

Signaling of Cell Death and Cell Survival Following Focal Cerebral Ischemia: Life and Death Struggle in the Penumbra

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Abstract. Focal ischemia by middle cerebral artery occlusion (MCAO) results in necrosis at the infarct core and activation of complex signal pathways for cell death and cell survival in the penumbra. Recent studies have shown activation of the extrinsic and intrinsic pathways of caspase-mediated cell death, as well as activation of the caspase-independent signaling pathway of apoptosis in several paradigms of focal cerebral ischemia by transient MCAO to adult rats and mice. The extrinsic pathway (cell-death receptor pathway) is initiated by activation of the Fas receptor after binding to the Fas ligand (Fas-L); increased Fas and Fas-L expression has been shown following focal ischemia. Moreover, focal ischemia is greatly reduced in mice expressing mutated (nonfunctional) Fas. Increased expression of caspase-1, -3, -8, and -9, and of cleaved caspase-8, has been observed in the penumbra. Activation of the intrinsic (mitochondrial) pathway following focal ischemia is triggered by Bax translocation to and competition with Bcl-2 and other members of the Bcl-2 family in the mitochondria membrane that is followed by cytochrome c release to the cytosol. Bcl-2 over-expression reduces infarct size. Cytochrome c binds to Apaf-1 and dATP and recruits and cleaves pro-caspase-9 in the apoptosome. Both caspase-8 and caspase-9 activate caspase-3, among other caspases, which in turn cleave several crucial substrates, including the DNA-repairing enzyme poly(ADP-ribose) polymerase (PARP), into fragments of 89 and 28 kDa. Inhibition of caspase-3 reduces the infarct size, further supporting caspase-3 activation following transient MCAO. In addition, caspase-8 cleaves Bid, the truncated form of which has the capacity to translocate to the mitochondria and induce cytochrome c release. The volume of brain infarct is greatly reduced in Bid-deficient mice, thus indicating activation of the mitochondrial pathway by cell-death receptors following focal ischemia. Recent studies have shown the mitochondrial release of other factors; Smac/DIABLO (Smac: second mitochondrial activator of caspases; DIABLO: direct IAP binding protein with low pI) binds to and neutralizes the effects of the X-linked inhibitor of apoptosis (XIAP). Finally, apoptosis-inducing factor (AIF) translocates to the mitochondria and the nucleus following focal ischemia and produces peripheral chromatin condensation and large-scale DNA strands, thus leading to the caspase-independent cell death pathway of apoptosis. Delineation of the pro-apoptotic and pro-survival signals in the penumbra may not only increase understanding of the process but also help to rationalize strategies geared to reducing brain damage targeted at the periphery of the infarct core.

Key Words: Apoptosis; Bax; Bcl-2; Caspase; Fas; Focal ischemia; Smac/DIABLO.

INTRODUCTION

Brain ischemia is a major catastrophic event that may lead to cell death in the vulnerable areas. Focal brain ischemia is most commonly the result of the occlusion of one of the carotid or the vertebral arteries or their branches and is usually caused by thrombosis or embolism. Experimental models of focal ischemia may be produced by ligation or by intra-luminal occlusion of the middle cerebral artery (MCAO) in rats and mice.

Focal permanent or transient MCAO and reperfusion produce massive cell death in the central core of the infarction. Yet the effects of ischemia and reperfusion depend, among other elements, on the duration of MCAO and the extent of reperfusion.

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Neurons in the central core of the infarction die by necrosis, which is characterized by the sudden failure of cellular energy, and swelling and rupture of the organelles. It has been considered that necrosis is a paradigm of passive cell death, as opposed to apoptosis or active cell death, in which cellular organelles merely break down. However, necrosis occurs in a scenario that permits the uncontrolled function of several proteases, calpains, and other enzymes that disrupt protein assemblies. Moreover, distinct proteins that control DNA repair in physiological conditions turn into cell killers under determinate pathological settings. Poly(ADP-ribose) polymerase-1 (PARP-1) binds to DNA strand breaks and metabolizes β -nicotinamide adenine dinucleotide (NAD^+) into polymers of ADP-ribose; thus, poly(ADP-ribose)ylation at specific sites facilitates the recruitment of the genomic repairing complex and has a key role in DNA redressing (1). However, enhanced PARP-1 activity and poly(ADP-ribose)ylation after focal ischemia promote cell death by NAD^+ and ATP depletion; PARP gene disruption or drug-dependent PARP inhibition generate resistance to cerebral ischemia (2, 3). These findings point to the likelihood that necrosis is a more complex phenomenon than conventionally sanctioned. Therefore, insights geared to increased understanding of the activation of

certain cell death signals that occur in necrosis will offer promise of the control of the cell death cascade following oxygen deprivation and ATP depletion in the infarct core.

Definition of Penumbra

Functional studies have identified the penumbra as a rim of tissue that is hypo-perfused around the ischemic core. It has been shown that blood flow in the penumbra is too low to maintain electrical activity but is sufficient to preserve ion channels (4). This area may also be visualized by positron emission tomography and magnetic resonance imaging-based techniques such as diffusion and perfusion-weighted imaging (5).

In addition to suffering from very sub-optimal perfusion levels, cells in the penumbra are subject to deleterious factors produced by neighboring cells, including various cellular responses to glutamate and aspartate release in the ischemic core leading to excitotoxicity. These include spreading depression, nitric oxide and reactive oxygen species causing oxidative stress, inflammatory cytokines, adhesion molecules, and metalloproteinases that facilitate the penetration of leukocytes through blood vessels, and endothelins that may increase vasoconstriction and consequent supplemental hypo-perfusion (5, 6).

Although many factors involved in necrosis may act in the penumbra, several metabolic reactions are different in the infarct core than in the penumbra. Early responses to focal ischemia include reduction of protein synthesis and induction of 70-kDa heat shock (hsp-70) mRNA (7). Permanent abolition of protein synthesis occurs in the infarct core and is associated with cell death, whereas recovery of protein synthesis helps cell survival. Heat shock proteins have a function in protein-protein interactions, such as protein folding, assembly, secretion, and degradation. HSP-70 is expressed in the cytoplasm at very early stages following stressful stimuli; it then extends to dendrites and finally to axons. HSP-70 has also been detected in microglial cells at the borders of the infarct (7). Moreover, HSP-70 down-regulates apoptosis (8). Interestingly, the chance of HSP-70 mRNA translation to HSP-70 protein is restricted to the neurons and glial cells in the penumbra, suggesting that the putative effects of HSP-70 can be expected at the periphery of the infarct core. In keeping with the suspected protective role of HSP-70 in the penumbra, mice over-expressing rat heat shock protein 70 are protected against cerebral infarction (9).

Proteins coded by proto-oncogenes, such as Fos, are involved in the regulation of gene transcription rates and participate in crucial cellular responses as they trigger cytoplasmic signals to the nucleus, where they interact with the DNA. Neurons in the central nervous system express c-fos in response to cerebral ischemia. Yet the role of c-fos mRNA induction following brain ischemia is controversial (10). It has been asserted that continuous

c-Fos expression precedes programmed cell death *in vivo* (11). However, c-fos mRNA is induced even after a transient episode of focal ischemia that has no lethal effects on the brain (12). Moreover, c-Fos protein is expressed in neurons of the penumbra. Many of these cells co-localize inducible HSP-70 protein, but the targets of c-Fos, acting on AP-1 sequences in DNA, differ from those of HSP-70 that influence protein assembly.

At short times following focal ischemia, neurons in the penumbra initiate an active process of signals opposing cell death, and they stop dying (13–15). Yet susceptibility to brain ischemia in the penumbra varies from one region to another, and brainstem neurons may survive for longer periods than cortical neurons in the penumbra, which may die after 6 hours (h) of reperfusion (6). Further interest in the penumbra is based on the fact that an infarct appears to grow with time at the expense of the penumbra, and that neurons in the penumbra are susceptible to internal and external stimuli that permit the delineation of strategies geared to reducing the infarct size.

Limitations of the Term Apoptosis Applied to Cells Dying in the Penumbra

Numerous studies using combined *in situ* end labeling of nuclear DNA fragmentation and DNA gel electrophoresis methods have detected DNA fragmentation in several ischemia models in the adult and developing brain (16). The method of *in situ* end-labeling of nuclear DNA fragmentation, formerly used as a marker of apoptosis, decorates in particular scattered neurons at the periphery of the infarct core at early stages following MCAO, thus suggesting apoptosis as the form of cell death in the penumbra (17, 18). Yet the number of cells stained with this method augments with time in the infarct core, thus indicating that the *in situ* method has the capacity to decorate any cell bearing nuclear DNA breaks (Fig. 1). In addition, no morphological evidence of classical apoptosis (i.e. early peripheral chromatin condensation, extremely dark and compacted nuclei, and formation of apoptotic bodies) has been detected following focal ischemia in the adult rodent brain (19). Furthermore, DNA fragmentation following ischemia, as seen on gel electrophoresis of isolated DNA, differs from classical apoptosis, as the bands are larger than those multiples of 180–200 base pairs resulting from unique endonuclease activation in apoptosis (20). Therefore, the term apoptosis, based on morphological and DNA ladder patterns, offers little help in understanding the metabolic events that occur in the penumbra following ischemia. Moreover, the term apoptosis is becoming progressively confusing because of the accumulation of unrelated properties that have been added to the classical concept (21). Nevertheless, classical apoptosis and cell death in the penumbra have in common sophisticated cellular

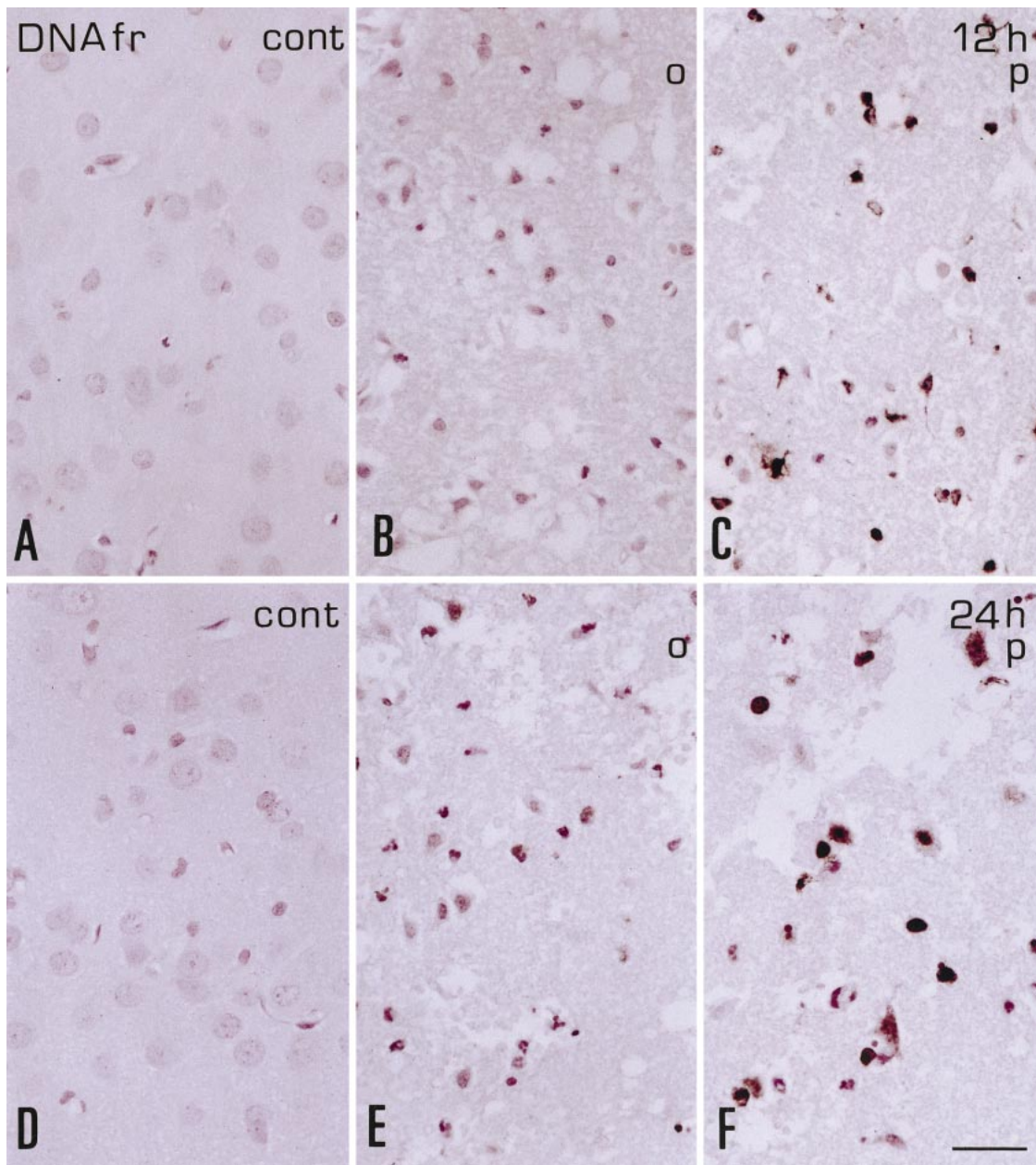


Fig. 1. Nuclear DNA fragmentation, as revealed with the method of in situ end-labeling of nuclear DNA fragmentation, at 12 h (A–C) and 24 h (D–F) following permanent MCAO in the rat. Positive cells are seen in the penumbra at 12 h (C), and in the infarct core and penumbra at 24 h (F). No positive cells are seen in the infarct core at 12 h in spite of the prevalent shrunken appearance (B). No positive cells are found in the contralateral cortex (A, D). Paraffin sections, Apoptag: in situ detection kit (Oncor, Lab Clinics, Barcelona, Spain), slightly counterstained with hematoxylin. Abbreviations: cont: contralateral; o: infarct core; p: penumbra. Scale bar = 25 μ m.

responses that are shared with programmed cell death during normal development.

Crucial proteins commanding programmed cell death in mammals are expanded families of proteins that are homologous to products regulating programmed cell death in *Caenorhabditis elegans*. These include the Bcl-2 family, caspases, and apoptosis protease-activating factor-1 (Apaf-1). These proteins are also activated in

several in vitro models of induced apoptosis and following ischemia in the penumbra.

The *bcl-2* family of proto-oncogenes, the mammalian counterpart of *ced-9* that negatively regulates cell death in *C. elegans*, encodes specific proteins that can either protect against or activate programmed cell death in several physiological and pathological conditions. Bcl-2 promotes cell survival, whereas Bax accelerates apoptotic

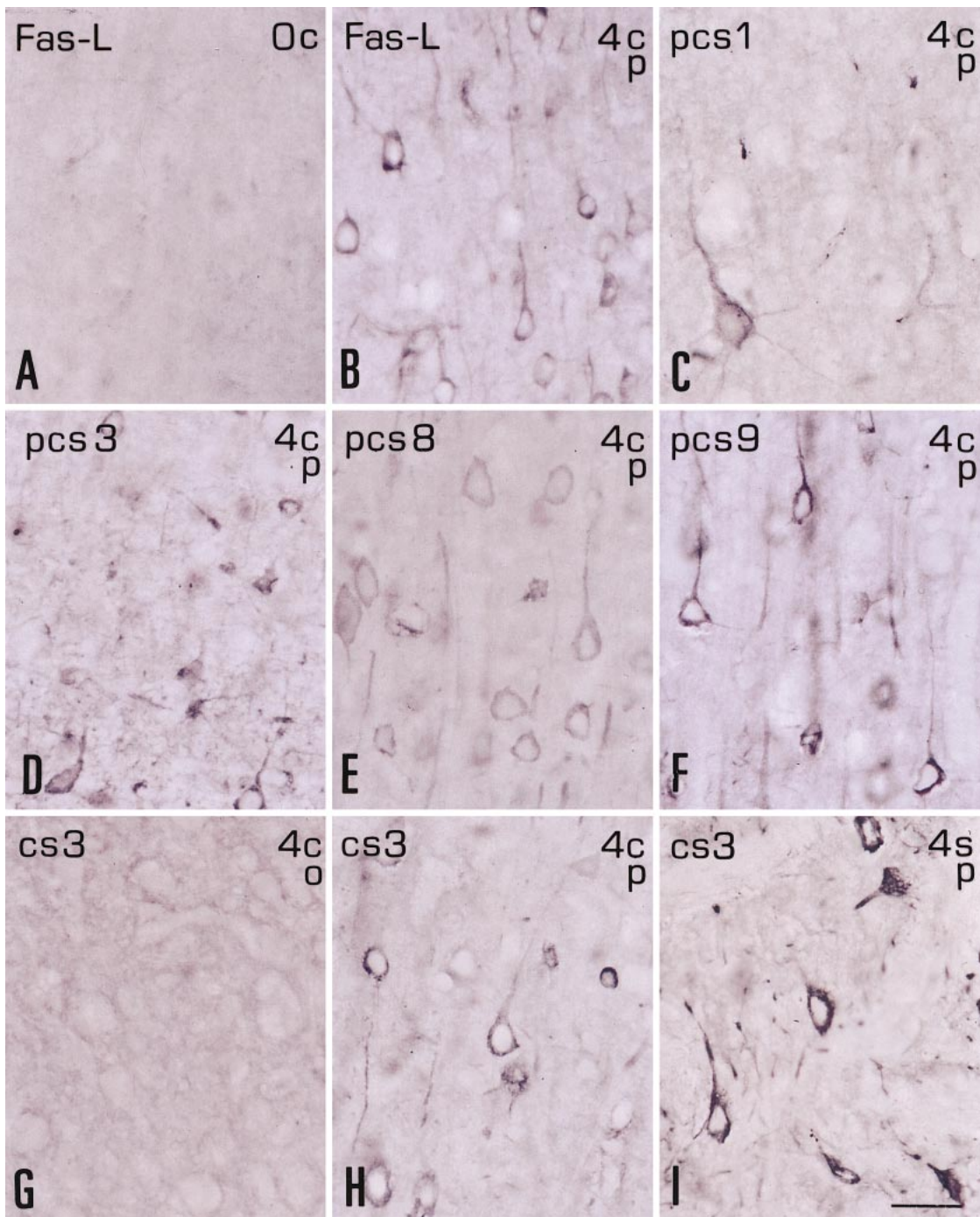


Fig. 2. Fas-L immunoreactivity in the cerebral cortex of control (A) and ischemic rats following 1 h of MCAO and 4 h of reperfusion (B). Marked increase in Fas-L immunoreactivity is seen in neurons in the penumbra when compared with the lack of immunostaining in control rats. Increased pro-caspase-1 (C), pro-caspase-3 (D), pro-caspase-8 (E), and pro-caspase-9 (F) immunoreactivity is found in the cytoplasm of neurons in the penumbra at 4 h following reperfusion. This contrasts with the absence of pro-caspase expression in the infarct core (G). Cleaved (active) caspase-3 immunoreactivity decorates the cytoplasm of cortical (H) and striatal (I) neurons in the penumbra 4 h after reperfusion. 0: control; 4: four h of reperfusion; c: cortex; s: striatum; o: core; p: penumbra. Vibratome sections without hematoxylin counterstaining. Antibodies Fas-L: sc-834, dilution 1:50 (Santa Cruz Biotechnology, Quimigranel, Madrid, Spain); pro-caspase-1: sc1218, dilution 1:1,000; pro-caspase-3: sc-1225; pro-caspase-8: sc-6134; pro-caspase-9: sc-8297, all with dilution of 1:100; active caspase-3 (17kDa): 9661S, dilution 1:50 (Cell Signaling, Izasa, Madrid, Spain); avidin-biotin-peroxidase (ABC) method; peroxidase reaction visualized with diaminobenzidine, NH_4NiSO_4 and H_2O_2 . Scale bar = 25 μm .

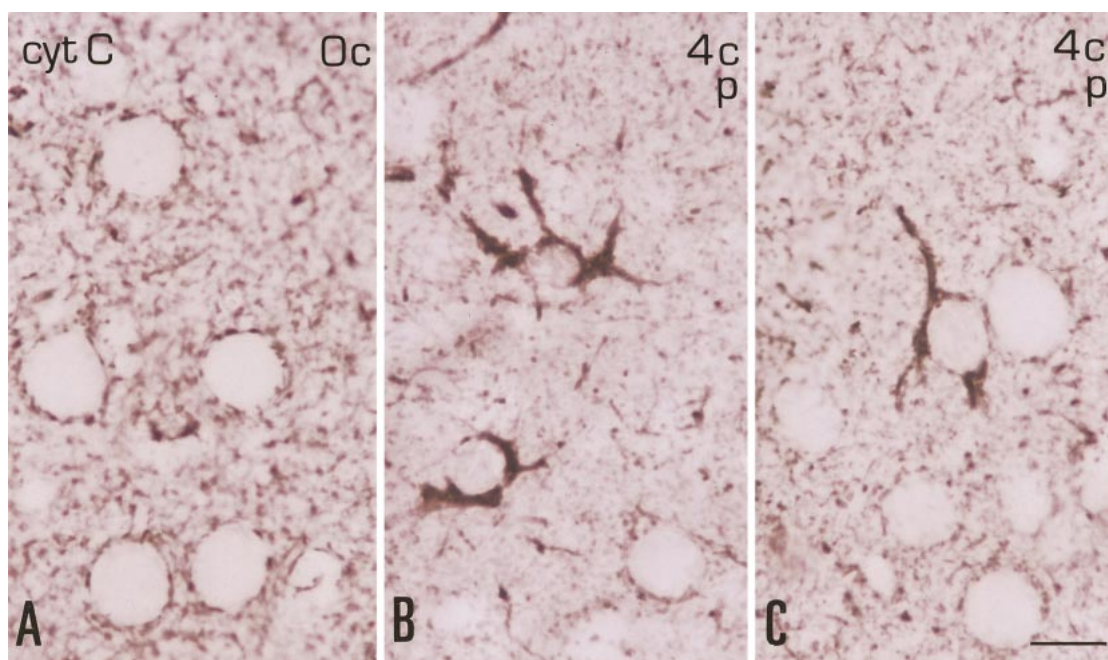


Fig. 3. Cytochrome c expression in the cerebral cortex of control rats shows a fine granular precipitate in the cytoplasm of neurons and neuropil (A). Strong homogeneous precipitate is seen in neurons of the penumbra following 1 h of MCAO and 4 h of reperfusion (B, C), a pattern consistent with cytochrome c release from the mitochondria to cytosol. 0: control; 4: four h of reperfusion; c: cortex; p: penumbra. Vibratome sections without hematoxylin counterstaining. The cytochrome c monoclonal antibody was obtained from Pharmingen (Becton Dickinson, Madrid, Spain), dilution 1:100. Scale bar = 10 μ m.

cell death. The *bcl-2*-related gene *bcl-x* encodes 2 proteins: Bcl-xL, which inhibits cell death, and Bcl-xS, which inhibits cell survival (22, 23).

Caspases are cysteine proteases constitutively expressed as zymogens or pro-caspases that, after activation, can cleave substrates (including pro-caspases) at aspartic acid residues. The family of caspases corresponds to the expanded representation of death-promoting *ced-3* in *C. elegans*. Caspases can be divided into 2 groups: upstream instigators (caspase-8, -9, and -10), the cascade initiators that can activate other caspases; and downstream terminators that cleave essential cellular substrates, thus destroying the cell (caspases-3, -6, and -7). Caspase-1, -2, -4, -5, -11, and -12 can act as initiators and executioners (24, 25).

Apaf-1 is the mammalian homologue of the *C. elegans* pro-apoptotic *ced-4* gene. Apaf-1 is a key protein of about 130 kDa in the apoptosome. Interactions with other proteins, particularly with cytochrome c and pro-caspase-9, activate caspase-3 and cell death (26, 27).

Basic Mechanisms of Programmed Cell Death: Signaling through Death Receptors and Caspase-Dependent Pathways

Programmed cell death depends on a complex program that, basically, may be initiated by external signals or by the activation of the mitochondrial pathway. Caspase-12 is an exception, as it appears to be activated following

endoplasmic reticulum stress. The extrinsic (death-receptor-mediated) pathway includes the Fas receptor, a surface receptor belonging to the tumor necrosis factor receptor family, which binds to the Fas ligand (Fas-L). The Fas/Fas-L signaling system activates Fas-associated death domain (FADD) and caspase-8, which in turn activates caspase-3 through an extrinsic pathway independent of mitochondria. The intrinsic (mitochondrial) pathway is initiated by Bax translocation to the mitochondrial membrane and competition with other members of the Bcl-2 family. This is followed by cytochrome c leakage to the cytosol, the binding of cytochrome c to Apaf-1, dATP and pro-caspase-9, and subsequent activation of caspase-9. Active caspase-9 cleaves pro-caspase-3 and other executioner caspases, thus leading to cell death. External stimuli also activate the mitochondrial pathway as caspase-8 may impact on cytochrome c release through cleavage of pro-apoptotic Bid, another member of the Bcl-2 family (28–30).

Caspases are effective in a large number of substrates, acting as scissors on structural and vital proteins, including PARP. In this respect, it is important to note that PARP-1 over-activation depletes NAD^+ and ATP, and it is associated with necrosis, whereas PARP-1 cleavage resulting in the products of 89 and 28 kDa is characteristic of caspase activation and programmed/apoptotic cell death (31).

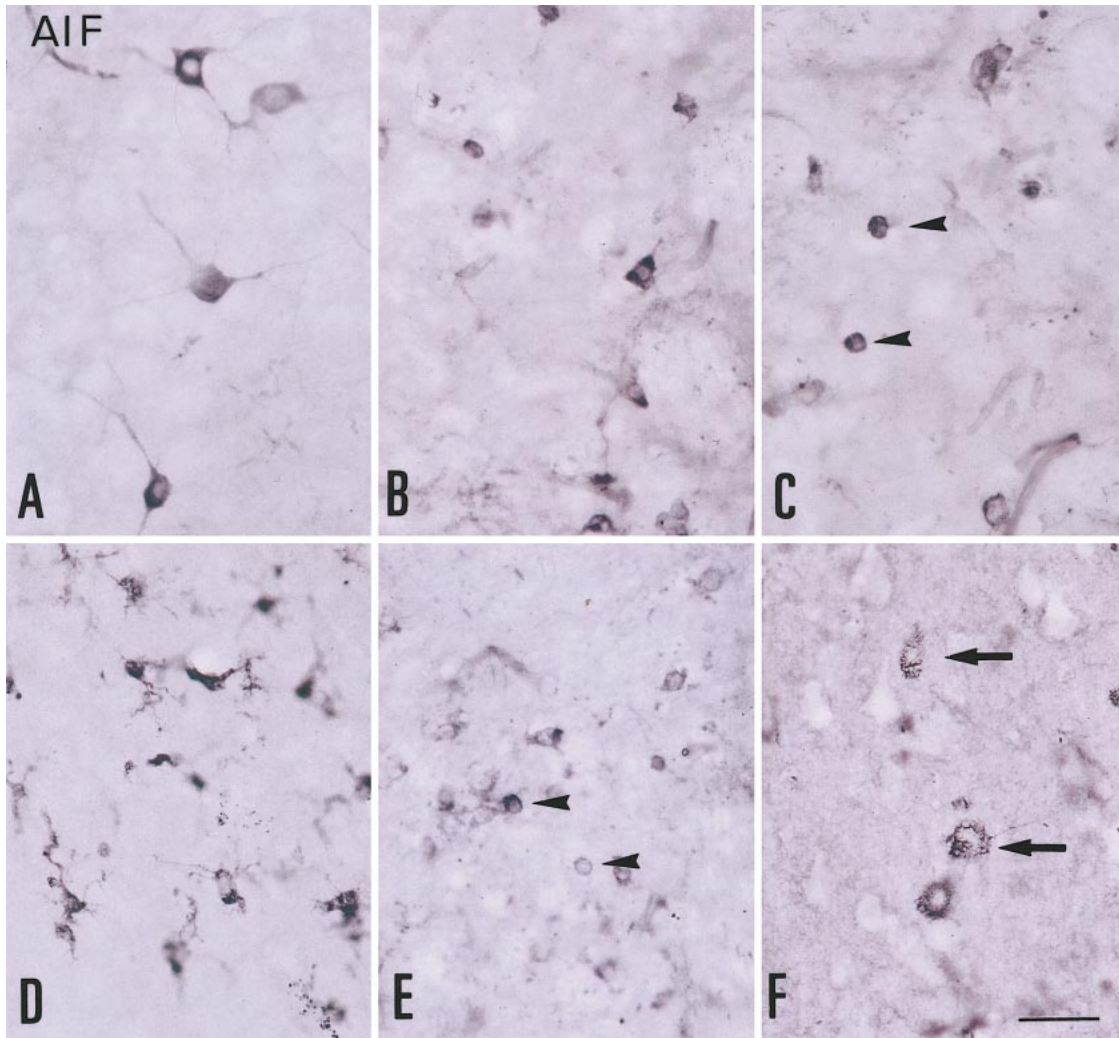


Fig. 4. Apoptosis-inducing factor (AIF) expression, following 1 h of MCAO and 4 h of reperfusion, in the penumbra of the cerebral cortex (**A, C**) and striatum (**B, E**). AIF immunoreactivity is observed in the cytoplasm and in the nuclei (arrowheads) of selected neurons. Clusters of glial cells in the underlying white matter are also stained with anti-AIF antibodies (**D**). Rarely, neurons show disrupted AIF immunoreactivity (**F**, arrows). Vibratome sections without hematoxylin counterstaining. The same findings are visualized with the antibodies AIF Ab-1 and Ab-2, dilution 1:100 (Oncogene, Bionova Científica SL, Madrid, Spain) directed against the carboxy and amino terminus domains of AIF, respectively. Scale bar = 25 μ m.

Caspases may also regulate apoptosis by alternative and complementary pathways. Caspase-2 may induce apoptosis by releasing cytochrome c and other pro-apoptotic proteins into the cytosol, thus indicating that caspase-2 is a direct effector of the mitochondrial apoptotic pathway (32). In addition to promoting the cleavage of pro-caspase-3 and activation of Bid and then of the mitochondrial apoptotic pathway, active caspase-8 translocates to the nucleus of apoptotic neurons in a rodent model of acute ischemia and inactivates PARP-2, another member of the poly(ADP-ribose) polymerase family involved in DNA repair (33).

The inhibitor of apoptosis (IAP) family includes the X-linked inhibitor of apoptosis (XIAP) that blocks cell death by repression of the terminal caspase cascade,

mainly caspase-3 and caspase-7 (34, 35). XIAP binds with the active caspase-9/Apaf-1 complex and blocks apoptosis (36). Yet XIAP is negatively regulated by Smac/DIABLO; Smac (second mitochondrial activator of caspases) or DIABLO (direct IAP binding protein with low pI) is released from the mitochondria during apoptosis and binds to IAP, thus preventing IAP inhibition of caspases (37, 38).

These observations demonstrate that apoptosome is a major complex that regulates cell death under appropriate conditions. Apaf-1, in the presence of cytochrome c and dATP, oligomerizes to form a large apoptosome complex of 700–1,400 kDa that recruits and processes caspase-9 to form a holoenzyme complex, which in turn recruits and activates caspase-3. The apoptosome is regulated by

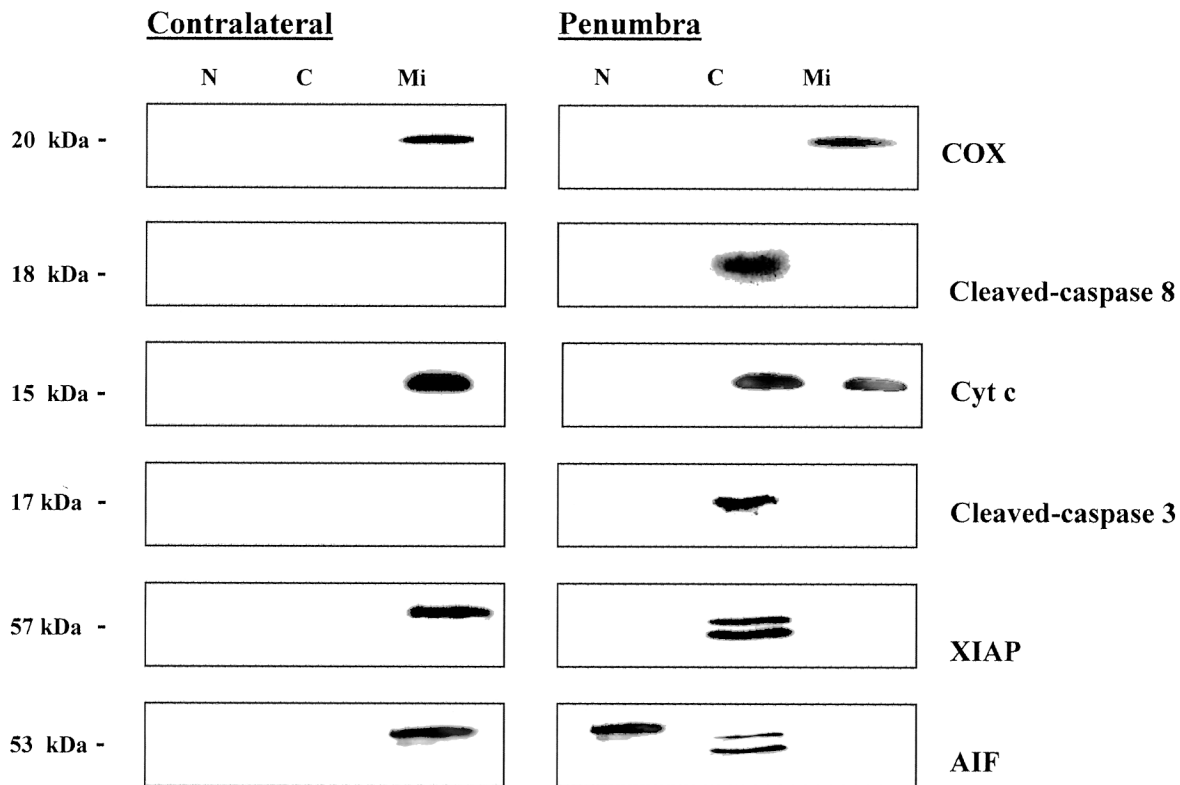


Fig. 5. Sub-cellular distribution in the nucleus (N), cytosol (C) and mitochondria (Mi) in the cerebral cortex of the penumbra, and in the contralateral cerebral cortex, following ischemia by transient MCAO in the adult rat. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (15% SDS-PAGE) shows cleavage of caspase-8 (18 kDa), cytochrome c (cyt c) release to the cytosol and caspase-3 activation (cleaved caspase-3 17 kDa) in the penumbra, after 1 h of ischemia and 4 h of reperfusion. In addition, X-linked inhibitor of apoptosis (XIAP) is translocated to the cytosol and cleaved in the penumbra at this particular time-point of reperfusion, thus probably indicating XIAP loss of function. Together, these findings indicate activation of the death-receptor and mitochondrial caspase-dependent cell death pathways. Finally, apoptosis-inducing factor (AIF) translocates from the mitochondria to the cytosol and nucleus in the penumbra, thus facilitating activation of the caspase-independent cell death pathway. The antibody to cytochrome oxidase subunit IV (COX) (Molecular Probes, Madrid, Spain), dilution 1:2,000, was used as a marker of mitochondrial protein loading in the gels. The cleaved caspase-8 (dilution 1:50), cleaved caspase-3 (dilution 1:50) and XIAP (dilution 1:100) antibodies were obtained from Cell Signaling (Izasa). The cytochrome c monoclonal antibody (dilution 1:500) was obtained from Pharmingen (Becton Dickinson), and the IAF (Ab-1) antibody (dilution 1:500) from Oncogene (Bionova Cientifica SL). After washing, the membranes were incubated with the secondary antibody labeled with horseradish peroxidase (Amersham Pharmacia Biotech, Barcelona, Spain) and then developed with the chemiluminescence ECL Western blotting system.

a variety of factors, including intracellular levels of potassium, heat shock proteins, IAPs, and Smac/DIABLO (39, 40). Interestingly, Smac/DIABLO release is inhibited by Bcl-2 and Bcl-xL, thus preventing inactivation of XIAP (41, 42).

Programmed/Apoptotic Cell Death Signaling through Caspase-Independent Pathway

In addition to the caspase pathway, there is apoptosis-inducing factor (AIF), a new caspase-independent death effector which, following apoptotic stimuli, is released from the mitochondrial inter-membrane space to the cytosol and then to the nucleus where it causes chromatin condensation and large scale DNA fragmentation (43–45). The mitochondrio-nuclear redistribution of AIF is

prevented by a Bcl-2 protein specifically targeted at mitochondrial membranes, and AIF is neutralized by HSP-70 (44, 45). Recent studies have shown that PARP-1 activation signals AIF release from mitochondria (46). These exciting observations support a model in which PARP-1 mediates a caspase-independent pathway of programmed cell death.

Expression of Bcl-2 Family Members in Focal Cerebral Ischemia

Over-production of Bcl-2 in transgenic mice is associated with reduction of the infarct size following focal cerebral ischemia (47). Similarly, human Bcl-2 over-expression with herpes simplex virus vectors limits neuronal death in focal cerebral ischemia (48). Several studies

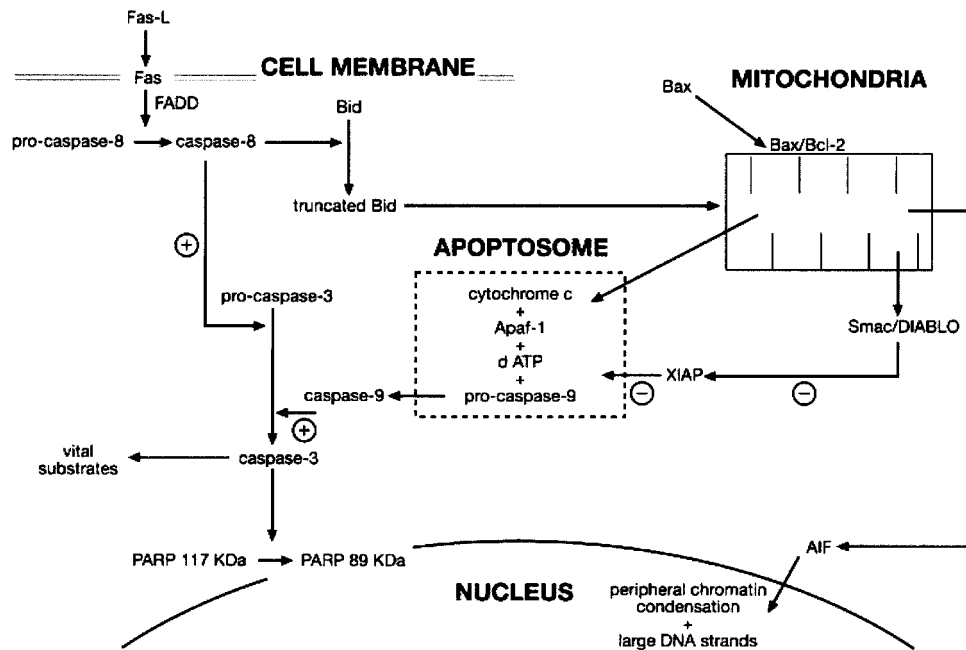


Fig. 6. Schematic representation of the death-receptor (extrinsic) and mitochondrial (intrinsic) caspase-dependent cell death pathways, and caspase-independent cell death signaling in the penumbra after transient focal cerebral ischemia and reperfusion. Abbreviations: FADD: Fas-associated death domain; Apaf-1: protease-activating factor-1; PARP: poly(ADP-ribose) polymerase; Smac: second mitochondrial activator of caspases; DIABLO: direct IAP binding protein with low pI; X-linked inhibitor of apoptosis; AIF: apoptosis-inducing factor.

in vivo have demonstrated modifications in the expression levels of members of the Bcl-2 family. Bax up-regulation has been found following ischemia (49) in association with Bcl-2 reduction in the infarct core (50). In contrast, the expression of Bcl-2 is increased in neurons of the penumbra (51). Yet co-localization of Bcl-2, Bax, and other members of the family (Bcl-x) in vulnerable neurons makes it difficult to define the exact expression level of each one of these factors required to induce cell death or to rescue individual cells from dying in paradigms of focal cerebral ischemia (52).

Death Receptors in Focal Cerebral Ischemia

The involvement of the death-receptor pathway following ischemia has been suggested by the demonstration of Fas mRNA induction after ischemia in the adult murine brain, and increased Fas protein expression in vulnerable areas following hypoxic-ischemic injury in the developing rat brain (53). Furthermore, a marked increase in the expression of Fas-L is found in the penumbra neurons at short times following MCAO and reperfusion in the rat (Fig. 2A, B), thus strongly indicating increased expression of the main proteins that initiate the Fas/Fas-L signaling pathway. More impressively, infarct resulting from focal cerebral ischemia is greatly reduced in mice expressing mutated (nonfunctional) Fas (54, 55). Increased pro-caspase-8 (56) and, especially, caspase-8 immunoreactivity has also been shown to occur after focal ischemia in the rat (57).

Activation of Caspase-Dependent Pathways of Apoptosis in the Penumbra

Increased pro-caspase-1, -3, -8, and -9 immunoreactivity is seen in the penumbra following permanent (56) and transient focal ischemia (Fig. 2C–F) in the adult rat. Mice deficient in interleukin-1 converting enzyme, ICE or caspase-1, are resistant to ischemic brain damage. However, implication of caspase-1 is probably dependent on the effects of caspase-1 on pro-inflammatory cytokines following ischemia rather than on the direct activation of the apoptotic pathway. Involvement of the mitochondrial pathway of apoptosis in cerebral ischemia is supported by cytochrome c release from the mitochondria to the cytosol following focal cerebral ischemia and reperfusion (58, 59) (Fig. 3). Cytochrome c release is coincidental with Bax translocation and activation of caspase-9 (59, 60), and this is followed by caspase-3 activation and caspase-3 expression in penumbra neurons (Fig. 2G, H), along with the formation of caspase-3-dependent products (89 kDa) of PARP (56, 61). Moreover, inhibition of caspase-3 reduces brain damage following focal stroke (15, 16, 61). Taken together, these observations indicate activation of caspase-dependent pathways of cell death in the penumbra following transient focal ischemia.

However, other studies have shown no evidence of activation of pro-caspase-3 or cytochrome c release to the cytosol in an experimental model of permanent focal ischemia (62). Consistent with these findings, administration of a specific caspase inhibitor does not have a

neuroprotective effect in this model of focal ischemia (62). The discrepancy between this and previous observations may be related to the extension and intensity of neuronal damage and to the predominance of necrosis over caspase-mediated cell death following permanent ischemia when compared with ischemia and reperfusion. Unfortunately, none of these experimental designs examines perfusion rates following stroke, thus making evaluation of the penumbra size according to functional criteria impossible.

Finally, truncated Bid (p15) translocates to the mitochondria in a murine model of focal ischemia, thus making activation of the mitochondrial pathway of apoptosis by cell-death receptor signals possible. Moreover, cerebral infarction and apoptosis are markedly reduced in the penumbra following MCAO in Bid-deficient mice (63).

Activation of the Caspase-Independent Pathway of Apoptosis in the Penumbra

In addition to activation of the caspase pathway of apoptosis, transient focal ischemia by MCAO is followed by increased AIF expression in the cytoplasm of scattered neurons and glial cells in the penumbra at 4 h after reperfusion (Fig. 4). Individual cells in the same area exhibit strong AIF immunoreactivity in the nucleus, thus suggesting nuclear translocation. Translocation of AIF from the mitochondria to the nucleus precedes chromatin condensation and DNA fragmentation in several paradigms (43–45). Disrupted positive cells are seldom observed, indicating that cells expressing AIF are indeed susceptible to cell death in the penumbra.

Further Complex Interactions around the Mitochondria

Recent Western blot studies in our laboratory have shown, in addition to cytochrome c release to the cytosol and caspase-3 activation, cleavage of caspase-8 in the penumbra (Fig. 5), thus indicating activation of the cell-death receptor transmitter and downstream activator of executioner caspases following ischemia. This is accompanied by Smac/DIABLO release to the cytosol, and XIAP cleavage in the penumbra following transient focal ischemia and reperfusion in the adult rat (Fig. 5), thus indicating blocking of pro-survival signals and loss of inhibition of the caspase-dependent apoptotic pathway at very precise time-points in the penumbra following MCAO. Finally, AIF translocation from the mitochondria to the cytosol and nucleus is further supported by sub-fraction studies and Western blot analysis (Fig. 5).

Concluding Comments

Taken together, these observations indicate complex reactions in the penumbra at short times following transient focal ischemia and reperfusion by MCAO in rats

and mice. Compiled findings support the notion of concomitant activation of the cell-death (extrinsic) and mitochondrial (intrinsic) pathways of apoptosis, as well as promotion of the mitochondrial release of downstream activators (i.e. cytochrome c) of executioner caspases through Bid. In addition, increased expression and nuclear translocation of AIF supports activation of the caspase-independent pathway of apoptosis in the penumbra. These observations are summarized in Figure 6. Considering the complexity of the scenario in the penumbra may help to increase understanding of the process, and to delineate strategies geared to reducing brain damage in the penumbra.

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