MOLECULAR PATHOPHYSIOLOGY OF STROKE

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Cerebral ischemia occurs when the amount of oxygen and other nutrients supplied by blood flow is insufficient to meet the metabolic demands of brain tissue. In ischemic stroke, the blood supply to the brain is disrupted by cerebrovascular disease. For decades, extensive research and clinical approaches to combat stroke have focused on the vascular aspects of cerebral ischemia. Therapeutic advances, including carotid endarterectomy, thrombolytic therapy, anticoagulation for cardiogenic stroke, antiplatelet agents, and the treatment of risk factors such as hypertension and hyperlipemia, have had significant effects on the morbidity and mortality of stroke.

The final event in cerebral ischemia is the death of neurons, resulting in irreversible loss of neurologic function. The advent of animal and tissue culture models of ischemia has led to many new insights into the mechanisms by which ischemic neurons die. If ischemia is complete and prolonged, neuronal death is inevitable. However, it has become increasingly clear that many secondary biochemical changes that exacerbate injury occur in response to the initial insult. In models of cerebral ischemia in rodents, as much as 50% or more of ischemic brain may be spared from infarction by preventing these secondary biochemical events. Understanding of the mechanisms by which neuronal cell death takes place has resulted in a number of therapeutic strategies that aim to prevent secondary biochemical changes and thus decrease the damage that results from cerebral ischemia. These basic mechanisms may also have relevance to other neurodegenerative diseases associated with excessive neuronal death.

This chapter summarizes many of the mechanisms that have been demonstrated to exacerbate the neuronal death caused by hypoxia and hypoglycemia. Ischemic neuronal death may involve the activation of enzymes and receptors that are constitutively expressed in brain. These existing receptors and enzymes do not require energy or the synthesis of new protein to exacerbate *necrotic* cell death. New evidence suggests that ischemic injury may also be exacerbated by the inducible proteins that mediate *programmed* cell death. These mechanisms are appealing targets for therapeutic intervention because they may occur hours or days after the initiation of ischemia.

CELL DEATH: NECROSIS VERSUS PROGRAMMED CELL DEATH

It has been observed that an orderly expression of new gene products is required to produce programmed cell death during development of the roundworm Caenorhabditis elegans (1). This observation has led to intense interest in the hypothesis that the expression of similar death-promoter genes could be important in the pathogenesis of human disease (2). Support for this hypothesis is derived from the existence of oncogenes, death-regulating genes that are either deleted or overexpressed in cancer. Genetic mechanisms that control cell death are clearly relevant to mitotic cells in development, cancer, and the maintenance and turnover of regenerating adult tissues. Neurons may also die by these mechanisms. A classic example is the withdrawal of nerve growth factor from dorsal root sympathetic neurons that results in delayed death, which requires the transcription of new messenger RNA (mRNA) and the synthesis of new proteins (3). Before their death, these neurons undergo morphologic changes associated with apoptosis (4), a term originally used to describe the morphologic characteristics associated with programmed cell death.

Programmed cell death is a mechanism by which the organism can remove unnecessary or redundant cells during development or in mature tissues where cell turnover is required. Programmed cell death has several key characteristics: (a) The death process is active, and the expression of new proteins is often involved. (b) Cellular energy stores

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	Necrosis	Programmed Cell Death
Process	Passive	Active
Energy failure	Primary	Secondary
Protein translation	Blocked	Exacerbates cell death
Morphology	Coagulative necrosis	Apoptosis
DNA fragmentation	None or random, resulting in either no migration or a smear on DNA gels	Occurs at histosome boundaries, resulting in multiples of 400 base fragments producing laddering on DNA gels
Inflammation	Prominent	Little or none

TABLE 92.1. CHARACTERISTICS OF NECROSIS AND PROGRAMMED CELL DEATH

are normal until the final stages of cellular death; therefore, energy failure is a late, secondary event in programmed cell death. (c) The activation of endonucleases results in numerous double-stranded DNA breaks at the boundaries between histosomes. The result is DNA fragments in multiples of 400 base pair size that produce characteristic "laddering" on DNA gels. (d) Morphologic changes characteristic of apoptosis, including cytoplasmic and nuclear budding ("apoptotic bodies"), are present. (e) In contrast to necrosis, programmed cell death results in neuronal death with little or no accompanying inflammation. Thus, "collateral damage" to neighboring cells is avoided.

In contrast to programmed cell death, necrotic cell death is characterized by energy failure, which results in inhibition of protein synthesis. Therefore, new gene products may not be expressed. Histologic characteristics of necrotic cell death are cytoplasmic and nuclear swelling, loss of integrity of cell organelles, rupture of the cell membrane, and dissolution of all cell structures. *In vivo*, necrotic cell death is often accompanied by intense inflammation with recruitment of inflammatory cells. This inflammatory response can injure adjacent normal cells. The characteristics of programmed cell death and necrosis are summarized in Table 92.1.

NATURE OF NEURONAL DEATH IN CEREBRAL ISCHEMIA

In ischemia, a mismatch between energy supply and demand may result in energy failure. Without adequate energy, protein synthesis cannot occur, and the genes that execute programmed cell death may not be expressed. The predominant histologic feature of stroke is infarction. Infarction is synonymous with necrosis (i.e., cytoplasmic swelling, dissolution of organelles and plasma membranes, and inflammation are present). In the permanent middle cerebral artery occlusion model in the rat, loss of glucose utilization is rapid and complete within a few hours (5),

with little time or energy available for the synthesis of new gene products. Thus, one would surmise that necrosis is the primary mode of cell death in this model.

Under other circumstances, however, cerebral ischemia may produce neuronal death with many of the characteristics of programmed cell death. In models of transient ischemia, for example, energy failure is transient, and neuronal death develops more slowly than in permanent focal ischemia, with many features of apoptotic cell death. Under these circumstances, cleavage of genomic DNA into fragments of various sizes on DNA gels, characteristic of programmed cell death, occurs (6,7). However, the most convincing evidence that the production of new gene products may be important in the pathogenesis of neuronal death after transient ischemia is that protein synthesis inhibitors block delayed death of neurons (8–10). Thus, depending on the duration and severity of ischemia, stroke may produce cell death with features of necrosis or apoptosis.

MECHANISMS OF NECROTIC CELL DEATH

The primary pathologic mechanism in stroke is the depletion of energy stores; however, considerable evidence indicates that excitatory amino acids (EAAs) exacerbate ischemic injury. EAAs such as glutamate are released by approximately 40% of all synapses in the central nervous system (11). Under physiologic conditions, EAAs participate in many neurologic functions, including memory, movement, sensation, cognition, and synaptic plasticity (12,13). However, EAAs can also have a pathologic effect. EAA-mediated toxicity was first demonstrated by Olney and co-workers (14) by peripheral administration of an EAA agonist that selectively killed neurons in the arcuate nucleus of the hypothalamus. These neurons contain high concentrations of glutamate receptors. Choi (15) demonstrated that micromolar extracellular glutamate and other EAAs produce rapid increases in intraneuronal cytosolic Ca²⁺ concentrations.

This increase in intracellular calcium concentration is rapidly lethal to primary neuronal cultures. The importance of calcium entry and excitotoxicity is supported by data demonstrating a direct correlation between extracellular calcium stimulation and neuronal death following exposure to glutamate (16).

The increase in intraneuronal Ca²⁺ in response to extracellular EAAs in vitro is mediated by the opening of a receptor-gated ion channel, the N-methyl-D-aspartate (NMDA) channel (17). The NMDA channel, named after its highestaffinity ligand, primarily gates calcium entry into the neuron. Treatment with antagonists that compete with glutamate and other EAAs for the receptor (competitive NMDA antagonists) or antagonists that bind to the ion channel itself (noncompetitive antagonists) can block calcium entry into neurons and prevent cell death induced by glutamate (18,19). Glycine is a co-agonist that is required in addition to glutamate to open the NMDA Ca2+ channel (20). Antagonists that bind to the glycine site on the NMDA receptor also block excitotoxicity in vitro (21). In addition to rescuing cells from EAA toxicity by blockade of the EAA receptors, it is possible to rescue neurons in culture by removal of extracellular calcium and sodium from the culture media following glutamate exposure (18). Conversely, inhibition of the sodium-calcium exchanger that normally facilitates extrusion of calcium results in an increase in neuronal death (18,22). Similarly, dantrolene, which attenuates decompartmentalization of intracellular stores of calcium, can reduce glutamate neurotoxicity in cortical neurons (23). Finally, neurons containing high concentrations of calciumbinding proteins, such as calbindin or parvalbumin, are relatively resistant to excitotoxic injury (24,25). These data provide compelling evidence that EAA-induced increases in intracellular Ca^{2+} are toxic to neurons in culture.

Compelling evidence is also available to indicate that excitotoxicity mediated by the NMDA receptor contributes to injury from cerebral ischemia in vivo. A rapid and large increase in the concentration of extracellular amino acids can be monitored by microdialysis after cerebral ischemia (26). Although NMDA antagonists are not effective in global ischemia models in which temperature is carefully controlled (27), a large number of studies have found that they decrease infarction volume in both permanent and temporary middle cerebral artery occlusion models in rodents (28). Blocking the translation of a gene that encodes a subunit of the NMDA receptor with intraventricular injection of antisense oligonucleotides also decreases infarction volume after middle cerebral artery occlusion in the rat (29). These data and many other studies support the hypothesis that excitotoxicity contributes to ischemic injury in vivo.

Several calcium-dependent or calcium-induced enzymes mediate the toxic effects of increased intracellular calcium (Fig. 92.1). These include nitric oxide synthase, cyclooxygenase, phospholipase A₂, and calpain 1. Calpain 1 is a calcium-activated protease that has been specifically linked to glutamate receptors in the rat hippocampus (30). Calpain 1 participates in the conversion of xanthine dehydrogenase to xanthine oxidase, which metabolizes xanthine to its reactive oxygen species, superoxide (31). Similarly, phospholipase A₂ is activated by calcium and facilitates the release of arachidonic acid from injured cell membranes (32). Arachi-

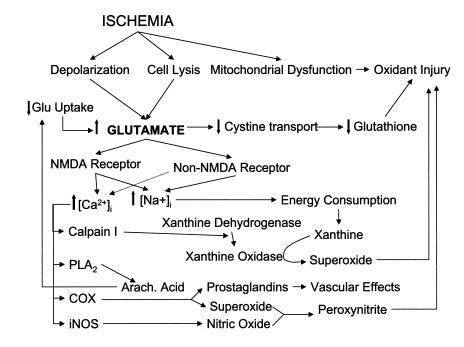


FIGURE 92.1. Schematic diagram illustrating the mechanisms by which ischemia and excitotoxicity injure neurons.

donic acid is then metabolized by the enzyme cyclooxygenase into a prostaglandin, PGH₂. The cyclooxygenase enzyme may produce a superoxide ion as a by-product of arachidonic acid metabolism (33). In addition, intracellular calcium can activate calcium-dependent isoforms of nitric oxide synthase to produce nitric oxide (34). The nitric oxide then combines with the superoxide produced as the by-product of cyclooxygenase, xanthine oxidase, or other sources to form the highly reactive species peroxynitrite, which exacerbates tissue damage (35). Therefore, EAA-mediated elevation of intracellular calcium concentrations activates both cyclooxygenase and nitric oxide synthase, which then synergistically contribute to ischemic brain injury (36).

Extracellular EAAs may activate other receptors besides the NMDA channel. EAA receptors can be categorized as ionotropic or metabotropic receptors. Ionotropic receptors are coupled directly to membrane ion channels, whereas metabotropic receptors are coupled to G proteins and modulate intracellular second messengers such as inositol triphosphate, calcium, and cyclic nucleotides. More than 20 genes have been identified that encode subunits of these receptors. The subunits combine in a variety of confirmations to yield receptors with specific pharmacologic and electrophysiologic characteristics (37). Ionotropic receptors can be categorized based on their sensitivity to the selective agonists NMDA, AMPA (α-amino-3-hydroxy-5-methyl-4isoxasole-proprionic acid), and kainate. The ionotropic receptors depolarize membranes by facilitating an influx of positively charged ions. The NMDA receptor facilitates an influx of both sodium and calcium, whereas the non-NMDA receptors (AMPA and kainate receptors) primarily facilitate an influx of sodium. However, some of the kainate and AMPA receptors are comprised of subunits that allow calcium permeability (38). This may be relevant to ischemic injury because in neurons after cerebral ischemia, glutamate receptor 2 (GR₂), a subunit necessary for non-NMDA receptors to exclude Ca²⁺, is relatively depleted (39). Accordingly, these non-NMDA subunits may become calciumpermeable after ischemia. The metabotropic receptors may also increase intracellular calcium by mobilizing calcium from stores in the endoplasmic reticulum. Studies with antagonists of the metabotropic receptor show that, depending on their subunit specificity, some, but not all, drugs of this class are neuroprotective in models of focal ischemia (40, 41).

In addition to the direct downstream effects of enzymes that are activated by elevation of intracellular calcium, a number of complex interactions and positive feedback loops augment the contribution of EAAs to ischemic brain injury. For example, free arachidonic acid can potentiate NMDAevoked currents in neurons (42) and inhibit reuptake of glutamate by astrocytes (43). In addition, platelet-activating factor, a phospholipase A2 metabolite, can stimulate the release of glutamate (44). Acidotic conditions favor the release of free iron, which can then participate in the metabolism of peroxide into the hydroxyl radical (Fenton reaction) (45). In addition, glutamate can interfere with the function of the cystine transporter. Inhibition of the cystine transporter results in decreased intracellular concentrations of glutathione and diminished intracellular endogenous antioxidant stores (46).

In vivo, excitotoxicity may be ameliorated by additional strategies besides inhibition of the NMDA receptor (Fig. 92.2). Glutamate release into synaptic cleft, where it interacts with EAA receptors, is primarily mediated by the release of glutamate from the synaptic pool. Thus, a large component of excessive neuronal excitation may be the result of synaptic release of EAAs. Neuronal depolarization of presynaptic neurons in turn depends on activation of non-NMDA receptor-gated channels and other depolarizing neurotransmitter receptors. The excitatory action of depolarizing neurotransmitter receptors is countered by hyperpolarizing receptor-gated ion channels, such as the GABA (γ-

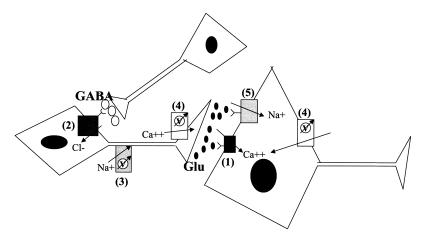


FIGURE 92.2. A simplified neuronal circuit diagram illustrating the ion channels that determine the synaptic release of glutamate and intraneuronal Ca^{2+} concentrations in response to ischemia. 1, *N*-methyl-b-aspartate (NMDA) receptor-gated ion channel; 2, α -aminobutyric acid (GABA) receptor-gated Cl-channel; 3, voltage-dependent Na^+ channel; 4, voltage-dependent Ca^{2+} channel; 5, non-NMDA receptor-gated ion channel.

aminobutyric acid) receptor. Propagation of the action potential induced by depolarization of the neuronal cell body requires voltage-dependent sodium channels. Finally, the release of glutamate itself depends on P- and Q-type voltagedependent calcium channels. Glutamate release into the synaptic cleft can bind to the NMDA receptor and open the calcium channel. As a result, calcium enters the cell driven by its concentration gradient. However, intraneuronal calcium may increase by other mechanisms. Postsynaptic voltage-dependent calcium channels may allow calcium entry into the neuron when cells are depolarized, and glutamate released into the extracellular cleft may activate non-NMDA receptor-gated channels and depolarize the neuron. Also, Na⁺ may enter the cell via the NMDA receptor-gated channel and depolarize the neuron. Thus, excitotoxicity may be ameliorated at a number of sites in vivo.

Many drugs that can inhibit excitotoxicity at each of these steps have been developed. GABA agonists such as clomethazole have been shown to be neuroprotective in vivo and are currently undergoing clinical trials (47,48). In rodent models of stroke, BW1003,619 and phosphenytoin prevent prolonged opening of the voltage-dependent sodium channel, ameliorate increases in extracellular glutamate, and decrease infarction volume (49-51). Drugs that prevent prolonged opening of P- and Q-type calcium channel antagonists are also neuroprotective in animal models of stroke (52). In contrast to their very limited effects in primary neuronal tissue culture models, non-NMDA antagonists are very effective in both global and focal ischemia models in rodents. Indeed, such agents have a longer time window of efficacy than do NMDA antagonists when administered after the onset of ischemia (53,54). Likewise, voltage-dependent calcium channel antagonists are not effective in vitro; however, the voltage-dependent calcium channel antagonist nimodipine is effective in reducing infarction volume in temporary focal ischemia in rats (55).

Blockade of excitotoxicity via all these pharmacologic strategies has proved effective in temporary focal ischemia models in rodents, the model that most closely resembles human stroke. Unfortunately, results with these agents in human trials have to date been very disappointing, for several possible reasons. First, drugs that affect neurotransmission in the brain have many undesirable side effects, which in turn have led to reductions to doses that may have been ineffective. Side effects include effects on respiration and cardiac rhythm. In addition, agents that directly antagonize the NMDA receptor may injure a circumspect population of neurons in the cingulate and retrosplenial cortex in rodents (56), and may induce hallucinations and psychosis in humans (57). Another obvious reason for the lack of efficacy in these drugs in clinical trials is the time interval between the onset of ischemia and the administration of drug. When given before the onset of ischemia, these treatments can spare 50% or more of ischemic rat brain tissue from eventual infarction. When given after the onset of ischemia, they are progressively less effective; however, such agents are effective up to 2 hours after the onset of middle cerebral artery occlusion in the rat. In the clinical trials, most patients were enrolled 6 to 12 hours after the onset of ischemia, long after the time that these drugs were effectively administered in animal studies.

Whatever the reason for the failure of these anti-excitotoxic drugs in human trials, it has become clear that it may be more practical to select treatment approaches that target mechanisms that are active at longer intervals after ischemia. Accordingly, efforts to understand the delayed mechanisms of neuronal injury have been increased, in particular the role of programmed cell death in ischemic neurons.

MECHANISMS OF PROGRAMMED CELL DEATH

Many of the key molecular events in programmed cell death have now been determined (Fig. 92.3). Just as calcium entry into the neuron is a key step in excitotoxicity, the release of cytochrome c from the mitochondria is a key event in initiating apoptosis in many cell types. Cytosolic cyto-

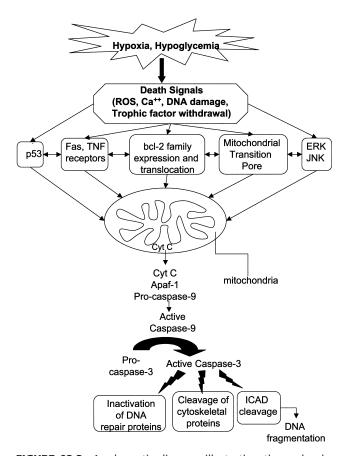


FIGURE 92.3. A schematic diagram illustrating the molecular mechanisms that control programmed cell death.

chrome *c* complexes with APAF-1 and procaspase 9 (58). As a result, procaspase 9 is cleaved into its active form, caspase 9. Caspase 9 then cleaves and activates other caspases, including caspase 3.

Caspases are a family of proteases that play a key role in executing programmed cell death. They were first identified by their homology with CED3, the key gene that irreversibly commits neurons in *C. elegans* to programmed cell death. A dozen mammalian caspases have been identified that have a variety of roles in executing programmed cell death and other cellular functions. Among the caspases, caspase 3 has the closest homology with CED3 and appears to play a key role as the final committed step in programmed cell death. Caspase 3 executes programmed cell death via cleavage of many other proteins. These proteolytic targets of caspase 3 include cytoskeletal protein(s), DNA repair proteins such as PARP, and other proteins (59). Caspase 3 also cleaves ICAD, an inhibitor of CAD, an endonuclease that cleaves DNA between histosomes. The result is cleavage of DNA between histosomes, a hallmark of programmed cell death

The egress of cytochrome ^c from the mitochondria into the cytosol is controlled by several mechanisms. Genes of the bcl-2 family play an important role in controlling cytochrome c egress. Anti-apoptotic bcl-2 family members, such as bcl-2 itself and bcl-x-long, inhibit the egress of cytochrome ^c (61,62). Pro-apoptotic members of the bcl-2 family, such as bcl-x-short and bax, can form dimers with themselves or with anti-apoptotic bcl-2 family members. The balance between the pro-apoptotic and anti-apoptotic bcl-2 family proteins in the mitochondrial membrane determines whether permeability of the membrane will increase to allow egress of cytochrome c into the cytoplasm. Under some circumstances, cytochrome c exits the mitochondria via the mitochondrial permeability transition pore. This pore can open in response to prolonged depolarization, produced by such stimuli as an increase in intracellular calcium (63). Furthermore, pro-apoptotic bcl-2 family members such as bax may also interact with this pore (64). However, bcl-2 family members themselves may form pores in membranes (65), and some evidence indicates that bax induces egress of cytochrome c from the mitochondria independently of the mitochondrial permeability transition pore (66). Initiation of the mitochondrial apoptosis is also controlled by expression and translocation of other numerous bcl-2 family members. For example, translocation of bax from the cytosol to the mitochondria initiates programmed cell death (67). Bad is phosphorylated before being translocated to the mitochondria (68). More than 20 additional proteins are found in the bcl-2 family, including many that are also involved in mitochondrial homeostasis. Thus, a key event in apoptosis, egress of cytochrome c from the mitochondria, is controlled by bcl-2 family proteins.

The molecular mechanisms by which programmed cell death is initiated are numerous and complex. Programmed

cell death may be activated via cell surface receptors, including the Fas receptor and tumor necrosis factor- α (TNF- α) (69,70). Activation of these receptors triggers activation of caspase 8, which in turn cleaves the bcl-2 family protein bid (71). The cleaved bid then translocates from the cytoplasm to the mitochondria, where it initiates cytochrome c egress (72). Other mechanisms by which the initiation of programmed cell death is controlled include the ERK (externally regulated kinase) and JNK protein kinase cascades (73). Finally, DNA base oxidation and other DNA damage may initiate programmed cell death via expression of the p53 transcription factor. These and other mechanisms may be involved in the initiation of programmed cell death in ischemic neurons.

PROGRAMMED CELL DEATH AFTER CEREBRAL ISCHEMIA

Evidence indicates that many of the mechanisms that initiate programmed cell death are activated in ischemic neurons under certain conditions. The mRNA of the Fas ligand is induced by forebrain ischemia (74). Expression of the Fas ligand and associated proteins and infarction volumes was smaller in LPR mice that expressed a dysfunctional Fas ligand than in wild-type controls (75). The Fas receptor is also up-regulated after cerebral ischemia in rat brain (76). TNF-α mRNA transcription is induced as an early response after cerebral ischemia (77). Expression of the TNF receptor is also increased after cerebral ischemia (78). TNF-binding protein, a protein that binds and inhibits TNF- α , reduced infarction volume after middle cerebral artery occlusion in rats (79). However, ischemic injury was exacerbated in TNF- α -receptor null mice, which suggests that TNF signaling pathways may instead have beneficial effects in ischemic injury under some circumstances (80). Caspase 8, which is activated by both the Fas and TNF receptors, is expressed and activated after cerebral ischemia (81). Changes in expression and activity of both the ERK and JNK kinase pathways occur following cerebral ischemia. The M-terminal kinases of c-Jun are activated after ischemia and phosphorylate c-Jun (82). The increased expression of ERK after focal ischemia and inhibitor of NEK-1, another kinase in the ERK pathway, protect the brain against focal cerebral ischemia (83,84). Single-stranded DNA damage is an early event in cerebral ischemia reperfusion injury and may trigger expression of p53 (85,86). Expression of p53 is induced after cerebral ischemia (87).

A number of studies in cerebral ischemia support a role for bcl-2 family genes in controlling ischemic neuronal death. In rodent models of ischemia, anti-apoptotic members of the bcl-2 family, including bcl-2 and bcl-x long, are expressed in neurons that are ischemic yet survive. Expression of pro-apoptotic members of the family, such as bax, is increased in neurons that are ischemic and die, such as

CA1 hippocampal neurons in models of global ischemia (88,89). Overexpressing anti-apoptotic members of the bcl-2 family protects neurons against ischemia. Transgenic mice that overexpress bcl-2 in neurons have a smaller infarction volume after temporary focal ischemia than do wild-type controls (90). Similarly, overexpression of bcl-2 by means of herpes simplex viral vectors protects neurons against ischemia in vivo (91,92). These studies show that overexpression of bcl-2 protein before ischemia is neuroprotective. To address whether bcl-2 that is endogenously expressed after ischemia has a protective role, antisense oligonucleotides were used to prevent translation of bcl-2 induced after ischemia. Rats treated with bcl-2 antisense oligonucleotides had a larger infarction volume than did rats treated with sense oligonucleotides or vehicle after temporary focal ischemia. These results suggest that expression of endogenous bcl-2 increases survival of ischemic neurons (93).

Abundant *in vivo* evidence also suggests that caspase activity exacerbates ischemic injury. Transgenic mice that express a dominant negative mutation of caspase 1 had smaller infarctions than did their wild-type litter mates (94). Furthermore, intraventricular infusion of peptide inhibitors of caspases decreased infarction volume in rats subjected to temporary middle cerebral artery occlusion. These peptide inhibitors of caspases also blocked damage in response to injection of excitotoxins (95). Caspase 3 mRNA and protein expression is induced in CA1 neurons after global ischemia. Caspase 3 is activated, and treatment with a specific peptide inhibitor of caspase 3 ameliorated neuronal death in the global ischemia model (96). These and other studies support a role for caspases in ischemic neuronal injury.

CONCLUSION

If ischemia is of long duration or severe, death is rapid and necrotic. However, if ischemia is transient or incomplete, the genes that execute programmed cell death may be activated. Which process occurs depends on the duration and severity of the ischemic insult (97,98). In rodent models of ischemia, the duration and severity of ischemia are controlled, so one can produce ischemic neuronal death with either characteristic. Indeed, a spectrum of biochemical and morphologic changes occurs with characteristics of both death processes. In the human disease, the duration and severity of ischemia depend on the exact location of the arterial occlusion and whether or not reperfusion occurs. Reperfusion can occur spontaneously or as the result of thrombolytic therapy. Just as in the animal models, arterial occlusions produce regions where blood flow is nearly completely absent, and also surrounding zones with incomplete ischemia. Therefore, transient and incomplete ischemia occurs in the human disease and so provides the conditions necessary for programmed cell death.

Our knowledge of the mechanisms by which ischemic

neurons die has increased considerably. It is now clear that the toxic effects of EAAs exacerbate injury resulting from ischemia. Antagonizing excitotoxicity via a variety of approaches can ameliorate injury in animal models of ischemia; however, these treatments appear to be too toxic and are effective for too short an interval after the onset of ischemia to be practical treatments in humans. When ischemia is transient or less severe, programmed cell death is activated. These events occur hours or days after the onset of ischemia and thus may be more practical targets for treatment. Further work is needed to determine the most effective and practical therapeutic strategies to prevent neuronal death after ischemia.

REFERENCES

- 1. Hengartner MO, Horvitz HR. *C. elegans* cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* 1994;76:665–676.
- 2. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;267:1256–1262.
- Martin DP, Schmidt RE, DiStefano PS, et al. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J Cell Biol* 1988;106: 829–844.
- 4. Saunders J Jr. Death in embryonic systems. *Science* 1966;154: 604–612.
- Shiraishi K, Sharp FR, Simon RP. Sequential metabolic changes in rat brain following middle cerebral artery occlusion: a 2-deoxyglucose study. *J Cereb Blood Flow Metab* 1989;9:765–773.
- MacManus JP, Buchan AM, Hill IE, et al. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci Lett* 1993;164:89–92.
- Li Y, Sharov VG, Jiang N, et al. Ultrastructural and light microscopic evidence of apoptosis after middle cerebral artery occlusion in the rat. Am J Pathol 1995;146:1045–1051.
- 8. Goto K, Ishige A, Sekiguchi K, et al. Effects of cycloheximide on delayed neuronal death in rat hippocampus. *Brain Res* 1990; 534:299–302.
- Tortosa A, Rivera R, Ferrer I. Dose-related effects of cycloheximide on delayed neuronal death in the gerbil hippocampus after bilateral transitory forebrain ischemia. *J Neurol Sci* 1994;121: 10–17.
- Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 1993;24:2002–2008.
- 11. Fonnum F. Glutamate: a neurotransmitter in mammalian brain. *J Neurochem* 1984;42:1–11.
- 12. Gasic GP, Hollmann M. Molecular neurobiology of glutamate receptors. *Annu Rev Physiol* 1992;54:507–536.
- Lipton SA, Kater SB. Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci* 1989;12: 265–270.
- 14. Olney JW, Ho OL, Rhee V. Cytotoxic effects of acidic and sulphur-containing amino acids on the infant mouse central nervous system. *Exp Brain Res* 1971;14:61–76.
- Choi DW. Ionic dependence of glutamate neurotoxicity. J Neurosci 1987;7:369–379.
- Ogura A, Miyamoto M, Kudo Y. Neuronal death *in vitro*: parallelism between survivability of hippocampal neurones and sustained elevation of cytosolic Ca²⁺ after exposure to glutamate receptor agonist. *Exp Brain Res* 1988;73:447–458.

- 17. Choi DW, Koh JY, Peters S. Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci* 1988;8:185–196.
- Hartley DM, Choi DW. Delayed rescue of N-methyl-D-aspartate receptor-mediated neuronal injury in cortical culture. J Pharmacol Exp Ther 1989;250:752–758.
- 19. Choi DW. Methods for antagonizing glutamate neurotoxicity. Cerebrovasc Brain Metab Rev 1990;2:105–147.
- Thomson AM. Glycine modulation of the NMDA receptor/ channel complex. Trends Neurosci 1989;12:349–353.
- 21. Smith SE, Meldrum BS. The glycine-site NMDA receptor antagonist, r-(+)-cis-beta-methyl-3-amino-1-hydroxypyrrolid-2-one, l-687,414 is anticonvulsant in baboons. *Eur J Pharmacol* 1992; 211:109–111.
- Andreeva N, Khodorov B, Stelmashook E, et al. Inhibition of Na⁺/Ca²⁺ exchange enhances delayed neuronal death elicited by glutamate in cerebellar granule cell cultures. *Brain Res* 1991; 548:322–325.
- Frandsen A, Schousboe A. Dantrolene prevents glutamate cytotoxicity and Ca²⁺ release from intracellular stores in cultured cerebral cortical neurons. *J Neurochem* 1991;56:1075–1078.
- Sloviter RS. Calcium-binding protein (calbindin—D28 kd) and parvalbumin immunocytochemistry: localization in the rat hippocampus with specific reference to the selective vulnerability of hippocampal neurons to seizure activity. *J Comp Neurol* 1989; 280:183–196.
- Weiss JH, Koh J, Baimbridge KG, et al. Cortical neurons containing somatostatin- or parvalbumin-like immunoreactivity are atypically vulnerable to excitotoxic injury in vitro. Neurology 1990;40:1288–1292.
- Benveniste H, Drejer J, Schousboe A, et al. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem* 1984;43:1369–1374.
- Buchan A, Li H, Pulsinelli WA. The N-methyl-D-aspartate antagonist, mk-801, fails to protect against neuronal damage caused by transient, severe forebrain ischemia in adult rats. J Neurosci 1991;11:1049–1056.
- McCulloch J. Excitatory amino acid antagonists and their potential for the treatment of ischaemic brain damage in man. Br J Clin Pharmacol 1992;34:106–114.
- Wahlestedt C, Golanov E, Yamamoto S, et al. Antisense oligodeoxynucleotides to NMDA-R₁ receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions. *Nature* 1993;363:260–263.
- Siman R, Noszek JC, Kegerise C. Calpain 1 activation is specifically related to excitatory amino acid induction of hippocampal damage. J Neurosci 1989;9:1579–1590.
- 31. Stark K, Seubert P, Lynch G, et al. Proteolytic conversion of xanthine dehydrogenase to xanthine oxidase: evidence against a role for calcium-activated protease (calpain). *Biochem Biophys Res Commun* 1989;165:858–864.
- 32. Chan PH, Fishman RA, Schmidley JW, et al. Release of polyunsaturated fatty acids from phospholipids and alteration of brain membrane integrity by oxygen-derived free radicals. *J Neurosci Res* 1984;12:595–605.
- 33. Kukreja RC, Kontos HA, Hess ML, et al. PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. *Circ Res* 1986;59:612–619.
- Vane JR, Mitchell JA, Appleton I, et al. Inducible isoforms of cyclooxygenase and nitric oxide synthase in inflammation. *Proc Natl Acad Sci USA* 1994;91:2046–2050.
- Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am J Physiol 1996; 271:C1424–C1437.

- Nogawa S, Forster C, Zhang F, et al. Interaction between inducible nitric oxide synthase and cyclooxygenase 2 after cerebral ischemia. *Proc Natl Acad Sci USA* 1998;95:10966–10971.
- 37. Nakanishi S. Molecular diversity of glutamate receptors and implications for brain function. *Science* 1992;258:597–603.
- Hollmann M, Hartley M, Heinemann S. Ca²⁺ permeability of Ka-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* 1991;252:851–853.
- Pellegrini-Giampietro DE, Zukin RS, Bennett MV, et al. Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proc Natl Acad* Sci USA 1992;89:10499–10503.
- Bond A, Ragumoorthy N, Monn JA, et al. Ly379268, a potent and selective group II metabotropic glutamate receptor agonist, is neuroprotective in gerbil global, but not focal, cerebral ischaemia. Neurosci Lett 1999;273:191–194.
- Lam AG, Soriano MA, Monn JA, et al. Effects of the selective metabotropic glutamate agonist Ly354740 in a rat model of permanent ischaemia. *Neurosci Lett* 1998;254:121–123.
- Miller B, Sarantis M, Traynelis SF, et al. Potentiation of NMDA receptor currents by arachidonic acid. *Nature* 1992;355: 722–725.
- Volterra A, Trotti D, Cassutti P, et al. High sensitivity of glutamate uptake to extracellular free arachidonic acid levels in rat cortical synaptosomes and astrocytes. *J Neurochem* 1992;59: 600–606.
- Clark GD, Happel LT, Zorumski CF, et al. Enhancement of hippocampal excitatory synaptic transmission by platelet-activating factor. *Neuron* 1992;9:1211–1216.
- Ying W, Han SK, Miller JW, et al. Acidosis potentiates oxidative neuronal death by multiple mechanisms. *J Neurochem* 1999;73: 1549–1556.
- 46. Murphy TH, Miyamoto M, Sastre A, et al. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 1989;2:1547–1558.
- 47. Snape MF, Baldwin HA, Cross AJ, et al. The effects of chlormethiazole and nimodipine on cortical infarct area after focal cerebral ischemia in the rat. *Neuroscience* 1993;53:837–844.
- 48. Wahlgren NG, Ranasinha KW, Rosolacci T, et al. Clomethiazole acute stroke study (CLASS): results of a randomized, controlled trial of clomethiazole versus placebo in 1,360 acute stroke patients. *Stroke* 1999;30:21–28.
- Graham SH, Chen J, Sharp FR, et al. Limiting ischemic injury by inhibition of excitatory amino acid release. J Cereb Blood Flow Metab 1993;13:88–97.
- Smith SE, Lekieffre D, Sowinski P, et al. Cerebroprotective effect of BW619C89 after focal or global cerebral ischaemia in the rat. *Neuroreport* 1993;4:1339–1342.
- 51. Boxer PA, Cordon JJ, Mann ME, et al. Comparison of phenytoin with noncompetitive *N*-methyl-D-aspartate antagonists in a model of focal brain ischemia in rat. *Stroke* 1990;21:III47–III51.
- Goldin SM, Subbarao K, Margolin LD, et al. Neuroprotective use-dependent blockers of Na⁺ and Ca²⁺ channels controlling presynaptic release of glutamate. *Ann NY Acad Sci*1995;765: 210–229.
- Sheardown MJ, Suzdak PD, Nordholm L. AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 hours. *Eur J Pharmacol* 1993; 236:347–353.
- Buchan AM, Lesiuk H, Barnes KA, et al. AMPA antagonists: do they hold more promise for clinical stroke trials than NMDA antagonists? *Stroke* 1993;24:I148–I152.
- 55. Kawaguchi K, Henshall DC, Simon RP. Parallel dose–response studies of the voltage-dependent Na⁺ channel antagonist

- BW619C89 and the voltage-dependent Ca²⁺ channel antagonist nimodipine in rat transient focal cerebral ischaemia. *Eur J Pharmacol* 1999;364:99–105.
- Olney JW, Labruyere J, Wang G, et al. NMDA antagonist neurotoxicity: mechanism and prevention. *Science* 1991;254: 1515–1518.
- 57. Woods JH, Koek W, France CP. Behavioral effects of NMDA antagonists.
- Li P, Nijhawan D, Budihardjo I, et al. Cytochrome c and DATPdependent formation of APAF-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997;91:479–489.
- Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997;326:1–16.
- Enari M, Sakahira H, Yokoyama H, et al. A caspase-activated DNAase that degrades DNA during apoptosis and its inhibitor ICAD. *Nature* 1998;391:43–50.
- 61. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* 1997;275:1129–1132.
- 62. Kluck RM, Bossy-Wetzel E, Green DR, et al. The release of cytochrome *c* from mitochondria: a primary site for bcl-2 regulation of apoptosis. *Science* 1997;275:1132–1136.
- Hirsch T, Marzo I, Kroemer G. Role of the mitochondrial permeability transition pore in apoptosis. *Biosci Rep* 1997;17:67

 76.
- 64. Narita M, Shimizu S, Ito T, et al. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. *Proc Natl Acad Sci USA* 1998;95:14681–14686.
- 65. Antonsson B, Conti F, Ciavatta A, et al. Inhibition of bax channel-forming activity by bcl-2. *Science* 1997;277:370–372.
- 66. Eskes R, Antonsson B, Osen-Sand A, et al. Bax-induced cytochrome c release from mitochondria is independent of the permeability transition pore but highly dependent on Mg²⁺ ions. J Cell Biol 1998;143:217-224.
- 67. Wolter KG, Hsu YT, Smith CL, et al. Movement of bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997; 139:1281–1292.
- 68. D'Agata V, Magro G, Travali S, et al. Cloning and expression of the programmed cell death regulator bad in the rat brain. *Neurosci Lett* 1998;243:137–140.
- Nagata S. Apoptosis regulated by a death factor and its receptor: fas ligand and fas. *Philos Trans R Soc Lond B Biol Sci* 1994;345: 281–287
- Beutler B, Bazzoni F. TNF, apoptosis and autoimmunity: a common thread? *Blood Cells Mol Dis* 1998;24:216–230.
- Yin XM, Wang K, Gross A, et al. Bid-deficient mice are resistant to fas-induced hepatocellular apoptosis. *Nature* 1999;400: 886–891.
- 72. Gross A, Yin XM, Wang K, et al. Caspase cleaved bid targets mitochondria and is required for cytochrome c release, while bclxl prevents this release but not tumor necrosis factor-r1/fas death. J Biol Chem 1999;274:1156–1163.
- 73. Seger R, Krebs EG. The MAPK signaling cascade. *FASEB J* 1995; 9:726–735.
- 74. Matsuyama T, Hata R, Yamamoto Y, et al. Localization of fas antigen MRNA induced in postischemic murine forebrain by *in situ* hybridization. *Brain Res Mol Brain Res* 1995;34:166–172.
- Martin-Villalba A, Herr I, Jeremias I, et al. Cd95 ligand (fas-l/apo-1l) and tumor necrosis factor-related apoptosis-inducing ligand mediate ischemia-induced apoptosis in neurons. *J Neurosci* 1999;19:3809–3817.
- 76. Felderhoff-Mueser U, Taylor DL, Greenwood K, et al. Fas/cd95/ apo-1 can function as a death receptor for neuronal cells in vitro and in vivo and is upregulated following cerebral hypoxic—

- ischemic injury to the developing rat brain. *Brain Pathol* 2000; 10:17–29.
- Wang X, Yue TL, Barone FC, et al. Concomitant cortical expression of TNF-alpha and IL-1 beta MNRAs follows early-response gene expression in transient focal ischemia. *Mol Chem Neuropathol* 1994;23:103–114.
- 78. Botchkina GI, Meistrell ME 3rd, Botchkina IL, et al. Expression of TNF and TNF receptors (p55 and p75) in the rat brain after focal cerebral ischemia. *Mol Med* 1997;3:765–781.
- Nawashiro H, Martin D, Hallenbeck JM. Neuroprotective effects of TNF binding protein in focal cerebral ischemia. *Brain Res* 1997;778:265–271.
- 80. Bruce AJ, Boling W, Kindy MS, et al. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nat Med* 1996;2:788–794.
- 81. Velier JJ, Ellison JA, Kikly KK, et al. Caspase-8 and caspase-3 are expressed by different populations of cortical neurons undergoing delayed cell death after focal stroke in the rat. *J Neurosci* 1999; 19:5932–5941.
- 82. Herdegen T, Claret FX, Kallunki T, et al. Lasting N-terminal phosphorylation of c-jun and activation of c-jun N-terminal kinases after neuronal injury. *J Neurosci* 1998;18:5124–5135.
- Alessandrini A, Namura S, Moskowitz MA, et al. Mek1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. *Proc Natl Acad Sci USA* 1999;96:12866– 12869.
- 84. Kitagawa H, Warita H, Sasaki C, et al. Immunoreactive akt, pi3-k and erk protein kinase expression in ischemic rat brain. *Neurosci Lett* 1999;274:45–48.
- 85. Chen J, Jin K, Chen M, et al. Early detection of DNA strand breaks in the brain after transient focal ischemia: implications for the role of DNA damage in apoptosis and neuronal cell death. *J Neurochem* 1997;69:232–245.
- 86. Canman CE, Chen CY, Lee MH, et al. DNA damage responses: p53 induction, cell cycle perturbations, and apoptosis. *Cold Spring Harb Symp Quant Biol* 1994;59:277–286.
- 87. Chopp M, Li Y, Zhang ZG, et al. P53 expression in brain after middle cerebral artery occlusion in the rat. *Biochem Biophys Res Commun* 1992;182:1201–1207.
- 88. Chen J, Zhu RL, Nakayama M, et al. Expression of the apoptosiseffector gene, *bax*, is up-regulated in vulnerable hippocampal CA1 neurons following global ischemia. *J Neurochem* 1996;67: 64–71.
- 89. Krajewski S, Mai JK, Krajewska M, et al. Upregulation of Bax protein levels in neurons following cerebral ischemia. *J Neurosci* 1995;15:6364–6376.
- Martinou JC, Dubois-Dauphin M, Staple JK, et al. Overexpression of bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* 1994; 13:1017–1030.
- 91. Linnik MD, Zahos P, Geschwind MD, et al. Expression of bcl-2 from a defective herpes simplex virus-1 vector limits neuronal death in focal cerebral ischemia. *Stroke* 1995;26:1670–1674.
- 92. Lawrence MS, McLaughlin JR, Sun GH, et al. Herpes simplex viral vectors expressing bcl-2 are neuroprotective when delivered after a stroke. *J Cereb Blood Flow Metab* 1997;17:740–744.
- Chen J, Simon RP, Nagayama T, et al. Suppression of endogenous bcl-2 expression by antisense treatment exacerbates ischemic neuronal death. *J Cereb Blood Flow Metab* 2000;20:1033–1039.
- 94. Hara H, Fink K, Endres M, et al. Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ice inhibitory protein. *J Cereb Blood Flow Metab* 1997;17: 370–375.
- 95. Hara H, Friedlander RM, Gagliardini V, et al. Inhibition of interleukin 1beta converting enzyme family proteases reduces is-

- chemic and excitotoxic neuronal damage. *Proc Natl Acad Sci USA* 1997;94:2007–2012.
- Chen J, Nagayama T, Jin K, et al. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci* 1998;18:4914– 4928
- 97. Ankarcrona M, Dypbukt JM, Bonfoco E, et al. Glutamate-in-
- duced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* 1995;15:961–973.
- 98. Bonfoco E, Krainc D, Ankarcrona M, et al. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA* 1995;92: 7162–7166.