INTRODUCTION

Brain ischemia refers to a neurological condition that brain blood flow is insufficient to meet metabolic demand. There are two kinds of brain ischemia: (1) focal ischemia in which ischemia is confined to a specific region of the brain and (2) global ischemia, which affects the entire area of brain or forebrain tissue. Focal brain ischemia is a subtype of stroke along with subarachnoid hemorrhage and intracerebral hemorrhage. Global brain ischemia may occur in many pathological conditions, such as cardiorespiratory arrest.

Necrosis

Necrotic cell death or necrosis is an accidental type of cell death in living tissue and always caused by pathological factors, such as energy failure after brain ischemia. Cell death due to necrosis is always passive, and thus does not need activation of a particular cellular signaling pathway. A typical necrotic process due to brain ischemia encompasses swelling of the cell and subcellular organelles, followed by multiple organelle damage, the loss of cell membrane integrity, and an uncontrolled release of cellular contents into the surrounding extracellular space (Fig. 43.1). As a result, necrosis usually initiates an inflammatory response in the surrounding areas of cell injury, which may be contained locally. However, severe necrosis may also lead to systemic inflammation in the remote organs, such as thymus, spleen, and small and large intestines. The systemic inflammatory response may be mediated by the circulating inflammatory signals, such as damage-associated molecular pattern molecules released from necrotic cell or tissue. Untreated necrosis can lead to a buildup of decomposing dead tissue and cell debris at or near the site of the cell death, resulting in tissue infarction.

Apoptosis

Apoptotic cell death or apoptosis is an active cellular process, can occur naturally, and requires activation of particular apoptotic pathways in multicellular organisms. In contrast to necrosis, which is a form of accidental cell death, apoptosis is physiologically required for tissue or organ formation during development or lifecycle. Therefore, the naturally occurring apoptosis is evolutionarily designed to provide beneficial effects to the organism by eliminating unwanted cells. The morphological characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation, and formation of apoptotic body (Fig. 43.2). Unlike necrosis, apoptosis usually does not initiate the inflammatory responses as apoptotic bodies can be quickly removed by phagocytes [1]. Apoptosis occurs naturally and extensively during normal brain development, which is critical for the establishment of a definitive pattern of neuronal connections. However, some pathological conditions may accidentally initiate the same apoptotic pathways to induce unwanted cell death. For example, in immature or developing neurons, an episode of brain hypoxia-ischemia (HI) turns on the apoptotic pathways to induce neuronal death [2].

Apoptotic pathways can be divided into caspase-dependent or caspase-independent. The caspase-dependent pathway can also be classified into the extrinsic and intrinsic pathways. The extrinsic pathway is mediated by the so-called death receptors including tumor necrosis factor (TNF) receptors, Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptor. The specific binding
FIGURE 43.1  Electron micrographs of cortical neurons from a sham-operated rat (A) and rats subjected to 120 min of focal ischemia followed by 1 h (B) and 24 h (C) of reperfusion (Hu et al., 2001), and a postnatal 26-day rat subjected to 30 min of hypoxia-ischemia followed by 48 h of recovery (D) [5]. (A) In sham, ribosomal rosettes (arrows), the endoplasmic reticulum (ER), mitochondria (M), nucleus (N), and Golgi (G) are normally distributed; (B) at 1 h of reperfusion, ribosomes are clumped into aggregates (arrowheads). Golgi apparatus disappears to form vacuoles (V). The ER and mitochondria (M) are severely swollen. The nucleus (N) seems not changed; (C) At 24 h of reperfusion, a necrotic neuron shows membrane damage (arrows), shrunken nucleus with clumped tigroid chromatin, irregular amorphous organelles, and vesicular structures and vacuoles; (D) an acute necrotic neuron shows “burst” to release its entire content.

FIGURE 43.2  Electron micrographs of dentate gyrus (DG) neurons from a postnatal 7-day rat pup subjected to 60 min of hypoxia-ischemia (HI) followed by 48 h of recovery [5]. (A) A normal DG neuron from the contralateral hemisphere after HI and (B) apoptotic DG neurons from the ipsilateral hemisphere after HI. The apoptotic neurons have classical apoptotic ultrastructural features including condensed nucleus as a dark mass ball (N), formation of filament bundles (arrows), and a phagocyte, probably microglia (M), attaching to the apoptotic neuron. The phagocyte contains both primary and secondary lysosomes (arrowheads). Scale bar = 2 μm.
of Fas-ligand, TNF-α, or TRAIL to their corresponding receptors leads to recruitment of the adaptor-protein Fas-associated death domain or complex-I/II to activate caspase-8, followed by activation of caspase-3. In the intrinsic apoptotic pathway, release of cytochrome c from mitochondria leads to activation of caspase-9. Activated caspase-9 works together with apoptotic factor-1 (Apaf-1) to activate caspase-3. Activated caspase-3 executes apoptosis via cleaving specific substrates, such as caspase-activated DNase (CAD) and poly(ADP-ribose) polymerase (PARP) to induce apoptosis. The caspase-independent apoptotic pathway is mediated via apoptosis-inducing factor (AIF). AIF normally resides just behind the mitochondrial outer membrane. When a mitochondrion is damaged, AIF may be released to the cytoplasm and then translocate to the nucleus to induce DNA fragmentation and chromatin condensation, resulting in apoptosis. In different cell types and conditions, increasing number of other factors, such as B-cell lymphoma 2 (Bcl-2), Bcl2-associated X protein (BAX), Bax-like BH3 protein (BID), Bcl-2 homologous antagonist/killer (BAK), or Bcl-2-associated death promoter (BAD), B-cell lymphoma-extra large (Bcl-Xl), are also involved in the apoptotic pathways, suggesting that the apoptotic processes are considerably more complicated and diversified than the simplified caspase-dependent and caspase-independent pathways described earlier.

Despite the widespread use of apoptosis or necrosis, in some cases, dying cells may have molecular and morphological overlap features of necrosis and apoptosis, which has occasionally been referred to as “necroptosis” and “apoptosis-like cell death.”

### Autophagy

Autophagy is the cellular machinery of late endosome/lysosomal degradation of unnecessary or dysfunctional cellular components. There are three basic types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is the process in which aberrant cellular components or organelles are sequestered by double-layer membranes to form autophagosomes (APs), and then the APs fuse with lysosomes to degrade the AP’s cargo. Microautophagy is mediated by direct lysosomal engulfment of aberrant cytoplasmic contents. Chaperone-mediated autophagy refers to the chaperone-dependent selection and delivery of a particular group of aberrant cytosolic proteins to lysosomes for degradation.

Macroautophagy is the major type of autophagy, thus commonly referred to as autophagy (hereafter). Fig. 43.3 shows simplified basic steps of the (macro) autophagy pathway, consisting of: (1) AP formation; (2) AP-to-lysosome fusion to form autophagolysosome or simply autolysosome (AL); and (3) lysosomal degradation of AP and its cargo by hydrolases. The biochemical cascade for AP formation begins with ATG1 (ATG=autophagic gene-related protein). Activated ATG1 facilitates incorporation of two key ATG complexes into a double-membrane cistern known as phagophore: (1) ATG12-ATG5 complex and (2) ATG8-phosphatidylethanolamine (PE) complex. In mammalian cells, ATG8 is also known as microtubule-associated protein 1A/1B-light chain 3-I (LC3-I), whereas ATG8-PE

**FIGURE 43.3** (Top) Simplified basic steps of the autophagy pathway consisting of autophagosome (AP) formation, AP-to-lysosome fusion, and AP and its cargo degradation. (Bottom) Electron micrographs of autophagic ultrastructures of hippocampal neurons from rats subjected to 15 min of ischemia followed by 4 h of reperfusion. (A) The arrow denotes a double membrane cistern or phagophore. (B) An AP containing dilated endoplasmic reticulum with ribosomes. (C) An autophagolysosome (AL) with partially digested cellular structures. ATG, autophagic gene-related protein; LC3, light chain 3. Scale bar = 0.2 μm.

IV. MOLECULAR MECHANISMS
is the active form and referred to as LC3-II. After incorporation of ATG12-ATG5 and LC3-II complexes, the phagophore is able to grow to envelope aberrant cellular components and organelles, and eventually becomes an AP. Therefore, the electron microscopy manifestation of double membrane structures containing cellular components and organelles represents the gold standard for quantification of cellular APs [3]. An AP is then merged with a late endosome/lysosome to form an AL for bulk degradation of both AP and its cargo content. Autophagy is a very sophisticated nonstop life-sustaining process to main cell homeostasis, which is active under normal conditions and further enhanced in response to cellular stress [4].

IV. Molecular Mechanisms

APOPTOSIS, NECROSIS, AND AUTOPHAGY AFTER BRAIN ISCHEMIA

Neuronal apoptosis has been repeatedly and consistently observed in neonatal HI models [1,5]. However, it is still a subject of debate about the role of apoptosis in the etiology of neuronal death in adult brain after ischemia. It may be important to understand neuronal death modes after brain ischemia as therapeutic intervention may target key molecular events involved in the particular cell death process. It is generally held that persistent or permanent brain ischemia will lead to typical necrotic cell death, followed by inflammatory responses. Neuronal necrosis often occurs in the core area after focal brain ischemia. However, after a brief episode of global cerebral ischemia followed by reperfusion and in the penumbra after focal ischemia, some neurons may die selectively, whereas the others, as well as glial and vascular cells may be preserved, i.e., the selective neuronal death. It remains controversial whether selective neuronal death in the penumbra after focal ischemia, or some population of neurons (e.g., hippocampal CA1 pyramidal neurons) after transient global ischemia, is apoptotic or necrotic, or a combination of both types of cell death.

Since the discoveries of apoptotic genes and mechanisms about two decades ago, selective neuronal death after brain ischemia has usually been deciphered as the apoptotic type. Many studies have reported that pro-apoptotic genes, such as caspases, BAX, BID, BAK, and BAD, and antiapoptotic genes, such as Bcl-XL and Bcl-2, are up- or downregulated in brain tissue samples after ischemia. Release of cytochrome c from mitochondria, upregulation of Apaf-1, activation of CAD and PARP, and translocation of AIF into the nuclei after brain ischemia have also repeatedly been reported.

However, the hypothesis of apoptotic neuronal death after brain ischemia has constantly been challenged by a considerable number of observations. First, it has been consistently and repeatedly observed that the morphological feature of selective neuronal death in mature brain is not apoptotic, but rather necrotic (Fig. 43.1 [2,5–7]. Second, assays of internucleosomal DNA damage with TUNEL (transferase-mediated dUTP nick end labeling) or electrophoretic DNA ladder have very frequently been used to identify apoptosis in many brain ischemia studies, but both assays are not apoptosis specific [8,9]. This is because both TUNEL and DNA ladder assays reflect DNA damage that occurs also in necrotic neurons. As a result, both TUNEL staining or electrophoretic DNA ladder are also positive in necrotic cells. Therefore, TUNEL positive and appearance of the DNA ladder may be insufficient evidence to conclude that selective neuronal death is apoptotic.

The active caspase-3 assays may be relatively specific for caspase-dependent apoptosis. Several studies show that, in neonatal brain, almost all dead neurons after HI are positive for active caspase-3 immunostaining, but it is extremely hard to find active caspase-3-positive dead neurons in the ipsilateral hippocampus of mature brain after HI (Fig. 43.4A). As controls, active caspase-3 is negative in the contralateral hippocampus [2,5]. These results are consistent with the fact that typical morphological features of apoptosis can be seen in immature neonatal neurons after HI (Fig. 43.2), but only necrotic morphological features can be observed in mature neurons after ischemia (Fig. 43.1). Furthermore, along with brain maturation, the mRNA of caspases-3 is gradually and dramatically reduced in brain samples, suggesting that caspase-3 may not even be expressed in neurons when brain is matured (Fig. 43.4A,B). Other apoptosis-related mRNAs, such as caspase-2, Bcl-2, and BAX, are also markedly reduced during brain maturation, whereas Fas, Fas-ligand (FASL), and Bcl-x L/S are not obviously changed (Fig. 43.4B). Two housekeeping genes L32 and GADPH are unchanged (Fig. 43.4B). These studies suggest that the caspase-3-dependent apoptotic machinery may be faded out or significantly diminished in neurons during brain maturation.

Several factors may be accountable for the apoptotic and necrotic neuronal death discrepancy in the literature. For example, many studies were carried out using neuronal culture ischemia-like models, such as hypoxia, hypoglycemia, oxygen glucose deprivation, or oxidative stress. However, primary cultured neurons may not represent mature, rather than developing neurons, as primary neuronal cultures are mostly derived either from rodent E18 embryos or from newborns. This is consistent with the fact that active caspase-3 is highly positive in 7-day primary neuronal cultures after exposure to oxidative stress inducer tert-butyl hydroperoxide (Fig. 43.4C), whereas active caspase-3-positive neurons are extremely hard to find in P60 mature neurons after HI (see Fig. 43.3A). Furthermore, ischemic brain injury is not limited...
to neurons, but may also occur in astrocytes, oligodendroglia, microglia, and invading leukocytes (e.g., macrophages, neutrophils, and lymphocytes). These nonneuronal cells should have strong intrinsic apoptotic machinery and may undergo apoptotic cell death after brain ischemia. Moreover, adult brain contains some progenitor cells and immature neurons mostly located in the subventricular zone and the subgranular zone of dentate gyrus. These immature cells in adult brain should still have built-in apoptotic machinery and thus may undergo apoptosis after brain ischemia. This view is supported by the observation that caspase-3-positive cells may very occasionally be found in the subventricular and subgranular zones of adult brain after global brain ischemia or HI (Fig. 43.4D).

**The Prosurvival and Prodeath Role of Autophagy After Brain Ischemia**

Evolutionarily, autophagy is a prosurvival mechanism for cleaning up cellular “garbage” to maintain cell homeostasis. Therefore, autophagy is constitutively required for neuronal survival. This is supported by key autophagy component knockout mouse studies, in which autophagy deficiency leads to accumulation of APs and neurodegeneration [10]. Numerous compelling pharmacological studies have also indicated that impairment of the autophagy pathway results in accumulation of APs, aberrant organelles, and cell death [11]. These studies support the prosurvival role of autophagy in pathological conditions.

Although controversial, it has repeatedly been suggested that the excessive autophagy activity may contribute to selective neuronal death after brain ischemia, the so-called autophagic cell death (ACD). The term “autophagic cell death or ACD” was originally coined in the 1970s in a purely morphological context to refer to dying cells containing numerous ALs, and was, in fact, not to suggest autophagy inducing cell death [12]. The prodeath hypothesis suggests that excessive activation of autophagy might induce cell death via overdigestion of cellular contents or by activation of apoptotic enzymes. An argument against
ACD may be that the large-scale accumulation of APs in dying cells may not be the cause of cell death, but rather reflects a cellular attempt to remove aberrant components and organelles or interruption of the late steps of the autophagy pathway after brain ischemia [13]. Morphological and histochemical studies so far did not prove a causative relationship between the autophagic process and cell death. ACD may also be defined as a type of cell death that can be suppressed by specific inhibition of the autophagy pathway. Although some pharmacological studies have shown that inhibition of autophagy reduces cell death, none of the presently available pharmacological agents are exclusively for inhibition of autophagy. Even some key autophagic proteins like LC3, ATG5, and ATG12 have autophagy-independent functions, including the role in cell death, endocytosis, and immunity-related GTPase trafficking [14,15]. For these reasons, the presence of ACD in pathological conditions remains elusive.

The inconsistent conclusions drawn from previous studies about the role of autophagy in selective neuronal death after brain ischemia may also be owing to misunderstanding of the experimental results. First, increases in AP numbers or the level of LC3-II protein are often misinterpreted as upregulation of autophagy in pathological conditions. In the field of tissue ischemia and reperfusion, several studies show that an increase in LC3-II protein level after brain ischemia is not because of upregulation of autophagy activity, but rather a result of impairment of the later lysosomal degradation step of the autophagy pathway or disruption of autophagic flux [11]. Autophagic flux refers to the complete dynamic process of the entire autophagy pathway including AP formation, AP fusion with late endosome/lysosome, and late endosome/lysosomal degradation of AP and its cargo. An increase only in the LC3-II level is insufficient to suggest upregulation of autophagy because it is only an upstream component of the entire autophagy pathway. In many cases, an increase in the LC3-II level is caused by impairment of lysosomal degradation of AP. For example, overproduction of aberrant cellular substances and damaged organelles after brain ischemia may overwhelm the downstream lysosomal degradation of AP, resulting in an increase in the LC3-II level in brain tissue samples [13]. Accordingly, the use of the LC3-II level as an autophagic activity marker must be complemented by assays to determine the overall autophagic flux, to permit a correct interpretation of the results [4]. Second, some studies use nonspecific autophagic agents or methods, and thus these studies may be inconclusive. Chemical inhibitors for autophagy are widely used for studying autophagy, but none of the current chemical agents are specific for autophagy [14]. Third, various cell types, such as neurons, astrocytes, oligodendroglia, microglia, and invading leukocytes (e.g., macrophages, neutrophils, and lymphocytes) may contribute to the changes in the tissue autophagic activity after brain ischemia. Therefore, change in the tissue LC3-II level may reflect a net result of mixed changes among diverse cell types after brain ischemia. Autophagy is likely to be activated in microglia and invading inflammatory cells to digest engulfed contents. Inhibition of autophagy might reduce the activity of phagocytes attacking ischemic tissues. Therefore, studying of the entire autophagy process, rather than a small portion of it (e.g., increase in LC3-II level), and dissecting the autophagy changes in different brain cell types may be critical to understanding the real role of autophagy in selective neuronal death after brain ischemia.

### MECHANISMS OF SELECTIVE NEURONAL DEATH AFTER BRAIN ISCHEMIA

Much brain ischemia research has been focused on the studies of the selective neuronal death, probably because selective neuronal death is more likely rescued or prevented in the ischemic penumbra than the pan-necrosis in the ischemic core after focal brain ischemia. Significant progress has been made toward our understanding of the underlying mechanisms of selective neuronal death after brain ischemia. In addition to the apoptotic cell death pathways and autophagy, many hypotheses have been postulated, including excitotoxicity, calcium influx, acidosis, inhibition of protein synthesis, excessive production of reactive oxygen species, subcellular organelle damage or dysfunction, endoplasmic reticulum stress, stress response, protein misfolding and aggregation, and so forth. Earlier electron microscopic studies show that electron-dense deposits are accumulated in living CA1 pyramidal neurons destined to undergo selective neuronal death after transient cerebral ischemia [6,16]. At that time, the identity and molecular composition of these deposits were unknown. Hu and colleagues have carried out a series of morphological, biochemical, and molecular studies demonstrating that these electron-dense deposits are protein aggregates made of misfolded proteins [11]. Protein aggregates are clumped together with organelle membranes, which become the foremost subcellular structures in neurons before selective neuronal death occurs after brain ischemia. Therefore, protein aggregation and multiple organelle failure may contribute to selective neuronal death after brain ischemia [11].

In summary, ischemic stroke leads to necrotic neuronal death in the ischemic core area and selective neuronal death in ischemic penumbra. It remains controversial whether selective neuronal death after a brief episode of global cerebral ischemia and in the ischemic penumbra after focal ischemia is apoptotic or necrotic, or a combination of both types of cell death. Autophagy
in neurons is likely an indispensable step for removing toxic substances and damaged organelles after brain ischemia. Autophagy in inflammatory cells, however, might be a double-edged sword; overactivation might make inflammatory cells capable of attacking repairable brain tissue especially at the early stage after ischemic stroke.

References

CHAPTER
44
Mechanisms of Glial Death and Protection

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INTRODUCTION

Cerebrovascular diseases cause tissue damage to both gray and white matter, which contribute about half of the CNS volume and differ in structure and cellular composition. White matter exclusively contains axons and their glial cell partners including fibrous astrocytes, oligodendrocytes (myelinating and nonmyelinating), and microglia. Gray matter harbors neurons and is rich in protoplasmic astrocytes, which shape synaptic transmission as they partner with nerve endings and postsynaptic elements to form the tripartite synapse.

Pharmacological developments of potential treatments for stroke have failed in clinical trials because they typically aimed at protecting neurons from postischemic damage and neglected glial cells, especially