JCB Comment

Endocytosing the death sentence

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A series of recent studies have suggested that endocytosis of the mannose-6-phosphate receptor (MPR)* might play a critical role in delivering the death signal to cells targeted for destruction by the immune system (for review see Barry and Bleackley, 2002). These studies have raised a number of controversial issues regarding the trafficking of proteins from the plasma membrane of the target cell to their substrates in the cytosol. In this issue, Trapani and colleagues examine the death of cells in which endocytosis of the MPR is blocked and show that the death signal is delivered effectively in the absence of MPR endocytosis (Trapani et al., 2002, this issue). How then is the death sentence delivered?

The immune system clears viral infections and tumorigenic cells by regulated secretion of soluble proteins leading to rapid apoptosis of the targets. This "lethal hit" is delivered by either cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells, both of which undergo regulated secretion of specialized lysosomes containing the proteins required to initiate cell death. The key soluble proteins in this pathway are the serine protease granzyme B, which cleaves substrates in the cytosol of the target (initiating apoptosis), and the pore-forming protein perforin, which is required to deliver granzyme B to the target cell cytosol.

When perforin was initially identified in the 1980s, it was found to bear a high degree of similarity to the pore-forming C9 component of complement and, like the membrane attack complex, perforin was shown to be able to insert into lipid bilayers and form 15-nm diameter pores in membranes (for review see Lowin et al., 1995). These findings led to a model of cell mediated lysis involving the formation of a perforin pore at the plasma membrane through which granzymes could be delivered to the cytosol. Over the last few years, this model has been challenged by observations that granzyme B can enter target cells by receptor-mediated endocytosis and then be released into the cytosol by "sublytic" (defined as causing <10% 51Cr release, a measure of plasma membrane permeability) levels of purified perforin. Several studies demonstrated that granzyme B was innocuous after endocytosis via the MPR until released from the endosomes by an agent, such as adenovirus, listeriolysin O, or streptolysin, with the ability to disrupt the endosomal membrane and deliver granzyme B to the cytosol (Froelich et al., 1998). Furthermore, the low doses of perforin, pneumolysin, or streptolysin required to trigger granzyme release from the endosomes did not permit detectable diffusion of proteins significantly smaller than 32-kD granzyme B (Browne et al., 1999). Do these results then imply that perforin is disrupting endosomal membranes rather than forming a pore at the plasma membrane?

The paper by Trapani and colleagues addresses the role of MPR and the pool of endocytosed granzyme B by using expression of dominant–negative dynamin mutants to block endocytosis. Their results are clear. Target cells are killed equally well whether endocytosis of granzyme B by the MPR is blocked or not, demonstrating that endocytosis by MPR is not necessary for target cell death, and the large pool of granzyme B, which can enter the endocytic pathway, need not play a role in this process. Additionally, the authors reexamine what was until now the most convincing evidence in favor of a role for MPR in target cell death, namely that MPR-overexpressing cells were more rapidly rejected than cells lacking MPR after allotransplantation (Motyka et al., 2000). This study shows that both cell types are completely eradicated. Surprisingly, the same rejections were observed in perforin-deficient mice, demonstrating that cell death was not occurring via the perforin/granzyme-mediated pathway, but rather by antibody-mediated responses, in part against the overexpressed human MPR.

Is there then any role for MPR in the uptake of granzyme B and the delivery of the apoptosis signal? Trapani et al. note that some granzyme B can be taken up into the cell via micropinocytosis and do not completely rule out a role for release of granzyme B from this pathway. But it is also worth outlining the other reasons that MPR binds granzyme B, as well as the current gaps in any argument requiring endosomal disruption as a means of delivering granzyme B to the cytosol. Newly synthesized granzyme B, like lysosomal hydrolases, is sorted to the secretory lysosomes of CTLs and NK cells via the MPR (Griffiths and Isaaz, 1993). Like many of the lysosomal hydrolases, some of the newly synthesized granzyme B is secreted constitutively and can then be taken up by MPR on the cell surface and targeted to the lysosomes by endocy-

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*Abbreviations used in this paper: CTL, cytotoxic T lymphocytes; MPR, mannose-6-phosphate receptor; NK, natural killer.

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tosis. Interestingly, CTL derived from patients with I-cell disease which lack the phosphotransferase required to add the M6P kill targets as efficiently as wild-type CTL, again demonstrating that MPR uptake is not required.

But perhaps the greatest hole in the model invoking endocytic uptake and release has been the lack of demonstration of holes in the endosomes. In general, the limiting membranes of endosomes are vital for keeping lumenal and cytosolic proteins separate, and this divide is sacrosanct. In dendritic cells of the immune system, the presentation of antigens taken up by MHC class II on the cell surface and presented by MHC class I—a process termed "cross-priming"—seems likely to involve a step involving endosome-to-cytosol transport (Watts, 1999). It is possible to see horse radish peroxidase taken up via macropinocytosis released into the cytosol of dendritic cells (Norbury et al., 1995). However, cross-priming is highly restricted to dendritic cells and macrophages, and the release of endocytosed proteins into the cytosol of other cell types has not been observed (Rodriguez et al., 1999). Several viruses and bacteria encode proteins that are able to disrupt endosomal membranes and deliver these pathogens to the cytosol after endocytic uptake. Adenovirus encodes specialized proteins for disrupting endosomal membranes, as it can indeed deliver granzyme B from an endosome to the cytosol when supplied exogenously with granzyme B (Froelich et al., 1998). A number of bacterial toxins also seem to form pores in endosomal membranes, triggered by the acidic environment (Schiavo and van der Goot, 2001). Could perforin be forming a similar pore, polymerising in the endosome? Several lines of evidence rule this out. Perforin is stored in the CTL and NK cell lysosomes in an active conformation. Its activity is highly sensitive to pH, however, and drops sharply when the pH drops below pH 7 (Bashford et al., 1988, Kuto et al., 1989), explaining how perforin can be stored in its active form in the acidic lysosomes. However, this then makes it very difficult to argue that perforin acts in an acidic compartment in target cells, especially given that CTL can themselves be targets (Kupfer et al., 1986). One suggestion has been that perforin pores formed at the membrane might be endocytosed as an attempt by the target cell to repair the damage. Although target cells have been shown to be capable of recovering from CTL attack, the method of membrane repair is not clear and the studies of Andrews and colleagues suggest that membrane repair in many cell types occurs by fusion of lysosomes with the damaged membrane (Reddy et al., 2001), rather than endocytosis.

There still remains the argument about whether the perforin pore is big enough for granzymes to pass through. The same study that describes the small pores formed at low concentrations also describes the formation of pores large enough to transport granzyme B when high concentrations of perforin are used (Browne et al., 1999). Given the small cleft into which perforin is secreted at the immunological synapse during cell mediated lysis (Stinchcombe et al., 2001), it is entirely possible that the local concentrations of perforin at this point are indeed very high. One of the problems with studying precisely how these proteins are delivered to the target cell remains the impressive potency of this pathway. Studies on live cell killing demonstrate that very few granules need be secreted in order to destroy a target (Lyubchenko et al., 2001; Stinchcombe et al., 2001), making the task of the cell biologist wishing to follow the pathway of these proteins truly challenging.

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