Caspase Function in Neuronal Death: Delineation of the Role of Caspases in Ischemia

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Abstract: Cerebral ischemia is one of the major causes of morbidity and mortality in the Western world. Despite extensive research, adequate therapies are still elusive. Neuronal degeneration and death are hallmarks of stroke/ischemia. Understanding how the death machinery executes neuronal death in ischemia will provide therapeutic targets. Key to the death machinery are caspases: the family of cell death proteases. While much data has been published regarding caspase involvement in models of ischemia, the pathways have not been thoroughly defined. The specification of the caspases critical for death has been hampered by the use of non-specific reagents. Thus many conclusions about specificity are unwarranted. In this review we discuss how caspases can be measured and review the existing knowledge of the roles of specific caspases in ischemia. We also discuss approaches to determining the molecules that execute ischemic death.

Keywords: Caspases, neuronal death, apoptosis, ischemia, stroke, IAPs.

INTRODUCTION

Proper development and maintenance of an organism requires a delicate balance of life and death at the cellular level. Disruption of this balance can be catastrophic. Unopposed survival leads to the development of tumors and neoplasias. Unopposed death leads to degenerative diseases. In the nervous system, unchecked death has been implicated in many neurodegenerative diseases, including Alzheimers disease, Parkinsons disease, and stroke/ischemia. The key to the balance of life and death is exquisite regulation of the apoptotic machinery. This machinery, first identified in *C. elegans*, is highly conserved from worm to human [1]. Understanding the molecular details of death mechanisms will allow the development of specific therapeutic interventions in diseases where an imbalance of life and death has occurred.

THE CASPASE FAMILY OF PROTEASES

Essential components of the death machinery are the caspase family of proteases. Caspases are cysteine aspartases with a conserved QACXG motif at the active site. This family of proteases is central to the execution of cell death and a better understanding of how the various caspases work will enable understanding of how the death pathways are regulated and can be manipulated in disease. There are 13 mammalian caspases which can be classified based on structure [2], cleavage specificity [3], mode of activation [4] and presumed function [5]. In the years since caspases were first identified, huge amounts of data have been acquired about their putative function in a variety of death pathways. Much of this data has used reagents and measures that do not have the specificity suggested by the conclusions about these data. Moreover, in the last two years, studies of caspase activation show that initiator and effector caspases are activated differently [6] and have changed our view of how caspase activation can be measured. This means that much previous data needs to be reinterpreted. In this review we will attempt to present the current view of how caspase activation and activity can be measured and how this can be applied to the study of caspase pathways in neuronal death.

Classification of Caspases

Structural classification divides the caspases into two groups, the long prodomain containing initiators and the short prodomain containing effectors (Figure 1). The prodomains of initiator caspases contain either CARD or DED domains that can interact with death domain containing adaptor proteins. Functional classification has further subdivided the initiator group of caspases into death initiators (caspases-2, -8, -9 and -10) and inflammation mediators (caspases-1, -4, -5, -11) [7]. While the inflammation mediators are considered by many to be irrelevant to apoptosis, it is now evident that these caspases can mediate apoptosis either directly or through the production of cytokines [8, 9]. Several caspases are unique to humans (caspases-4, -5 and -10) while caspase-11 is found only in rodents [1]. The caspase-12 gene is found in rodents and humans but the protein is not expressed in most humans [10].

ACTIVATION OF CASPASES

Recent work suggests that classification based on mechanism of activation groups the caspases into long and short prodomain categories as well [6]. All caspases are synthesized as inactive zymogens that require activation. Early work suggested that cleavage was a required step for

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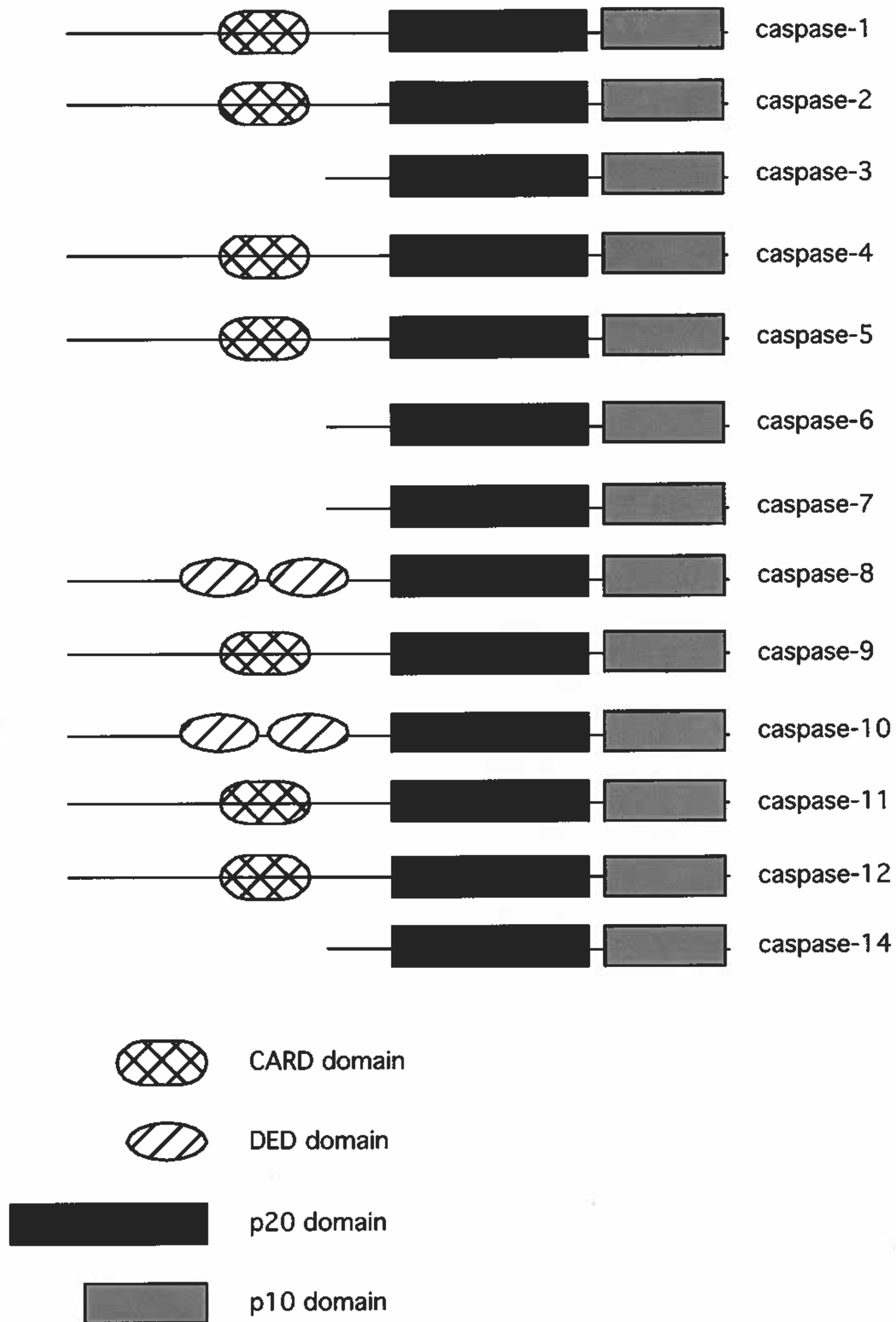
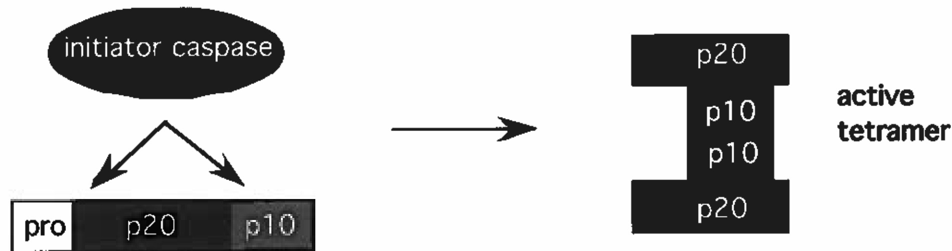


Fig. (1). Mammalian caspases. CARD = caspase recruitment domain, DED = death effector domain.

activation of all caspases [11]. However, work on caspase-9 indicated that this might not be true for all caspases: caspase-9 zymogen could have activity [12]. In the last two years it has become clear that the long prodomain containing caspases do not require cleavage for activation while caspases with short prodomains do [6, 13, 14]. Two methods of caspase activation are illustrated in Figure 2. The effector caspase zymogens exist as cytosolic dimers. Cleavage releases a large (p18-20) and small (p10-14) subunit from each dimer; these subunits combine to form a tetramer, which is the active enzyme. Cleavage can be by granzyme B, by another caspase, or by the caspase itself. Thus cleavage of effector caspases is a measure of activation. In contrast, caspases with long prodomains, initiator caspases, exist as monomers. Activation is by a proximity induced dimerization mechanism. Upon dimerization these caspases assume a conformational change resulting in an active enzyme without requiring cleavage. In the case of caspase-9,



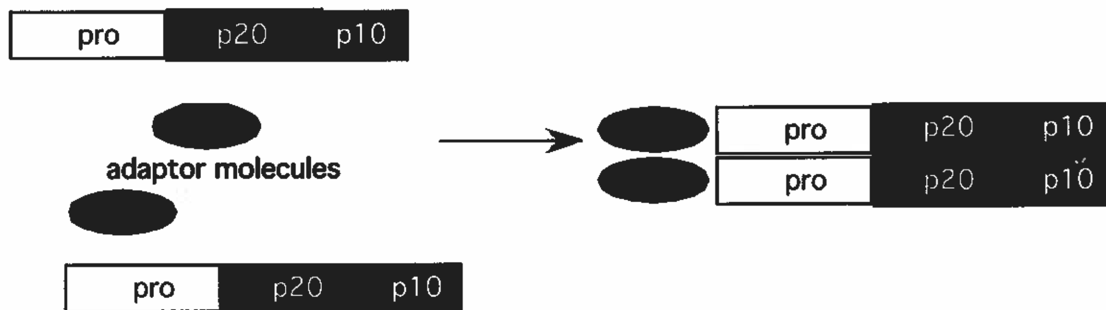


Fig. (2). Caspase activation mechanisms.

cleavage can potentiate inhibition of active caspase-9 by XIAP, an endogenous inhibitor of apoptosis [15]. Since initiator caspases are activated without cleavage, cleavage is not a valid measure of caspase activation. Many studies have employed measures of cleavage as synonymous with activation. How can activation be measured? For caspase-9, activation of caspase-3 is often used as a read-out. However, caspase-8 can also activate caspase-3. Thus, caspase-3 activation is not a specific measure of caspase-9 activation. Caspase-2 does not directly activate effector caspases. Studies of caspases-2 and -8 show that initial activation is via proximity dimerization and then the caspase cleaves itself to potentiate its activity [16, 17]. Therefore measures of cleavage can provide some indication of activation for these caspases, but cleavage is neither necessary nor sufficient for activation of initiator caspases [6].

Proximity induced dimerization can be effected by adaptor proteins that interact with the CARD and DED domains or potentially just by proximity of the monomers in the cellular environment [6]. There are specific adaptor proteins for several of the caspases. The best studied is APAF1, the adaptor protein for caspase-9 [18]. Release of cytochrome c from the mitochondria facilitates the assembly of the APAF-1-caspase-9 complex, the apoptosome, in an ATP-dependent manner. This complex can then activate effector caspases. RAIDD is a specific adaptor of caspase-2 and plays a role in caspase-2 activation during trophic deprivation mediated death of neurons [19, 20]. FADD is an adaptor of caspase-8, leading to formation of the death inducing signaling complex [21]. Two other "osomes" have been identified, the inflammasome, a complex that activates cytokine cleaving caspases [7, 22], and the piddosome, a complex that activates caspase-2 [23]. All the individual components of these structures have not been identified. The piddosome may be induced under toxic conditions that lead to induction of p53 which induces PIDD which binds to RAIDD to activate caspase-2. The roles of the inflammasome and piddosome in death have not been fully evaluated.

Regulation of Caspase Activity

There are different levels of caspase regulation to ensure that death is not accidental. When discussing caspase regulation it is important to distinguish between activation and activity. Activation refers to the conformational and structural changes that lead to the formation of the active enzyme; activity refers to the cleavage of substrates.

In addition to regulation of caspases by activation, there are also naturally occurring inhibitors and modulators of

inhibitors of caspases. S-nitrosylation of the active site cysteine of caspases by nitric oxide (NO) and peroxynitrite ($ONOO^-$) has been shown to reversibly inactivate multiple caspases, including caspases-1, -3, -7, -8 and -9 [24, 25] and is thought to be responsible for some of the neuroprotective effects of NO. The potential of NO to induce S-nitrosylation of caspases is felt to be due to factors that include the redox state of the cell [reviewed in [26]]. FLIP, a catalytically inactive structural homolog of caspase-8, can act either as an inhibitor or activator of caspase-8, depending on the expression level of FLIP [27]. Inhibition occurs at high expression levels by competition with caspase-8 for recruitment to the Death Inducing Signaling Complex (DISC). Activation occurs at low expression levels by the formation of heterodimers with caspase-8 that allow caspase-8 to assume an active conformation [28]. In mammals, the inhibitor of apoptosis protein (IAP) family of gene products are a highly conserved group of polypeptides which suppress apoptosis [29, 30]. Family members include cIAP1 and 2 and XIAP and their mouse orthologs MIAP1, 2 and 3, NAIP, and survivin [31]. The *in vivo* physiological function of IAPs in neuronal death has not yet been determined. Cell-free studies show that three of the IAPs (cIAP1/2 and XIAP) can bind to and inhibit caspases-3, -7, and -9 but do not bind to caspases-1, -2, -6, -8, or -10 [32-38]. cIAP1 interacts with TRAF2, part of the TNFR complex and can indirectly inhibit caspase-8 activity [39]. Binding of XIAP to caspase-9 requires cleavage of caspase-9 to reveal the XIAP binding site [15]. Thus, cleavage of caspase-9, rather than activating the enzyme, allows the inhibition of caspase-9 activity. IAPs can also be cleaved by caspases. XIAP is cleaved by caspases *in vitro* into 2 fragments, one (BIR1-2) specifically inhibits caspases-3 and -7 and the other (BIR-3-Ring) inhibits caspase-9 [35, 40], thus cleavage of IAPs by caspases may be an important part of the regulation of death. Two mammalian inhibitors of IAPs have been identified, Diablo/SMAC [41-44] facilitates caspase-9 and caspase-3 activity by blocking the inhibitory action of IAPs. HtrA2/Omi has both serine protease and IAP inhibiting activities [45-48]. Figure 3 schematically illustrates how the IAPs may regulate caspase activity.

There is also transcriptional regulation of some caspases. Methylation of the caspase-8 gene appears to be a mechanism for tumor development. The role of transcriptional regulation has not been well studied for most of the caspases; current knowledge is reviewed in [49] .

DEATH PATHWAYS

Different, specific caspase cascades are activated by different death stimuli [50]. In non-neuronal systems caspases have been organized into two pathways, the extrinsic, receptor mediated pathway and the intrinsic, post-mitochondrial pathway. These are shown in Figure 3. In each pathway a death stimulus leads to activation of an initiator caspase, which then leads to activation of executor caspases. Receptor pathways are mediated through the TNF super family. The prototype is FasLigand (FasL) mediated cell death [51]. FasL binds to the cell surface Fas receptor inducing recruitment of the death domain protein FADD and assembly of the DISC with activation of caspase-8 and subsequent activation of executioner caspases-3 and -7 and death. Other death receptors assemble similar complexes but the details may differ.

Intrinsic death pathways center on the mitochondrial release of apoptotic factors, including cytochrome c which interacts with APAF-1 in an ATP requiring assembly of the

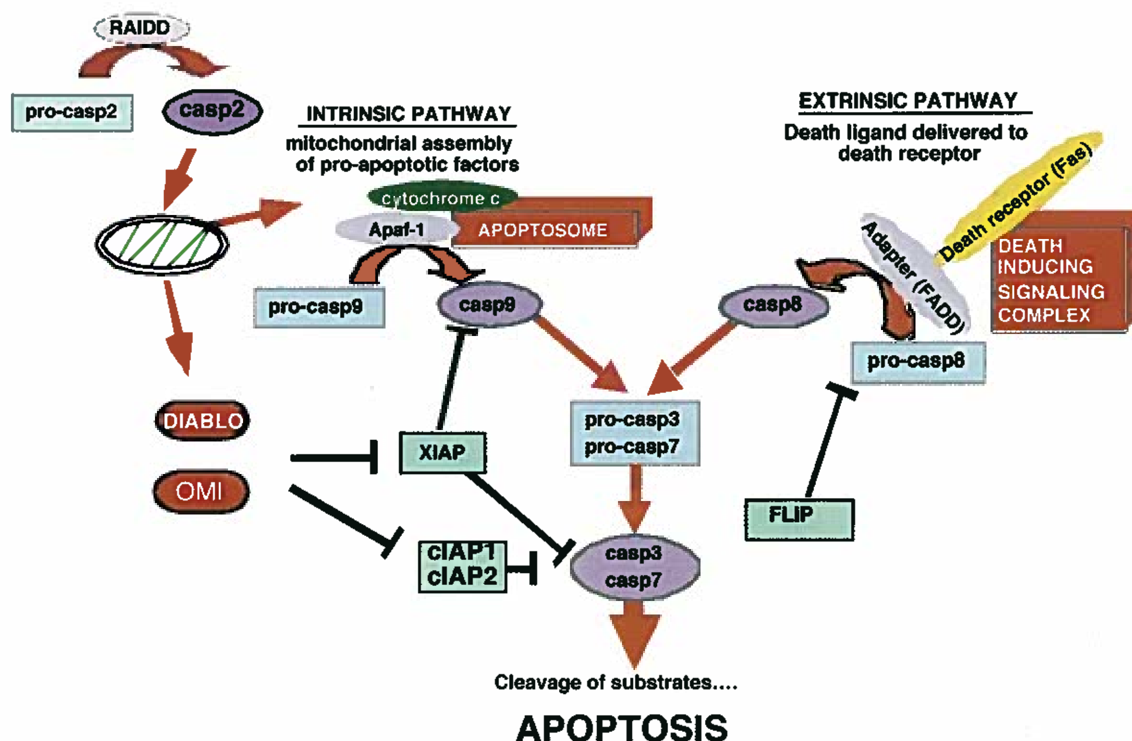


Fig. (3). Caspase pathways.

apoptosome, activating caspase-9 which then activates caspase-3 and -7 [50]. Data support a role for caspase-2 in permeabilizing mitochondria [52-55] which would place caspase-2 upstream of caspase-9, suggesting that there may be a cascade of initiators leading to executioner activation.

These pathways do not include any of the caspase-1 family caspases, or caspase-6, all of which have been implicated in neuronal death [8, 9, 50, 56, 57]. There also can be amplification loops: caspase-8 can cleave the BH3-only protein BID, leading to translocation of tBID to the mitochondria, release of cytochrome c and apoptosome assembly [58]. Caspase-2 can also act upstream of BID. There is also data to suggest that death can occur without mitochondrial involvement but that mitochondrial permeabilization potentiates death. In mammalian cells mitochondria appear to have an important function in cell death, either as a direct mediator of death or as an amplifier of death signals. The death pathways have not been as well studied in neuronal death. Morphologically, apoptotic death is not as clearly defined in neurons and dying neurons have been seen to have morphologic features of apoptosis and necrosis [59, 60]

Tools for Studying Caspase Function

What measures are available to determine if a specific caspase is critical for executing death? As noted in the preceding sections, many of the commercially available reagents may not provide data about specific caspases. In order to prove that a specific caspase is critical for death, it must be shown that ablation of that caspase prevents death. Evidence of activation and activity of that caspase with respect to the morphologic progression of death complements this data.

As discussed above, activation of effector caspases can be measured as cleavage to the p18/p12 peptides. Effector caspase activation can thus be detected in cell and tissues using antibodies which are specific for the cleaved caspase and do not detect the zymogen. There are antibodies available commercially for all of the effector caspases. It is important to check the specificity of the antibody in the system under study. There are also cleavage specific antibodies for several of the initiator caspases. Since cleavage is not required for activation, the usefulness of these reagents is limited. There is currently no good measure of activation of initiator caspases, as noted above. Activity measures usually rely on assays that use peptide pseudosubstrates. There are a variety of these substrates and many are marketed as specific for individual caspases. DEVD is used frequently as a measure of caspase-3 activity. Unfortunately, it is not specific for caspase-3 and, as used in the activity assay, detects most caspases. This lack of specificity is true, to varying degrees, for all the pseudosubstrates [3, 61]. The same peptides are also used as specific inhibitors of individual caspases and the same lack of specificity applies. For example, VDVAD, marketed as a specific inhibitor/substrate of caspase-2 is equally effective on caspases-3 and –7 [3, 61]. When evaluating published data, as well as when planning studies of caspases, it is important to understand the limitations of the reagents being used. Several of the peptide substrates have been adapted for use in live cells. As with the cell lysate activity assays, these will provide measures of general caspase activity but not of which specific caspases are active.

Another measure of activity is detection of cleavage of endogenous substrates of caspases. Caspase-1 processes pro-interleukin-1β (IL-1β) to mature IL-1β [62], which is secreted and can be measured in the media of cultured cell [9]. PARP is a death substrate, which is often utilized as a specific substrate of caspase-3 [63] but is also cleaved by other caspases [64]. LaminA is a specific substrate of caspase-6 [65].

This leaves few accurate measures of caspase activation or activity. What approach can be used to understand the caspase pathways? The most specific approach currently available is the molecular ablation of individual caspases. This can be done in cells and in animals. Caspase-null mice can be divided into those with devastating phenotypes (caspases-3, -8 and -9 null animals) and those with more subtle phenotypes (caspases-1, -2, -11, -12) [66]. The devastating phenotypes suggest an important role for these caspases during development. Lack of a profound developmental phenotype does not mean that a caspase has no function, rather that it is not as important during development but may have a role in maintaining homeostasis of the developed organism. There are also issues of redundancy of functions when one member of a multi-membered family is removed. A compensatory increase of caspase-9 has been shown in the neurons of caspase-2 null mice [67, 68]. The phenotype can also vary with the background of the mice. Original reports of caspase-3 null mice showed mice with abnormal neuronal development and neonatal lethality [69]. However changing the background of the caspase-3 null mouse has altered the phenotype to that of normal neuronal development in a mouse that matures and breeds [70]. This makes interpretation of the function of caspase-3 in neuronal development difficult.

The study of specific molecules has been greatly facilitated by RNA interference technology [71]. Small interfering RNA (siRNA) allow specific knockdown of targeted messages in mature neurons [72]. Studies suggest that siRNA may not have major advantages over antisense oligonucleotide knockdown [73], but the two approaches may be complementary. siRNA require the assembly of cellular machinery for their effect [74]. The machinery may be the rate limiting step in the use of multiple siRNA in one cell. However siRNA could be combined with antisense oligonuclotides, which do not use the same machinery, to specifically down-regulate multiple members of death pathways. In this way, individual molecules proposed to act in a death pathway can be specifically knocked down, both individually and in groups, to allow determination of function of the molecules in the pathway.

Caspases in Ischemia

Cerebral ischemia is one of the major causes of morbidity and mortality in the western world. Despite extensive research, adequate therapies are still elusive. The discovery of the caspase family of cysteine proteases was an important breakthrough for the understanding of the

molecular mechanisms of ischemic brain damage and opened a new area of possible therapeutic targets. Ischemia is an acute insult (hence the term stroke) that leads to chronic sequelae. Since it is acute and often clear when it occurs, the possibility of therapeutic intervention holds promise. Experimental research has shown numerous examples where interventions restraining caspase activity after ischemic episodes improve outcome. Thus, in rodent models of ischemia, prior administration of caspase inhibitors resulted in reduced infarct area and improved neurological outcome both after transient and permanent ischemia [75-80]. The neuroprotection of caspase inhibitors in ischemia is observed even when they are administered as late as 9 hours after the insult [79]. However, functional recovery is not always observed [81] and there are reports of a shift from apoptosis to necrosis after interventions that hamper the caspase pathways [82-84]. However, in neurons the morphologic differentiation between apoptosis and necrosis is not so clear.

A large literature exists concerning the role of caspases in ischemic neuronal death. Much of the work has looked at the appearance of cleaved caspases and measures of caspase substrate cleavage in various models. Multiple caspases have been implicated to play a role in ischemia model systems, including caspases-1, -3, -8, -9 and -11, discussed in more detail below. Work comparing caspase cleavage in the ischemic core with secondary cleavage in the penumbra found that caspases-1 and -8 were cleaved early in the core while caspases-9 and -3 were cleaved later in the penumbra, suggesting that different caspase cascades are activated during different phases of ischemia. Several studies have used caspase null animals to address the role of caspases. The involvement of caspase-3 in human brain ischemia is not yet clear: cleavage of caspase-3 and PARP are detectable in the brain hours after cardiac arrest, but not after atherothrombotic arterial occlusion [85, 86]. We will not present data using caspase pseudosubstrates and inhibitors because these data do not provide the specificity ascribed to them (see discussion of this above).

Caspase Involvement in Rodent Models of Ischemia

Caspases-1 and -11

One of the first implications of caspases in neurological diseases came from studies of caspase-1 in ischemic injury [87, 88]. Activation of caspase-1 leads to the generation of the pro-inflammatory IL-1β [62]. The caspases most closely related to caspase-1 are classified as the inflammatory caspases and have been considered as not directly related to the death pathways. However many recent studies questioned this view. The caspase-1 family members appear to be determinant elements in the death pathways induced by ischemia. Additionally, cytokines, such as IL-1β, have been shown to play a critical role in ischemia, so inflammatory caspases can play a double role – generating cytokines and potentiating death.

The expression of caspases-1 and -11 are increased after ischemia [89, 90]. Caspase-1 protein levels and activity are also increased in the ischemic brain [8, 76, 91-93]. The strongest evidence of the importance of caspase-1 and -11 in ischemic damage comes from genetically manipulated animals: mice expressing dominant-negative caspase-1 or mice deficient in caspase-1 or -11 showed significant reduction of the infarct volume and improvement of neurological deficits after ischemic injury [87, 88, 94-96]. Their inhibition or absence prevents activation of downstream caspases [8, 91]. This suggests that caspases-1 and -11 are apical elements in the ischemic death pathways.

Caspase-2

The data available about caspase-2 and ischemia suggest that this protease does not play an important role in this injury. In some studies, ischemia was shown to increase the expression and protein levels of caspase-2, but not in others [89, 93, 97-100]. Caspase-2 deficient mice are not protected from either transient or global ischemia [101] but it must be considered that these mice have an increase of caspase-9 and DIABLO/Smac in neurons that might obscure a dependence of ischemia on caspase-2 [67].

Caspase-3

Caspase-3 is the caspase that has been most studied in ischemia and strong evidence suggests that it significantly contributes to damage. Induction of caspase-3 messenger and protein levels has been found in different ischemic models in different species [81, 89, 91-93, 97, 100, 102, 103]. More significantly, caspase-3 cleavage and thus activation has been repeatedly observed after ischemia [91, 92, 104-108]. The recent development of viable mice lacking caspase-3 confirms its importance in damage: these mice showed reduced ischemic infarcts; however the mice still have cleavage of PARP that is felt to be mediated by caspase-8 [70].

Caspases-6 and -7

The effector caspases-6 and -7 are expressed in low levels in the brain (Van de Craen 1997) and thus it is assumed that they are minimally implicated in brain damage. But, as for most neurological injuries, their participation in brain ischemia has been poorly investigated. Ischemia induces an up-regulation of the mRNA of caspases-6 and -7 [89] and increases protein levels of caspase-6 [93]. No data is available about caspase-7 protein levels or about activation of these caspases in the ischemic tissue. Thus, the data with regard to actual roles for these caspases in ischemia are inconclusive.

Caspase-8

Ischemia leads to increased caspase-8 mRNA and protein levels [89, 93]. Cleavage of caspase-8 has been demonstrated in different ischemic models [91, 103, 108]. Ischemia also causes release of TNF-α by glia and neurons and up-regulation of Fas ligand and Fas receptor at the messenger and protein levels which may lead to assembly of the DISC and activation of caspase-8 [109-114]. Caspase-8 null animals die around embryonic day 12 [115] and thus cannot be studied for effects of ischemia in the adult. However, mice deficient in Fas ligand are resistant to ischemia [116]. Overall, the data about caspase-8 indicates that this protease participates in ischemic death pathways.

Caspase-9

Several studies indicate that the mitochondrial pathway contributes significantly to ischemic damage. Ischemia does

not up-regulate caspase-9 mRNA levels [89]. However there have been reports of an increase in cytochrome c release into the cytosol [102-104, 117-120], and apoptosome formation [117]. Numerous studies reported cleavage of caspase-9 after ischemia [117, 120, 121], but as discussed above caspase-9 cleavage is not required for activation. These data are suggestive of a role for caspase-9, but not conclusive. Caspase-9 null animals die before birth and thus are not available for studying adult ischemia.

Caspase-12

Caspase-12 is found in the ER and is thought to mediate ER stress induced death. Ischemia can induce caspase-12 mRNA and protein expression and cleavage, possibly as a consequence of ER stress [122, 123]. Caspase-12 does have a long prodomain and likely does not require cleavage for activation, but may utilize cleavage for potentiation of activity. Neurons with up-regulation of caspase-12 also show DNA fragmentation, suggesting that this protease may be involved in ischemic damage [123]. However, there are no reports indicating that absence or inhibition of this caspase is neuroprotective after ischemia. Therefore, if activation of caspase-12 is a consequence of stress in a dying cell or if it contributes to orchestrate the death is still unknown.

Role of IAPs in Cerebral Ischemia

A role for IAPs in ischemia is supported by the finding that surviving neurons in ischemic tissue upregulate the levels of XIAP and NAIP [124, 125]. Regulation of XIAP by release of Omi/HtrA2 during ischemia leading to a transient increase of an Omi-XIAP cytosolic complex has also been shown [126]. IAPs may influence the damage after ischemia by restricting the extent of damage by suppressing continuing caspase activation. IAPs can directly inhibit caspases-3, -7, and -9, providing a potential mechanism for therapeutically limiting cell death after ischemia. Experimental studies support this theory: overexpression of XIAP resulted in reduced caspase-3 activation, decreased number of cells with DNA damage, reduction in the infarct size and, most relevant, better neurological outcome [127, 128]. Overexpression of NAIP also reduces ischemic damage in the hippocampus [124]. Study of cIAP2 suggests that increased cIAP2 levels may be a key molecular mechanism underlying ischemic preconditioning [129].

Core *vs* Penumbra

Focal ischemia results in a rapid neuronal degeneration in the core of the lesion, where cerebral blood flow - values drop under 10%, whereas the surrounding tissue, the penumbra, with better but not enough perfusion to survive, degenerates over a longer time frame. The mechanisms of cell death are traditionally considered to be necrotic for the core and apoptotic for the penumbra. However, the dying neurons in the core do not show the morphological characteristics of necrosis and mixed features of both necrosis and apoptosis are observed [130]. Investigations show that caspases are activated in the ischemic core [91, 102, 108], supporting the view that active death mechanisms are implicated in the early ischemic damage. Interestingly, these studies involve different death cascades in the core and penumbra. In the core, the death receptor pathways linked to caspase-8 and caspase-1 are activated within 30 min after reperfusion, and are up-stream of caspase-3, while in the penumbra the mitochondrial pathway is also involved with a delayed timing [91, 104]. This reinforces the importance of studying the timing of apoptotic events.

Caspases Participate in Ischemic Preconditioning

The phenomenon in which brief periods of ischemia (ischemic preconditioning) confer strong neuroprotection to subsequent severe ischemic insult is known as ischemic tolerance [131, 132]. The molecular mechanisms underlying ischemic tolerance are not fully understood but there are indications that the caspase cascades are involved. In ischemic models of cultured neurons, treatment with a caspase inhibitor reduces the resistance to subsequent ischemia [133]. Ischemic preconditioning in rodents attenuates cytochrome c redistribution [134] and DEVD-ase activity [135] induced by a later severe ischemia. A recent study in rats reported that caspase-3 is activated in neurons that survive the second ischemic insult, but ischemic preconditioning promotes up-regulation of IAPs and blocks the mitochondrial release of Smac/Diablo, halting the caspase death cascade and enabling neurons to survive despite caspase-3 activation [129].

Approaches to Studying Caspases in Ischemia

In the preceding section we have presented the current knowledge about caspases in animal models of ischemia and this is illustrated in Figure **4**. Much of what is currently known is inferential and clearly many details of the pathways remain to be dissected out. There are several ways to obtain such information. Animal models provide the complexity that most closely resembles the human disease. However, this complexity can hamper the detailed study of molecular mechanisms. For the molecular dissection, cell models offer a system in which individual molecules can be manipulated and evaluated as etiologic factors in death. When a cell is slated to die, many events may occur that are consequences of the death program but not components of the death pathway. Elucidation of the actual death pathway requires the ability to correctly identify the causal death molecules. Accurate measures of these molecules, as discussed above for caspases, are essential. Also critical is establishing the time course of activation of the molecules with relation to the morphologic changes in the cell. In order for a molecule to be causal in the death pathway it must be activated at the appropriate time. It is also essential that ablation of the molecule in question prevents death. Current technology, particularly RNA interference, makes this relatively simple to do in cultured cells. Of course utility of cellular models requires that the models closely resemble the disease being modeled. The differences in the death mechanisms activated in the core and penumbral areas after ischemia suggest that it would be best to study each in a primary neuronal model.

When choosing models it is important to consider the overall relation of the cell model to the in vivo system. The model should show similar caspase dependence in a setting

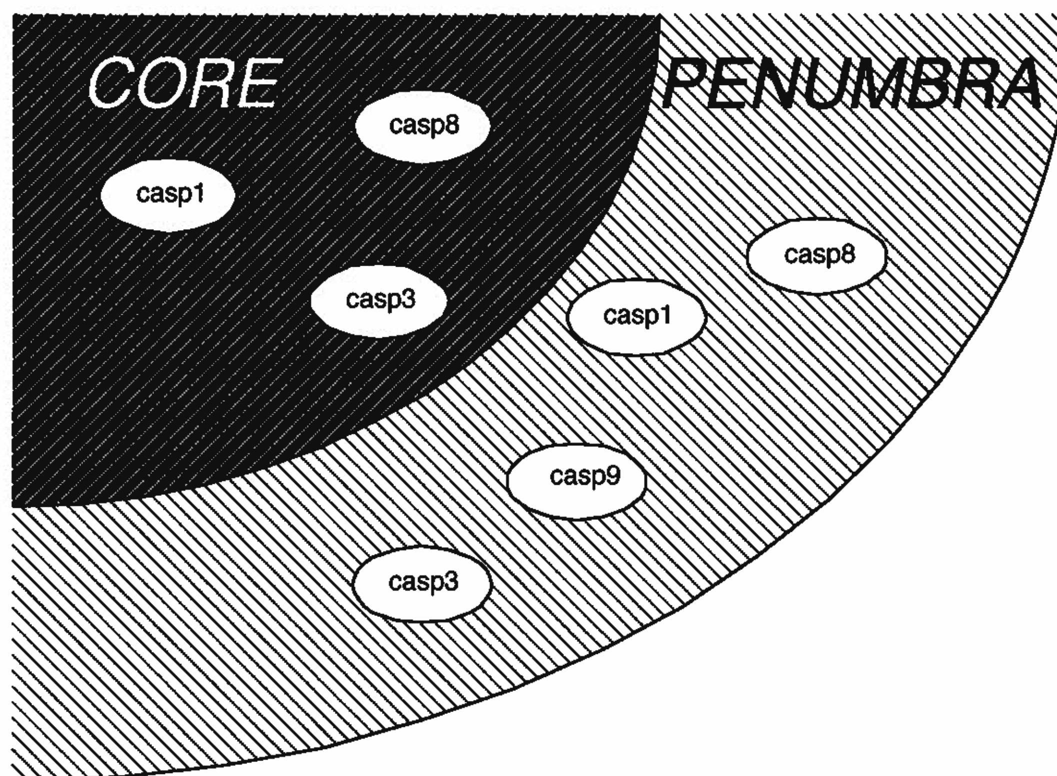


Fig. (4). Caspases in ischemia.

of death that is dependent on the other molecules that have been found to play critical roles in neuronal death after ischemia. These include IL-1β, NO and superoxide dismutase 1 (SOD1). Nitric oxide synthase (NOS) is the rate-limiting step in NO production and neuronal NOS null mice are resistant to ischemia [136] supporting a role for NO production in ischemia. IL-1β has also been proposed to contribute to neuronal death after ischemia. Studies show that postischemic infarct size and delayed neuronal death are decreased in IL-1β null mice [137, 138]. This supports studies showing that mice lacking caspase-1 are resistant to ischemia [94, 95]. There is also data suggesting a critical function for free radicals in the activation of caspases during ischemia. SOD is the cells' first line of defense against oxygen toxicity, catalyzing the dismutation of superoxide to hydrogen peroxide. Mice overexpressing SOD1 have a decrease in cleavage of caspase-8 and are resistant to ischemia [139] supporting a mechanism where superoxide generation leads to caspase activation and neuronal death after ischemia. Mice overexpressing SOD1 have recently been shown to have a block in the release of Omi/HtrA2 from the mitochondria after ischemia [126]. Mice lacking SOD1 have exacerbation of neuronal injury after ischemia [140].

A Primary Neuronal Culture Model of Ischemic Core Death

As shown in figure 4, ischemic core death proceeds with activation of caspase-1, as well as activation of caspase-3 and probable activation of caspase-8. We have studied a cellular model of down-regulation of SOD1 leads to a caspase-1 mediated death that requires production of NO and IL-1β [9, 72, 141]. Evidence suggests that caspase-9 is not involved in this pathway [142]. As noted above, SOD1 is among the key cellular enzymes by which neurons and other cells detoxify free radicals and protect themselves from damage [143, 144]. There is increasing evidence that formation of peroxynitrite from superoxide and NO is a major pathway of superoxide damage [141, 145-148]. SOD1 down-regulation leads to the generation of superoxide, and requires NO and IL-1β; other free radicals, such as hydroxyl radical, are not employed in this pathway [141]. Death induced by a decrease of SOD1 requires generation of IL-1β by caspase-1 [9]. IL-1β acts in an autocrine matter, interacting with the IL-1receptor to signal downstream components of the death pathway. This model allows investigation of the roles of nitric oxide and actions of IL-1β in death initiated by oxidative stress and mediated by caspase-1 activation. All of these elements have been implicated in ischemic death in vivo but their relation within the death pathway has not been established. It is unclear yet if caspase-8 participates in the SOD1 down-regulation mediated death. This primary model of neuronal death will allow delineation of the hierarchical relations of caspase-1 mediated death and provide the mechanism by which free radicals, NO and IL-1β activate effector caspases in the ischemic core.

A Primary Neuronal Culture Model of Penumbral Death

The appearance of penumbral death occurs after core events have started and shows activation of the intrinsic, mitochondrial death pathway with activation of caspases-9 and -3. A key distinction between the core and penumbral

death is the involvement of the mitochondrial death pathway. The cell model most commonly used for cerebral ischemia is oxygen glucose deprivation (OGD), where cultured brain cells are exposed to 5%CO_2/95%N_2 for different times, leading to cellular damage and death. Undoubtedly, caspases participate in OGD-induced death, as indicated by the ability of caspase inhibitors to prevent death [149]. However, which caspases are involved is not fully understood. There are indications that the mitochondrial pathway plays a role in the OGD induced death. Cytochrome c and Smac/Diablo are released into the cytosol. OGD also leads to cleavage of caspase-3 [149, 150] and neurons from caspase-3 deficient mice are less sensitive to OGD [70].

Limited knowledge is available about the roles of other caspases in OGD –induced death. Cleavage of caspases-1, -8, and Bid [8] but not caspases-6 or -7 [70] is detected after OGD. Cultured neurons from caspase-1 deficient mice are resistant to OGD and demonstrate inhibition of caspase-3 processing, BID cleavage and mitochondrial release of cytochrome C and Smac/Diablo [8]. Thus, it was suggested that caspase-1 is an upstream activator of the mitochondrial death pathway after OGD. This primary culture model provides a system where the hierarchical relation of the mitochondrial pathway to actions of caspases-1 and -8 can be determined.

Each of these models should provide molecular details about how the caspase pathways are regulated in neuronal death. These model systems are intentionally minimalist, composed of only neurons, so that the neuronal signaling pathways can be established. This information can then be used to study more complex systems, including glial-neuronal co-culture systems, brain slice models and the in vivo models in a systematic fashion. Clearly establishing the hierarchical components of the ischemic death pathways could ultimately lead to the design of specific therapeutic interventions for stroke/ischemia.

ABBREVIATIONS

CARD = Caspase recruitment domain

DED = Death effector domain

DISC = Death inducing signaling complex

FasL = Fas ligand

IAP = Inhibitor of apoptosis protein

IL-1β = Interleukin-1beta

OGD = Oxygen glucose deprivation

siRNA = Small interfering RNA

SOD1 = Superoxide dismutase 1

NO = Nitric oxide

NOS = Nitric oxide synthase

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