In a stroke lesion, there is a core of necrotic cell death surrounded by a zone of tissue at risk, termed the penumbra. In the penumbra, there is usually less severe tissue damage. Programmed cell death (PCD) pathways have been documented to be present in the penumbra. While many studies have used the term apoptosis as equivalent to PCD, the absence of complete biochemical and morphological characteristics of apoptosis, coupled with the ineffectiveness of caspase inhibitors, indicates that there are other important cell death pathways activated during stroke. A few caspase-independent cell death pathways have been identified and there are likely more to be described.

**Parthanatos**

Stroke injury leads to the activation of a cell death program, dependent on poly(ADP-ribose) polymerase-1 (PARP1) activation and culminating in apoptosis inducing factor (AIF) mediated cell death. Poly(ADP-ribose) (PAR) polymer is the death signal in this pathway and Thanatos is the Greek personification of death and mortality, hence, the name parthanatos. In this form of cell death that occurs in many different organ systems during ischemia-reperfusion injury, PAR polymer translocates from the nucleus to the cytoplasm and mitochondria. This leads to release of AIF from the mitochondria, an effect that is inhibited by poly(ADP-ribose) glycohydrolase (PARG), an enzyme that degrades PAR polymer. After cerebral focal ischemia reperfusion injury, PARP1 is expressed in the cytoplasm and mitochondria. This participation with calpain to release AIF has been documented to be present in the penumbra. Parthanatos is caspase-independent and is biochemically and morphologically distinct from apoptosis. Regulation of PAR signaling may be a useful therapeutic intervention to reduce stroke-mediated damage.

**Apoptosis-Inducing Factor**

Additional investigations this year have explored the role of AIF during stroke. In an elegant piece of work, investigators studied the actions of the calcium-dependent protease, calpain I, on the release of AIF. The role of calpains in mediating neuronal injury has been controversial, which may be, in part, due to nonspecific reagents and the variability of the experimental models. In this study, calpain I cleaves an N-terminal fragment from AIF, leading to its liberation from HSP10 in the mitochondria, which may then render AIF available for release from the mitochondria. Calpastatin, an inhibitor of calpain activity, was shown to provide neuroprotection, as was knockdown of AIF during cerebral transient ischemia, further supporting a role for calpain in the release of AIF and subsequent neuronal injury after stroke. The question that remains is whether calpain-mediated release of AIF is a parallel pathway independent of PAR or whether PAR participates with calpain to release AIF. There is suggestive data, in mouse embryonic fibroblasts, treated with DNA damaging alkylating agents, that PARP1 and AIF-dependent cell death may involve p53, Bax and calpains, but not Bak, caspases or PARP2. The study used a series of genetically modified cells and time course analysis to define a molecular signaling sequence of PARP1, calpain activation, Bax and AIF translocation in cell death due to DNA damage. These data are provocative, but the study was conducted in engineered mitotic cells. Further work in experimental neuronal systems is required.

Another player in the field of AIF release in stroke is cyclophilin A, a protein that is highly expressed in the cytoplasm and nucleus of neurons. Cyclophilin A has been implicated in various models of cell death and, in the Rice-Vannucci model of experimental ischemic stroke performed in 9 day old mice, cyclophilin A deficiency is neuroprotective and AIF does not translocate to the nucleus. These data suggest that cyclophilin A may physically interact with AIF after ischemic injury, but not under normal physiological conditions.

**Other Caspase-Independent Cell Death**

BNIP3 belongs to a growing family of mitochondrial death signals. It is not detectable in neurons under physiological conditions, but BNIP3 protein expression is induced by hypoxia. After 90 minutes of middle cerebral artery occlusion in rats, BNIP3 is upregulated 48 hours after focal brain ischemia reperfusion in ischemic tissue, but not in nonischemic tissue. Knockdown of BNIP3 expression in primary cortical cultures delays neuronal cell death after hypoxia;
however, a role for BNIP3 in oxygen-glucose deprivation, more commonly used in vitro as a culture model of middle cerebral artery occlusion, was not explored. It is possible that BNIP3 might mediate EndoG translocation from the mitochondria. However, there is concern regarding the specificity of the EndoG antibodies, which are not well characterized. It is difficult to understand how a matrix protein, such as EndoG, could have a specific release mechanism involving BNIP3 or whether translocation occurs after rupture of mitochondrial membranes.8

Energy
PARP1-dependent cell death is believed to result, at least partially, from NAD+ depletion. Both genotoxic stress and nutrient deprivation activate PARP1 and result in NAD+ depletion. However, mitochondrial NAD+ levels are not reduced following those stressors.9 Nampt, an enzyme involved in NAD+ biogenesis, protects cells from death by genotoxic stress, an effect that is mediated by regulating mitochondrial NAD+ levels. In addition, Nampt inhibited AIF translocation to the nucleus following genotoxic stress.9 These studies emphasize the importance of investigating the subcellular levels of NAD+ versus total levels in experimental models of stroke. It will be interesting to determine if Nampt plays a role in parthanatos after ischemia reperfusion injury in the brain and whether it has a neuroprotective action in stroke.

Endoplasmic Reticulum Stress and Autophagy
Endoplasmic reticulum (ER) stress induced neuronal cell death plays an important role in stroke pathophysiology.10 BBF2H7 is a novel ER stress transducer with interesting potential as a candidate for neuroprotection. BBF2H7 appears to be expressed in the peri-infarction zone after permanent focal brain ischemia, but not in the nonischemic neurons.11 In neuroblastoma cells, overexpression of BBF2H7 suppresses and conversely knockdown of BBF2H7 promotes ER stress-induced cell death. Taken together, these data suggest a role for BBF2H7 and ER stress in ischemic injury; however, changes in protein expression do not necessarily define a functional role in ischemic injury and may instead be bystander events triggered by severe stress. Additionally, the cell death assays were performed in tumor cell lines and have not yet been replicated in neurons or the brain. Thus, while intriguing, any interpretation must be cautiously made.

The role of autophagy in neuronal cell death is an emerging area of cell death research. Autophagy can be triggered by ER stress and oxidative stress, poising it to be a key player in ischemic cell death. Autophagy is a lysosomal pathway important for the turnover of organelles and proteins that can be induced in neurons.12 Autophagy has only recently been recognized as an important process that may be a key regulator of neurodegenerative processes. Recent studies implicate a role for autophagy in the prenumbra after experimental stroke. Beclin 1 and microtubule-associated protein 1 light chain 3, which are known to promote autophagy are induced in the penumbra of rats after 90 minutes middle cerebral artery occlusion.13 Both genetic disruption and chemical inhibition of autophagy inhibit necrosis induced by 40 minutes of hypoxia in C elegans.14 Taken together with the observation of autophagic morphology following the modified Levine/Vannucci procedure in adult mice,15 these early studies suggest that autophagic cell death may be a critically important form of cell death in the nervous system that has, until recently, been overlooked.

Summary
“Death Hath so Many Doors to Let Out Life.”
—John Fletcher & Francis Beaumont
While life and death are binary events, there are many different pathways for cells to die. We are just coming to the realization that there is more to cell death than apoptosis and necrosis, which implies that our understanding of cell death in the brain is severely restricted. Until we understand the primary pathways that result in cell death after ischemia, we will not be able to define new and effective therapies. Several advances this year are predictive of future studies. When AIF was first identified, it was thought that AIF mediated a very specific and restricted form of caspase-independent cell death. We are now finding that AIF belongs to the growing family of mitochondrial death effectors and that many different signaling events can lead to AIF release. AIF can be released as a commitment point to cell death, but it can also be released later in the cell death cycle. Nuclear AIF is a promiscuous marker for cell death; therefore, it is important to determine whether AIF translocation is a primary causal event in the cell death process by blocking AIF translocation. Additional work is necessary to fully understand the role of AIF in neuronal cell death. There is a growing number of caspase-independent cell death effectors being identified in the brain, but a lot of work is required in order to understand which are primary causal events, toward which therapeutics might be targeted, and which are secondary and perhaps less important events. Along these lines, PAR signaling appears to be an important primary event that would be amenable for therapeutic development. Lastly, autophagy is likely to be a very fruitful frontier for understanding the response of the brain to stress and injury. Cell death due to autophagy may turn out to be one of the more important cell death signaling events in the brain and is a relatively untapped area of research.

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