabilization (MOMP) and release of proteins such as cytochrome *c*, SMAC/Diablo and htRA2/Omi from the mitochondrial intermembrane space. In the cytosol, cytochrome *c* facilitates formation of an 'apoptosome complex' with APAF and caspase-9, which recruits and activates the executioner casapse-3. In support of a critical role for Bid, Bid-deficient cells are resistant to granzyme B-induced cell death and maintain their proliferative potential following treatment with granzyme B/perforin. Cells over-expressing Bcl-2, which prevents Bid-induced MOMP, are also resistant to granzyme B-induced cell death and proliferate following treatment with granzyme B/perforin even though Bid is still efficiently cleaved in these cells. It would therefore be interesting to know if α-tubulin cleavage by granzyme B is prominent in cells overexpressing Bcl-2. If α-tubulin is cleaved in these cells, yet they survive and proliferate, it would suggest that either the damage is reparable or that the effect of granzyme B-cleaved α-tubulin does not impair proliferation or promote cell death. The effect of α-tubulin cleavage on cell death may therefore become evident when studied in cells in which apoptotic pathways are impaired, such as cells over-expressing Bcl-2, and in conjunction with those in which caspase activity has been blocked.

Reduced expression of α-tubulin by siRNA caused little or no effect on killing by NK.[13] This is not entirely surprising given that knockdown of α-tubulin was incomplete, however, it remains possible that complete ablation of α-tubulin may cause disruptions to microtubule-dependent activities that confounds effects relevant during granzyme B-induced apoptosis. This leads to the question regarding whether α-tubulin cleavage and the corresponding microtubule stabilization is a dominant effect caused by generation of C-terminally truncated α-tubulin or if all of the cellular pool of α-tubulin must be cleaved in order to elicit the necessary effect during CL-induced apoptosis, whatever that effect turns out to be. If the former is the case (i.e. partial digestion of the cellular α-tubulin protein pool) the C-terminally truncated α-tubulin would represent a dominant form responsible for microtubule stabilization.

Cleavage of α-Tubulin and Cytoskeletal Rearrangement

Microtubules play a role in maintaining or altering cell shape. For example, upon initial CL/target cell interaction the microtubule organizing centre moves from the back of the CL toward the leading edge at the CL:target interface. This reorganization is likely to participate in orchestrating cytoskeletal rearrangements required for formation of a functional immunological synapse. It is therefore possible that granzyme B-mediated cleavage of α-tubulin causes cellular alterations in addition to inducing cell death. Indeed granzyme B has recently been shown to cleave extra cellular proteins such as vitronectin, fibronectin and laminin to induce tissue rearrangement without killing the target.[16] In contrast, Adrain *et al.*[13] showed that when cells were treated with lethal concentrations of granzyme B, inhibition of caspases prevented cellular rearrangements in short-term (>7 h) assays. It is of note that the cytoskeletal rearrangements reported by Buzza *et al.*,[16]

were observed in long-term assays (24 h), therefore the assays reported by Adrain *et al.*,[13] may have been long enough to observe granzyme B-induced apoptosis but not long enough to observe cytoskeletal effects in the absence of cell killing.

Microtubules are also involved in mitosis where they extend from spindle poles and capture sister chromatids to ensure efficient and equal separation of the genetic material into daughter cells. If chromosomes are misaligned due to disruption of the microtubule network, or if cells are treated with microtubule inhibitors, mitosis is arrested at the spindle checkpoint. Another possible effect of granzyme B-mediated cleavage of α-tubulin may therefore be to prevent cellular reorganization or to prevent proliferation of a tumour or virus infected cell. As discussed above, cells overexpressing Bcl-2 proliferate following treatment with granzyme B suggesting that α-tubulin does not prevent cell division, however, if the effect of α-tubulin cleavage is to temporarily retard cell division until caspases are activated, this effect may only be elucidated in cells in which apoptotic pathways are impaired.

Cleavage of α-Tubulin and Viral Clearance

Several granzyme B substrates, including α-tubulin, are also cleaved by caspases (Figure 1). It is not clear why this redundancy exists, but it is possible that cleavage of these proteins by granzyme B has immediate consequences that are required before caspases are activated. One such instance may be envisaged in viral clearance. The spread of viruses such as HIV and human T-cell leukemia virus type 1 (HTLV-1) is facilitated by the formation of a 'virological synapse', a specialized site of cell-to-cell contact that involves rearrangement of the microtubule cytoskeleton within an infected T cell. Movement of the microtubule organizing centre and Golgi apparatus to the site of cell-to-cell contact is thought to facilitate the secretion of virus in a manner analogous to T-cell delivery of granule contents to their targets, 'granule exocytosis'[17] or within the target cell.[18] Poxviruses also enter the cell via microtubules and replicate in viral factories near the nucleus. As poxviruses are large ($\sim$250–300 μm) and cannot rely on diffusion, they also use microtubules to exit the cell.[19] It would seem prudent for a CL to disable the viral escape machinery immediately upon detection of an infected cell rather than relying on caspases. It is therefore tempting to speculate that granzyme B mediated cleavage of α-tubulin may impair transmission of viruses either by altering microtubule polarity required for direction-dependent cellular events or by preventing the cytoskeletal rearrangement essential for viral transfer before activation of caspases occurs. In support of this, granzyme AB$-/-$ mice are more susceptible to infection with ectromelia (a poxvirus) than wild-type mice.[20]

Perspectives

The microtubule network controls many processes that are integral to the life of a cell including cell division and localization/transport of organelles. Microtubules are also essential for viral entry and escape during infection. As such, altering the microtubule network is likely to affect the target cell in many ways. Identification of α-tubulin as a substrate for granzyme B is therefore of great interest, however, there is clearly much work to be done to determine the consequences of α-tubulin cleavage on CL-induced cell death or on the mechanism by which the immune system deals with dangerous cells.

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