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Review

Excitotoxicity, Apoptosis, and Ischemic Stroke

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Introduction

Most strokes are caused by acute interruption of the brain arterial blood supply, typically by a thrombus or embolus, leading to tissue ischemia; the remainder are caused by blood vessel rupture, leading to hemorrhage. Two main approaches have emerged for the acute treatment of ischemic stroke. The first targets the insult itself, trying to enhance blood flow by lysing an offending arterial thrombus within hours of symptom onset, or by reducing the tissue back-pressure induced hours to days later by edema formation. Thrombolysis via the acute delivery of tissue plasminogen activator is now in widespread use in the United States, having been approved for such use by the US Food and Drug Administration in 1996. The second major therapeutic approach, neuroprotection, aims to reduce the intrinsic vulnerability of brain tissue to ischemia, a strategy that might be employed in both ischemic and hemorrhagic strokes, since the latter invariably entails an ischemic component. The central nervous system is far more vulnerable to ischemic insults than most other organs, so it seems plausible that the cellular underpinnings of this heightened vulnerability could be identified and blocked.

Substantial evidence has accumulated implicating glutamate neurotoxicity ("excitotoxicity") and attendant calcium overload as a prominent contributor to the vulnerability of the central nervous system to ischemic insults (Rothman and Olney, 1986; Choi, 1988). Ischemia triggers the excessive release of glutamate from presynaptic nerve terminals and astrocytes into the extracellular space, with consequent overstimulation of glutamate receptors, especially N-methyl-D-aspartate (NMDA) receptors. This receptor overstimulation leads to excessive influx of Ca^{2+} (and Na^{+}) through glutamate receptor-gated ion channels, followed passively by movements of Cl^{-} and water. The resulting combination of intracellular volume and Ca^{2+} overload induces lethal metabolic derangements, internal organelle swelling, and plasma membrane failure (Choi, 1995), usually consistent with necrosis (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; see below).

More recently, the idea has emerged that programmed cell death culminating in apoptosis may also contribute importantly to ischemia-induced central neuronal death (Choi, 1996; MacManus and Linnik, 1997; McIntosh *et al.*, 1998; Dirnagl *et al.*, 1999). A key issue then is the relationship between excitotoxic necrosis and apoptosis in central neuronal death after ischemic insults.

Apoptosis vs. Necrosis after Brain Ischemia

Apoptosis is the end result of a genetically-regulated program that induces cells to die in an "altruistic" fashion, with minimal release of genetic material and other pro-inflammatory intracellular constituents (Johnson and Deckwerth, 1993; Hengartner and Horvitz, 1994). Unlike necrosis, such programmed cell death is an active molecular process that often requires active transcription and translation for initiation (Schwartz and Osborne, 1993). In normal physiologic settings such as development, apoptosis has a characteristic morphological appearance, featuring chromatin condensation and aggregation to the nuclear margin, cell and internal organelle shrinkage, and fragmentation of the nucleus and cytoplasm into membrane-bound vesicles (apoptotic bodies) (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Conversely, light microscopic and ultrastructural studies have demonstrated that many neurons dying following ischemia (van Lookeren Campagne and Gill, 1996; Martin *et al.*, 1998; Colbourne *et al.*, 1999) exhibit features of necrosis. The implication of excitotoxicity in ischemic and traumatic neuronal death over the past decade has further strengthened this notion-as noted above, excitotoxicity preferentially induces necrosis.

A suggestion that apoptosis might be triggered by brain ischemia was provided by the demonstration that protein

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synthesis inhibitors reduced delayed CA1 neuronal death following transient global ischemia in rodents (Goto *et al.*, 1990; Shigeno *et al.*, 1990) and infarct volume after focal ischemia in rats (Linnik *et al.*, 1993). Additional support was provided by the presence of internucleosomal DNS fragmentation following global or focal ischemia (MacManus *et al.*, 1993; Tominaga *et al.*, 1993). While protein synthesis inhibitors produce broad alterations in cellular metabolism, and at submaximal doses can even enhance expression of the anti-apoptotic protein, Bcl-2 (Furukawa *et al.*, 1997), subsequent studies have strengthened implication of the apoptotic cascade. Activation of caspase-3 has been demonstrated in cortical neurons several hours after a focal ischemic insult (Namura *et al.*, 1998). Increased Bcl-2 expression was shown in neurons that survive focal ischemia (Chen *et al.*, 1995), while expression of the pro-apoptotic protein, Bax, was increased selectively in vulnerable CA1 neurons following transient global ischemia (Krajewski *et al.*, 1995). Furthermore, intracerebroventricular infusion of the caspase inhibitor (*N*-benzyloxycarbonyl-Asp(OMe)-Glu (OMe)-Val-Asp(OMe)-fluoromethylketone or z-DEVD. FMK) decreased infarct size after transient focal ischemia (Hara *et al.*, 1997), reduced hippocampal CA1 cell death in transient global ischemia (Chen *et al.*, 1998), and decreased TUNEL staining while improving neurological outcome after fluid-percussion injury (Yakovlev *et al.*, 1997). Transgenic overexpression of Bcl-2 (Martinou *et al.*, 1994) or its delivery via herpes virus vector (Linnik et al., 1995), were both found to reduce infarct volume in mice subjected to focal ischemia; and the survival of hippocampal CA1 neurons after transient global ischemia was enhanced in transgenic mice overexpressing Bcl-2 (Kitagawa *et al.*, 1998). In experiments in our lab, mice lacking the *bax* gene exhibited reduced infarct volume after transient middle cerebral artery occlusion (F. Gottron FJ and Choi DW, unpublished data).

Data obtained *in vitro* have complemented the evidence presented above. Cultured murine cortical neurons, transiently deprived of oxygen and glucose, normally die by excitotoxic necrosis, but after blockade of excitotoxicity with NMDA and AMPA/kainate antagonist drugs (and lengthening of the deprivation period to overcome the protective effect of these drugs), underwent apoptosis sensitive to z-VAD.FMK (Gottron *et al.*, 1997). While the neuroprotective effect of caspase inhibitor drugs against ischemic insults *in vivo* may be partly attributed to inhibition of caspase-1 (formerly interleukin-1β converting enzyme or ICE) and attendant reduction in interleukin-1β-mediated inflammation (Rothwell *et al.*, 1997), the neuroprotective effect of z-VAD in cell culture is unlikely to be explained by this mechanism as it was not reversed by the addition of a high concentration of interleukin-1β to the bathing medium (Gottron *et al.*, 1997).

Two explanations may reconcile evidence implicating apoptosis with morphological studies implicating necrosis after hypoxic/ischemic insults: 1) overlap and ambiguity in the criteria used to distinguish apoptosis from necrosis in many studies; and 2) the parallel occurrence of apoptosis and necrosis following ischemic insults. While morphological criteria were originally used to distinguish apoptosis from necrosis, recent explorations have fueled a growing consensus that these criteria, or indeed any single set of features, fail to make this distinction in a satisfactory fashion. Internucleosomal DNA fragmentation measured by agarose gel electrophoresis or TUNEL staining, once thought to be a biochemical signature of apoptosis, has also been noted in cells undergoing necrosis (Charriaut-Marlangue and Ben-Ari, 1995; Gwag *et al.*, 1997). Proteolytic pathways activated during necrosis can lead to the activation of enzymes capable of producing internucleosomal DNA fragmentation indistinguishable from those seen in apoptosis (Dong *et al.*, 1997). Perhaps overlap in the criteria used to distinguish apoptosis from necrosis is predictable from the single plausible assumption that many real world insults are capable of triggering both death pathways. The fact that a neuron has begun to undergo programmed cell death cannot provide a guarantee that its plasma membrane will not fail, leading ultimately to necrosis. Thus one might expect to find cells exhibiting specific features of apoptosis, for example chromatin condensation, cell shrinkage, internucleosomal DNA fragmentation or even conceivably activation of caspase-3, and yet also mitochondrial and cell body swelling, and resistance to anti-apoptotic interventions. Conversely, partial failure of organelle and plasma membranes, associated with some features of necrosis-like morphology, may not prevent a cell from successfully executing programmed cell death.

Furthermore, besides the possibility that a single cell may simultaneously undergo early apoptosis and necrosis events before one or another ultimately produces death, there is the plausible possibility that a single insult may induce some cells to undergo apoptosis, while others undergo necrosis. Such differential fates could reflect variations in insult intensity (for example, greater ischemia in the center of the ischemic zone compared to the penumbra), or heterogeneity in intrinsic (cell type or maturity, see below) or extrinsic (e.g., trophic environment or synaptic input) cell properties.

Excitoxicity: Necrosis vs. Apoptosis

The idea that excitotoxicity typically leads to necrosis is consistent with many *in vivo* and *in vitro* studies (Olney *et al.*, 1979; Schwob *et al.*, 1980; Lothman and Collins, 1981; Nadler, 1981; Schwarcz *et al.*, 1983; Sperk *et al.*, 1983; Schwarcz *et al.*, 1984; Ben-Ari, 1985; Choi, 1987; Koh *et al.*, 1990; Ishimaru *et al.*, 1999). Even low levels of excitotoxicity evoked over many hours caused cultured cortical neurons to undergo necrosis, leading my colleagues and me to favor the view that excitotoxicity preferentially induces necrosis (Gwag *et al.*, 1997).

However, there is clear evidence that exposure to excitotoxins can induce neurons to undergo apoptosis under

some conditions, particularly when exposure is mild (due to low levels of agonists, or low levels of receptor expression). Maturity may also be a factor, as young neurons are more prone to undergo apoptosis than older neurons (McDonald *et al.*, 1997; Cheng *et al.*, 1998). When cultured central neurons were briefly exposed to low excitotoxin concentrations, the majority of cells died in a delayed fashion with morphological features of apoptosis, including chromatin condensation and apoptotic bodies (Ankarcrona *et al.*, 1995; Bonfoco *et al.*, 1995). More recently, several investigators have provided molecular evidence favoring the occurrence of excitotoxin-induced apoptosis: exposure of cortical neurons to mild excitotoxic insults has been linked to caspase-3 activation (Du *et al.*, 1997; Tenneti and Lipton, 2000), and caspase inhibition has been shown to attenuate excitotoxin-induced apoptosis (Du *et al.*, 1997; Tenneti *et al.*, 1998; Hirashima *et al.*, 1999; Tenneti and Lipton, 2000), but not excitotoxin-induced necrosis (Gottron *et al.*, 1997; Tenneti *et al.*, 1998). Apoptotic morphology has also been demonstrated *in vivo*, following excitotoxin injections into the amgydala or striatum (Pollard *et al.*, 1994; Ferrer *et al.*, 1995; van Lookeren Campagne *et al.*, 1995; Qin *et al.*, 1996), although *in vivo*, apoptosis may be induced indirectly (induced by excitotoxic destruction of trophic inputs and consequent trophic factor depletion), rather than directly.

Activation of glutamate receptors might promote neuronal apoptosis non-specifically, inducing sufficient levels of cellular injury to activate cellular sensors linked to the apoptosis cascade. In addition or alternatively, there are three specific mechanisms by which ischemic glutamate receptor overactivation may promote neuronal apoptosis: early mitochondrial production of reactive oxygen species (ROS) (Dugan *et al.*, 1995; Reynolds and Hastings, 1995), reduction of intracellular K^+ (Yu *et al.*, 1997), and enhancement of toxic Zn^{2+} influx (Koh *et al.*, 1996; Choi and Koh, 1998). While high levels of toxic Zn^{2+} induce fulminant neuronal necrosis, lower levels can induce apoptosis (Manev *et al.*, 1997; Kim *et al.*, 1999; Lobner *et al.*, 2000).

Levels of Intracellular Calcium

In the ischemic brain, the excitotoxic overactivation of NMDA and calcium-permeable AMPA receptors leads to massive calcium influx; membrane depolarization induces further Ca^{2+} influx through voltage-gated Ca^{2+} channels and reverse operation of the Na^+/Ca^{2+} exchanger (Choi, 1988). The occurrence of cellular Ca^{2+} overload, and the neuroprotective benefits of NMDA or AMPA antagonists have been well-documented in ischemia models (Rothman and Olney, 1986) (Meldrum *et al.*, 1987; McCulloch, 1992). In contrast to the strong linkage between glutamate receptor activation, elevated $[Ca^{2+}]_i$, and consequent necrosis, the relationship between $[Ca^{2+}]_i$ and propensity to undergo apoptosis is controversial. Some studies have emphasized the possibility that increasing $[Ca^{2+}]_i$ may trigger apoptosis (for review, see (McConkey and Orrenius, 1996; Nicotera and Orrenius, 1998)). On the other hand, there is strong evidence indicating that there can be an inverse relationship between $[Ca^{2+}]_i$ and propensity to undergo apoptosis, especially in neurons. Leukemia cells (Genot, 1994) and lymphoma cells (Lam *et al.*, 1993) stimulated to undergo apoptosis, as well as apoptosis-prone cardiomyocytes derived from patients in congestive heart failure (Siri *et al.*, 1991; Narula *et al.*, 1999) exhibit reduced $[Ca^{2+}]_i$. Immature neurons prone to undergo apoptosis (see above) have lower levels of $[Ca^{2+}]_i$ than mature neurons (Koike and Tanaka, 1991). Neuronal apoptosis induced by growth factor withdrawal or serum deprivation (Koike *et al.*, 1989; Distasi *et al.*, 1998; Soler *et al.*, 1998; Yano *et al.*, 1998), by staurosporine (unpublished observation, Babcock DJ, Gottron FJ, & Choi DW), or by diphenylhydantoin (Yan *et al.*, 1995) is associated with reduced $[Ca^{2+}]_i$. Lowering extracellular Ca^{2+} or reducing the activation of membrane Ca^{2+} channels will induce apoptosis in pancreatic β cells (Mizuno *et al.*, 1998), spontaneously hypertensive rat aorta *in vivo* (Sharifi and Schiffrin, 1998), SH-SY5Y neuroblastoma cells (McGinnis *et al.*, 1999), cultured astrocytes (Chiesa *et al.*, 1998), dorsal root ganglia neurons (Tong *et al.*, 1996), and central neurons (Koh and Cotman, 1992; Ikonomidou *et al.*, 1999; Takadera *et al.*, 1999). Pharmacological agents that raise $[Ca^{2+}]_i$ such as thapsigargin (Lampe *et al.*, 1995; Levick *et al.*, 1995), voltage-gated Ca^{2+} channel agonists (Gallo *et al.*, 1987; Koike *et al.*, 1989; Galli *et al.*, 1995), or glutamate receptor agonists (Balazs *et al.*, 1988; Yan *et al.*, 1994; Rivera *et al.*, 1998) attenuate several types of neuronal apoptosis. With respect to ischemic insults, normal to low levels of $[Ca^{2+}]_i$ were found in CA1 pyramidal neurons susceptible to delayed death (likely apoptosis) 3 days after transient global ischemia (Connor *et al.*, 1999), and raising $[Ca^{2+}]_i$ attenuated ischemic apoptosis in cultured cortical neurons (unpublished observation, Babcock DJ, Gottron FJ, & Choi DW).

Further study will be necessary to clarify what is apparently a complex relationship between $[Ca^{2+}]_i$ and apoptosis. Possibly, this relationship may be different in different cell types, or following different types of insults. However, alternatively substantial conceptual convergence will emerge when $[Ca^{2+}]_i$ is defined with greater temporal and spatial resolution in various cell deaths. The idea of a "Ca^{2+} setpoint" (Koike *et al.*, 1989; Lee *et al.*, 1999) is attractive: where propensity to undergo apoptosis is inversely related to $[Ca^{2+}]_i$ at some critical regulatory timepoint, and hence promoted by calcium starvation. Among many possible mechanisms, increasing $[Ca^{2+}]_i$ may enhance the phosphorylation and inactivation of the pro-apoptotic protein Bad (Yano *et al.*, 1998), and Ca^{2+}-activated calpain may inactivate caspase-9, thereby inhibiting cytochrome c-induced caspase-3 activation (Chua *et al.*, 2000).

The postulate of a regulatory Ca^{2+} setpoint does not exclude the possibility that an early rise in $[Ca^{2+}]_i$ (e.g., due to minimally-toxic glutamate receptor stimulation) might drive the injury originally responsible for apoptosis initiation, or the

possibility that a late rise in $[Ca^{2+}]_i$ might constitute a signaling event mediating a portion of the apoptosis cascade, such as activation of an endonuclease responsible for internucleosomal DNA cleavage. Furthermore, the idea of a single level of $[Ca^{2+}]_i$ is an oversimplification; localized Ca^{2+} concentrations in various sub-cellular sites such as the endoplasmic reticulum may be especially important in regulating apoptosis.

Therapeutic Implications

If excitotoxic necrosis and apoptosis occur concurrently after ischemia or trauma, a combination of agents inhibiting both death pathways may be more neuroprotective than blocking either pathway alone. Indeed, the above discussion raises the unsettling perspective that the beneficial effects of anti-calcium strategies, such as NMDA antagonists, may be counterbalanced by deleterious enhancement of pathological apoptosis due to calcium starvation (Lee *et al.*, 1999). Pohl *et al.* (1999) have demonstrated that NMDA antagonists worsen the severity of apoptosis in a rat trauma model. Two in vivo studies to date have provided evidence for neuroprotective synergy between anti-excitotoxic and anti-apoptotic treatments. Du *et al.* (1996) demonstrated that co-administration of the NMDA antagonist, dextrophan, with the protein synthesis inhibitor, cyclohexamide, produced greater than 80% reduction in infarct volume. More recently, Ma *et al.* (1998) observed evidence of neuroprotective synergy between an anti-excitotoxic (MK-801) and an anti-apoptotic agent (z-VAD.FMK or z-DEVD.FMK) in a rat model of transient focal ischemia.

There may turn out to be multiple ways to achieve inhibition of both excitotoxic necrosis and apoptosis. As suggested by the two studies cited above, strong pharmacological blockade of apoptosis pathways may permit NMDA antagonists or other calcium-lowering strategies to be employed with relative impunity. Alternatively, identifying a temporal sequence of brain injury mechanisms may permit distinct targeting of anti-excitotoxic and anti-apoptotic treatments. For example, an acute ischemic or traumatic insult might initially induce a wave of intense excitotoxicity associated with calcium overload and necrosis, followed later by lower levels of glutamate receptor activation, a critical time window where $[Ca^{2+}]_i$ levels are low, and apoptosis. If these phases can be identified, for example *via* historical predictive data coupled with imaging techniques, one might devise a regimen where short acting glutamate antagonists are administered immediately, followed later by anti-apoptotic treatments--perhaps including even Ca^{2+}-entry promoting agents (as contrary to conventional wisdom as that latter manipulation would be). Another therapeutic strategy aimed at limiting ischemic apoptosis might include measures directed at limiting intracellular K^+ loss, for example by blocking neuronal delayed rectifier (I_K) channels. Such a strategy, like a Ca^{2+}-entry promoting strategy, would also be paradoxical in the context of conventional wisdom, as it could be predicted to increase circuit excitation and excitotoxicity, but might be employed in a targeted fashion or in conjunction with anti-excitotoxic treatments. Lastly, it may be possible to limit zinc toxicity, for example by limiting zinc release from presynaptic terminals, or zinc entry via channels or transporters; alternatively, zinc buffering or extrusion by the plasma membrane transporter, ZnT-1, might be enhanced.

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