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Commentary

Eaten Alive

Autophagy and Neuronal Cell Death after Hypoxia-Ischemia

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Hypoxic-ischemic injury to the brain is a major cause of morbidity and mortality in infants and adults. Despite advances in obstetric care, the incidence of cerebral palsy from perinatal hypoxia-ischemia remains at >2 per 1000 births,[1] and arterial ischemic stroke occurs in ~1 of 4000 term infants.[2] In the United States, stroke is the third leading cause of death in adults (Centers for Disease Control and Prevention National Center for Health Statistics), and hypoxic-ischemic brain injury can be prominent in survivors of cardiac arrest, the leading cause of death. As with any pathological insult, multiple pathways of injury and adaptation are activated after hypoxic-ischemic brain injury. Delineating positive and negative contributions of these pathways to neuronal cell loss is critical for development of neuroprotective therapies but can be exceedingly difficult to detangle. Dying neurons in the ischemic brain exhibit a range of morphologies ascribed to apoptotic, autophagic, and oncotic (necrotic) cell death pathways,[3] although the role of autophagy as a mediator of cell death has been controversial.[4,5] In this issue of *The American Journal of Pathology*, Koike and colleagues[6] present the first *in vivo* evidence of neuronal cell death requiring autophagy in the mammalian brain.

The Janus-Faced Role of Autophagy in the Brain

Autophagy is one of the most rapidly growing fields in biomedical research, undergoing an explosion of interest reminiscent of that exhibited for apoptosis in the 1990's.[7] Macroautophagy was traditionally thought of as a conserved, relatively nonselective response to nutrient stresses, involving sequestration of portions of the cytoplasm into membrane-bound vacuoles targeted for lysosomal degradation. More recently, it has become apparent that levels of autophagy are exquisitely regulated and that autophagy-related processes play central roles in development, aging, and the pathogenesis of cancer, neurodegeneration, and infectious diseases.[8–13]

The role of autophagy as a mediator of cell death has become generally accepted in some fields,[3,14] but alternative interpretations have been proposed.[4] Earlier studies used phosphoinositide 3-kinase inhibitors to inhibit and thus implicate stress-induced autophagy in a prodeath role.[15,16] Other studies used rapamycin to demonstrate an important beneficial role for autophagy in aggregate clearance in neurodegenerative conditions.[17] However, both classes of drugs may have diverse effects on cells that extend beyond autophagy regulation to include modulation of survival/death kinases by 3-methyladenine[15] and regulation of protein synthesis and differentiation through mTOR.[18] Thus, the issue of whether autophagy observed in dying cells reflects a death mechanism, failed adaptation, or epiphenomenon requires additional complementary approaches to establish causality.

The identification of autophagy (*Atg*) genes involved in specific ubiquitin-like conjugation reactions that are essential for extension of autophagic membranes has revolutionized the field,[7,19] allowing for development of specific markers for autophagic vacuole formation and maturation,[20] and providing molecular genetic tools for knocking out or knocking down essential *Atg* gene products. In brief, Atg7 acts as an E1-activating enzyme to produce high-energy thioester bonds involving two ubiquitin-fold proteins LC3/Atg8 and Atg12, which are transferred to their E2 enzymes Atg3 and Atg10, respectively. These E2-conjugating enzymes act to transfer LC3/Atg8 and Atg12 to phosphatidylethanolamine and Atg5, their respective targets at the growing autophagic membrane. Although complete knockout of Atg5 is required to abolish autophagy,[21] a reduction in the expression level of

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Atg proteins is sufficient to blunt robust autophagic vacuole induction observed during stress and injury. These knockdown studies have confirmed that certain forms of cell death require sufficient expression of Atg proteins to support stress-induced increases in autophagy, implicating autophagy in an active death-promoting role.[22–25]

On the other hand, knockout mouse studies targeting Atg5 or Atg7 elegantly demonstrate that neurons require at least basal levels of autophagy for maintenance of health and function *in vivo*.[26,27] The chronic absence of autophagy results in build up of ubiquitinated protein aggregates in neuronal populations, accompanied by neurodegeneration and death of the mice within the first 2 months of life. Interestingly, neuronal populations most heavily affected by inclusions are not necessarily the most susceptible to degeneration, suggesting that inclusions may represent some degree of success in adapting to the underlying stress. Pediatric neurodegeneration is also seen with lysosomal storage diseases, although the primary deficit here is not induction of autophagy, but inadequate lysosomal clearance of sequestered material. Regardless of mechanism, it is clear that both initiation and successful completion of autophagic degradation are essential for basal neuronal health.

In contrast to the beneficial roles of physiological autophagy, the first study using *Atg* gene knockdowns to show involvement of pathological autophagy in neuronal cell death was published a year ago.[25] Just as pharmacological inhibitors may influence pathways beyond autophagy, the possibility that a given Atg protein may regulate processes other than autophagy needs to be considered. In neuronal cells, RNAi knockdown of LC3, Atg7, or Atg5 conferred protection from cell death[25] and neurite degeneration in toxin and genetic models of Parkinson's disease,[28] and RNAi targeting Atg5 and beclin1 protected 661W cells from hydrogen peroxide injury.[29] As it is unlikely that multiple Atg proteins would have the same hypothetical nonautophagy function, these cell culture studies indicate an active role for autophagy in neurodegeneration and some forms of neuronal cell death.

The current study in this issue of the *AJP* demonstrates that autophagy is robustly increased during neonatal and adult brain hypoxia-ischemia *in vivo*.[6] Although caspase 3 is cleaved in ~35% of hippocampal pyramidal neurons during injury, neither caspase 3-deficient nor caspase-activated DNase (CAD)-deficient animals show any degree of protection, indicating the existence of additional cell death mechanisms. In these apoptosis-deficient animals, alternative apoptotic mediators such as caspase 7 and an unknown DNase appear to be induced, although the magnitude of the DNA laddering observed is slight compared to that exhibited by wild-type animals, suggesting that these compensations do not account for the degree of hippocampal loss exhibited in the injured hemisphere of apoptosis-deficient animals. Using conditional knockout mice deficient in brain Atg7, which show no signs of neurological abnormalities or inclusions until 3 weeks of age, Koike and colleagues[6] further show that P7 mice lacking Atg7 in neurons exhibit dramatic protection from neonatal hypoxia-ischemia up to 7 days after hypoxic-ischemic injury. These intriguing results represent the first *in vivo* evidence that autophagy actively contributes to brain injury and neuronal cell death.

Relationship between Autophagy and Apoptosis

The mythical god Janus not only is depicted with two faces, analogous to the prosurvival and prodeath roles of autophagy, but also functions as the god of gates, beginnings and endings. The study by Koike and colleagues[6] elegantly demonstrates a crucial role for the autophagy E1 enzyme Atg7 as the gatekeeker of hypoxic-ischemic neuron death mediated by both apoptotic and nonapoptotic mechanisms. There are multiple potential points of interaction between apoptosis and autophagy, and these have been the subject of an excellent recent review.[14]

Although type II or autophagic programmed cell death was originally described as a distinct morphological form of developmental cell death lacking apoptotic features, in many systems there are features of both autophagy and apoptosis,[30] with autophagy preceding apoptotic cell death. Although this is sometimes indicative of compensatory adaptation,[31] molecular genetic evidence from a variety of systems indicate that autophagy-dependent processes are sometimes necessary to trigger apoptotic cell death.[6,32]

Teleologically speaking, autophagy may function similarly to other well known stress response pathways to mediate either successful repair or cell suicide, both of which would be adaptive in a multicellular organism. Imbalances between the extent of autophagy induction and the ability of the cell to complete autophagic degradation and recycling/regeneration of cellular components would create a state of autophagic stress and eventually autophagic cell death.[11] Neuronal cells may be particularly susceptible to developing autophagic stress during aging, because of increased demand for autophagic clearance of damaged constituents, extensive dependence on anterograde and retrograde trafficking over long distances, and decreased degradative and biosynthetic reserves.[11] Autophagic stress attributable to excessive injury-induced mitochondrial degradation is associated with caspase-independent cell death pathways,[25,33,34] Autophagic stress attributable to impaired lysosomal function has also been shown to precede apoptosis *in vivo*.[35]

Dysregulation of calcium, as observed after acute or chronic autophagic stress,[36,37] may play a key role in switching between autophagy and apoptosis because calpain-cleaved Atg5 mediates apoptosis.[38] Although autophagy sometimes harnesses the apoptotic machinery for cell suicide, it is also clear that autophagic cell death can be executed independently of an intact apoptotic machinery.[22,23] Indeed, it has been proposed that autophagic cell death is important mainly in apoptosis-deficient cells.[4] Interestingly, mature neuronal cells tend to be relatively apoptosis-resistant, related to high expression of neuronal inhibitors of apoptosis.[39] For example, although numerous apoptotic profiles are observed in postmortem brain tissues after hypoxic-ischemic injury in

neonates, they are not readily observed in similarly injured adult brains (C.T.C., personal observation). Young rats injured by 6-hydroxydopamine show predominantly apoptotic morphology, whereas nonapoptotic cell death becomes prominent in 42-day-old rats subjected to the same insult.[40] Notably, Koike and colleagues[6] observed a greater degree of autophagy induction in adult mice subjected to hypoxic-ischemic injury, suggesting that autophagy-driven cell death may be even more significant in mature neurons.

Mitochondrial Mechanisms and Future Questions

Because the Atg7-deficient animals showed reductions in both caspase 3-dependent and -independent pathways of neuron death, continued investigation into molecular connections between autophagy and apoptosis is indicated. Mitochondria may very well play a key pivotal role, poised at the interface between life support and cell suicide. The proportion of the cellular mitochondrial complement that undergoes depolarization has been proposed to determine whether a stressed cell undergoes autophagy, apoptosis, or necrosis.[41] Mitochondria also play key roles in calcium buffering, phospholipid metabolism, and cell signaling, each of which can be important for regulation of autophagy and cell death.

Although the concept of autophagic stress assumes that dysregulation of a fundamentally cytoprotective response leads to detrimental consequences when autophagic demand cannot be balanced by cellular reserves,[11] it is also possible that the differences between physiological and pathological autophagy are mediated by fundamentally different pathways of induction. For example, a short mitochondrial form of $p19^{ARF}$ induces autophagy and caspase-independent cell death,[42] and autophagy appears to be induced by this mechanism to fulfill a death-promoting role from the beginning. Autophagy and mitochondrial degradation can proceed independently of beclin 1 in toxin models of Parkinson's disease,[25] suggesting fundamentally different upstream mechanisms and escape from potential beclin 1-Bcl-2-related regulation.

Mitochondrial oxidative stress is robustly induced in several brain pathologies associated with autophagy, including cerebral hypoxia-ischemia injury,[6,43] traumatic brain injury,[44] and Parkinsonian stresses.[25] Direct stimulation of mitochondrial oxidative stress could serve to bypass the requirement for beclin 1, given that mitochondrial reactive oxygen species have been shown to function downstream of beclin 1-phosphoinositide-3 kinase signaling.[45] Moreover, redox activation of mitochondrial pools of extracellular signal-regulated protein kinase contributes to toxicity,[46] and MAPK/ERK kinase inhibitors confer protection from autophagic cell death[25] and from hypoxic-ischemic, traumatic, and neurotoxic injuries.[47]

Another area for further study concerns the role of autophagy in white matter damage. Axonal tracts are susceptible to hypoxic-ischemic and traumatic injuries. Although not examined in the study by Koike and colleagues,[6] dysregulated autophagy is implicated in axonal degeneration.[48] Although neurite retraction may represent a short-term solution to promote survival of the neuron cell body, irreversible neurodegeneration is likely to result if excessive degradation cannot be balanced biosynthetically to re-establish function.

To summarize, a growing number of studies have used neuronal cells with reduced capacity for autophagy induction, and now mice deficient in brain Atg proteins, to show that autophagy can contribute to neurodegeneration and function as a gatekeeping mediator of apoptotic and nonapoptotic death pathways. Additional studies to address autophagy regulation under specific contexts, including oxidative neuronal injury, dysregulated calcium homeostasis, and neuron-specific compartments are required to facilitate potential future therapies to reduce neuropathological outcomes. If autophagic neurodegeneration and cell death result from imbalances between initiation and completion of autophagic recycling (autophagic stress), threshold-based therapies that strive to restore balance to the system without completely abolishing basal autophagy could be sought. If autophagic cell death results primarily from different mechanisms of activation, or simultaneous activation of adaptor systems that convert autophagy into a prodeath mechanism, threshold regulators may not be as effective as targeting the factors that determine commitment to neuron cell death.

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