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Autophagy during common bacterial and Viral infections of children

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Introduction

Autophagy is a well recognized survival mechanism by the cell. The central role of autophagy is to remove unwanted or excessive proteins from the cell and thereby maintain homeostasis within the cell. This process is also called macroautophagy. The organelles which sequester the excess proteins are called autophagosomes. Autophagosomes can be identified rather easily by transmission electron microscopy because their outer walls include a characteristic double membrane. Eventually the double-membraned autophagosome fuses with the single-membraned lysosome to form a single-membraned autolysosome.

In addition to macroautophagy, two other forms of autophagy are mentioned in the literature. These include microautophagy and chaperone-mediated autophagy. Since macroautophagy is the form of autophagy usually associated with infectious diseases, the other forms of autophagy will not be discussed in this article. Further information is available in recent reviews. In this primer on autophagy, I have selected articles that describe autophagy during infections common to children. Autophagy is now being recognized not only as a cellular survival mechanism but also as an increasingly important component of both the innate and adaptive immune responses to bacterial and viral pathogens common to children. Viruses which elicit autophagic responses are being used in clinical cancer therapeutic trials. Furthermore, defective autophagy may explain, at least in part, the pathogenesis of Crohn's disease.

Autophagosomes and cellular survival

Autophagy was first recognized as a survival strategy for cells during starvation. One of the most cited reports is entitled "The role of autophagy during the early neonatal starvation period" (1). In this study, the authors investigated the appearance of autophagosomes in embryonic and neonatal mice, especially as a response to starvation. In their protocol, the authors analyzed the organs of mice at multiple stages, including the 18-day embryo and the neonate at 0.5 hr, 3 hr, 6 hr, 24 hr, and 60 hr after birth. The authors observed what they called "massive autophagy" in the heart musculature within 3 to 6 hr after natural birth. The number of autophagosomes diminished to basal levels by 60 hr after birth. The authors postulated that autophagy was an immediate response to a severe period of starvation secondary to an abrupt interruption in the transplacental nutrient supply. They further postulated that neonates used amino acids produced by autophagy for energy homeostasis.

Autophagy itself occurs in a stepwise fashion(2,3). The first step includes vesicle formation and requires activation of the class III phosphatidyl-inositol 3-kinase. The kinase is associated with a protein complex containing an essential autophagy protein called Beclin. The origin of the vesicle may be the rough endoplasmic reticulum. The second step includes vesicle elongation, which is facilitated by a cytosolic microtubule-associated protein called light chain 3 (LC3). In turn, phosphatidyl-ethanolamine (PE) is added to LC3 to form a lipidated protein called LC3-PE or more commonly LC3-II. The LC3-II protein is incorporated into the nascent double-walled autophagosome, where it is readily detected by specific antibody probes. The autophagosome has a very distinctive appearance after immunostaining for LC3-II protein. When examined by fluorescence microscopy, the organelles have a punctate appearance within the cytoplasm and therefore are often referred to as puncta (Figure 1). Eventually, the autophagosome fuses with a lysosome. In the final maturation step, the inner membrane of the nascent autolysosome is degraded along with its distinctive LC3-II protein. Thus the puncta are no longer detectable by antibody probes.

Autophagy and bacterial infection

Macroautophagy can be a mechanism by which to engulf intracellular pathogens for degradation as part of the process of innate immunity. For example, *Streptococcus pyogenes* is engulfed by autophagosomes in some cells, after which the bacterial load is reduced by lysosomal degradation (4). Likewise, autophagy participates in the eventual lysosomal mediated destruction of *Mycobacterium tuberculosis* (5). More recent reports have described mechanisms by which the previously mentioned LC3 protein is involved in autophagy of bacteria. When bacteria are present in the cytosol, they become coated with polyubiquitin complexes. In turn, the ubiquitinated bacteria are recognized by other adapter proteins, which form a bridge with the LC3 protein. Once attached to LC3, the bacteria are escorted into the autophagosome/lysosome pathway, where they are destroyed.

Autophagy, innate immunity, and Crohn's disease

Knowledge about autophagy is rapidly becoming relevant to clinical pediatrics. In a recent study linking autophagy and innate immunity, investigators documented a previously unknown pathway by which cytosolic receptors called Nod1 and Nod2 recruit an autophagy response after bacterial invasion of a cell(6). Again, the authors used an LC3-II detection assay to measure autophagy. By counting LC3-II-positive puncta they showed that the Nod protein was involved in engulfing *Shigella* into autophagosomes. The authors went on to discover that the Nod protein interacts with a second protein called ATG16L1 at the cell membrane and that this interaction was critical for engulfment of bacteria by autophagy. Of great interest, a single nucleotide polymorphism in the ATG16L1 gene is an allele linked to susceptibility to Crohn's disease. When the authors repeated their bacteria-induced autophagy assays in human cells from Crohn's patients with this mutation, they found reduced autophagy after stimulation with bacteria. In a second series of experiments, the authors repeated these assays in a mouse model known to be deficient in Nod function. Again, autophagy of bacteria was reduced. These studies provide a link between Crohn's disease and a long suspected propensity for persistent enteric bacterial infection and that link is deficient autophagy(7).

Autophagy and adaptive cellular immunity

Autophagy may also be involved in adaptive immunity(8). Adaptive immunity to viruses involves both an antibody response and a cellular response. In turn, the cellular response can be mediated by either CD8+ or CD4+ lymphocytes. The cellular responses require that the viral antigen be presented by an MHC molecule. As a generalization, MHC class I molecules deliver viral peptides from the cytosol to the cell surface, where the complex is presented to a CD8+ lymphocyte. MHC class II molecules deliver peptides originating in cytoplasmic

vesicles to the cell surface, where the complex is recognized by CD4+ lymphocytes. For example, viral proteins engulfed by an autophagosome are transported into an autolysosome, where smaller peptides are generated. Subsequently, the smaller peptides are carried within the antigen presenting groove of the MHC class II molecule to the cell surface to engage CD4 + lymphocytes(3). The co-discoverers of the MHC restriction of antigen presentation to T lymphocytes, Rolf Zinkernagel from Switzerland and Peter Doherty from Australia, were awarded the Nobel Prize in Medicine in 1996(9).

Autophagy and viral infection

Autophagy has been extensively investigated in animal models of herpes simplex virus (HSV) infection as well as in human clinical cancer trials. Of note, the research centers around an intriguing HSV-1 gene called ICP34.5(10). This gene is often called the viral neurovirulence gene because its removal dramatically decreases the ability of HSV-1 to replicate in the mouse brain. HSV-1 mutant viruses lacking ICP34.5 have been used in experimental human cancer therapeutic studies, in which brain tumors have been inoculated(11). The goal is to destroy brain tumors by viral lysis while preserving normal brain tissue. These trials with engineered HSV vectors are continuing(12).

Of great interest, the neurovirulence of the HSV ICP34.5 gene is directly related to autophagy (13). Namely, ICP34.5 inhibits autophagy by binding to the Beclin protein (see step 1 above). A mutant HSV-1 containing ICP34.5 but lacking the Beclin binding site is less neurovirulent. In other words, replication of the mutant virus is inhibited by autophagy. Thus, autophagy appears to suppress the neurovirulence of HSV-1 infection in the normal brain of an animal model. In addition, autophagy is a mechanism by which the HSV-infected cell can present viral peptides for MHC II restricted killing by CD4 lymphocytes(14). In other words, autophagy is facilitating the adaptive immune response to limit HSV spread.

With regard to certain RNA viruses, such as hepatitis C virus, autophagy facilitates a remarkably different process, namely, autophagy is required for viral replication(15, 16).Although the precise mechanism is not yet defined, autophagy proteins may provide a supportive framework for initial translation of hepatitis C RNA at the earliest phase of infection. In short, this hepatitis virus has taken over a cellular process usually reserved for prolonging the life of an infected cell and instead converted the process into a mechanism for assembly of the agent infecting the cell.

Autophagy during varicella infection

Varicella-zoster virus (VZV) is a human herpesvirus, but the VZV genome lacks a gene similar to HSV ICP 34.5. Autophagy has been documented during VZV infection of cultured cells (17). To determine whether autophagy was a component of the pathogenesis of infection with community acquired chickenpox, we examined cells removed from the vesicular rash of children. When the immunostained vesicle cells were visualized by confocal microscopy, we were surprised at the ease with which autophagosomes were detected in the samples (Figure 1). Numerous autophagosomes were visualized within individual cells throughout the skin vesicles.

The meaning of the abundant autophagosome formation cannot be quickly determined. However, we have shown that normal skin tissues do not harbor easily detectable autophagosomes. The skin vesicle is the final site of VZV replication, after a viremia during which the virus exits the capillaries and enters the epidermis (18). The fact that autophagosomes were detected early in cells at the base of the skin vesicle confirms that the data in cultured cells accurately predicted the microenvironment in human tissues after varicella infection. Therefore, autophagosomes likely represent a mechanism for prolongation of cellular survival

following varicella and zoster infection, in other words, a form of the innate immune response. Varicella is among the first diseases in which autophagosome formation has been directly confirmed in samples from an infected human. In one sense, the vesicle has become a window by which to directly observe autophagy in a human.

Conclusion

Several investigators have proposed that autophagy is an ancient evolutionary mechanism for preservation of the host from attack by microbial pathogens(8). Varicella infections certainly would fit this model, since the primordial varicella virus emerged at least 60 million years ago (19). Autophagy also may play a role in the immune response to live attenuated viral vaccines, such as measles and varicella vaccines. What is of further interest to all pediatric infectious disease specialists is that a disease associated with defective autophagy has already been identified and that disease is a variant of Crohn's disease(20). At present, there is no single diagnostic test by which to easily measure autophagy. Instead, individual components of the stepwise autophagy pathway will require analysis. Nevertheless, in the coming years, other conditions associated with an impaired response to pathogens undoubtedly will be associated with defective autophagy mechanisms.

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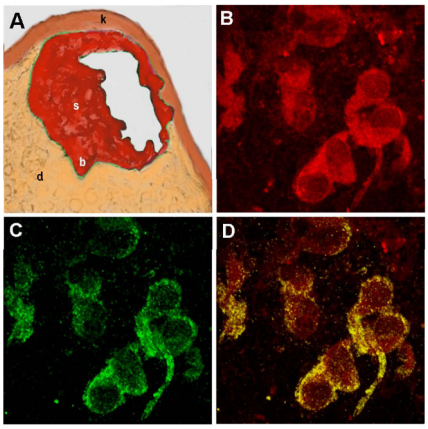

Figure 1.

Autophagosomes in cells removed from a varicella vesicle. Panel A represents a diagram of a vesicle, where d=dermis, b=basal layer, s=spinous layer and k=keratin layer. The red staining of the spinous layer represents epidermal cells that are infected within a varicella vesicle. Varicella infected cells were collected from a child with chickenpox, by pressing a glass slide onto the floor of a vesicle which had been unroofed. The glass slide contained about 10 cells. For further analysis, vesicular cells were fixed and then stained with anti-VZV mouse monoclonal antibody (Panel B, red color) and anti-LC3 rabbit antibody (Panel C, green color), before viewing with a confocal fluorescent microscope at 630×. Panel D (yellow color) is an image created by merging Panels B and C. The imaging in Panel C documents that vesicular cells contain numerous individual punctate autophagosomes (green dots). The imaging in Panel D documents that autophagy is occurring in the infected vesicular cells.