Is caspase-3 inhibition a valid therapeutic strategy in cerebral ischemia?

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Neurodegenerative diseases are characterized by progressive impairment of brain function as a consequence of ongoing neuronal cell death. Apoptotic mechanisms have been implicated in this process and a major involvement of caspase-3, a typical pro-apoptotic executioner protease, has been claimed. In this review, the role of caspase-3 in neuronal cell loss in animal models of stroke is discussed and critically evaluated. In summary, it is concluded that the biochemical evidence favoring caspase-3 as a therapeutic target in cerebral ischemia is not convincing, and the development of selective caspase-3 inhibitors for the treatment of human stroke must be viewed as high risk.

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▼ Stroke represents the third-largest cause of death and the leading cause of disability in the USA. Estimates indicate that stroke is responsible for half of all patients hospitalized for acute neurological disease. There are approximately 750,000 stroke cases per year in the USA alone, with four million survivors from the initial stroke. It has an estimated market of at least US$1 billion with an annual healthcare cost of about US$40 billion. Stroke represents a 'highly-unmet medical need' because there is currently no effective therapy available and the market size is continually growing because of the aging population. To date, the only approved treatment (in the USA alone) is thrombolysis using tissue-type plasminogen activator (tPA). This treatment is only applicable to a limited number of patients because of the inherent risk of hemorrhagic transformation and there is a narrow therapeutic window in which treatment is beneficial. Thus, neuroprotective agents with different modes of action and the ability to be used during an extended time window are urgently required.

The induction of stroke in animals leads to biochemical changes involving the excitotoxic cascade, increases in intracellular Ca^{2+}, changes in gene expression, inflammatory responses and, ultimately, cell death. Researchers have tried for many years to intervene at different stages of the cascade to achieve neuroprotection. Although robust data were generated from intervention studies using *N*-methyl-D-aspartate (NMDA) antagonists (for review see Ref. 1), dose-limiting side effects of these non-selective drugs have resulted in disappointing results in clinical trials (although the newer selective compounds do show more promise). In recent years, attention has focused on other mechanisms that might be involved in the ischemic cascade, and several groups have claimed a causative role of active caspase-3 in mediating apoptotic neuronal cell death in animal models of acute neurodegeneration, such as traumatic brain injury and ischemia. However, there is considerable controversy concerning the mechanisms of cell death occurring in these models. This article provides a critical review of selected studies that assess the significance of activated caspase-3 and/or apoptosis as major contributors to the brain damage and neuronal cell loss induced by global or focal ischemia.

Apoptosis, necrosis and the caspase cascade

According to the classical view of cell death, cells can die in two distinct ways – either apoptosis or necrosis. It is well established that apoptosis, also called programmed cell death, is a major pathway that regulates and controls cell death and tissue homeostasis

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during development and maturation[2,3]. Apoptosis is also very frequent in the healthy adult human body; approximately 100,000 cells die by apoptosis every second to regulate tissue homeostasis and the immune system[3]. Originally, apoptosis was defined by its characteristic ultrastructural changes associated with the elimination of dying cells in proliferating or differentiating cell populations, in the absence of a gross inflammatory response[4]. Typical hallmarks of apoptosis have been identified and are known to contribute to the apoptotic phenotype; these include cell shrinkage, membrane blebbing, intracellular cleavage of proteins that are essential for the correct function of the cell (e.g. proteins involved in cell stability or DNA repair), chromatin condensation and DNA fragmentation. The necrotic cell-death process markedly contrasts with the tightly controlled apoptotic mechanisms. Necrotic cells swell and rupture in an uncontrolled manner, and the cellular contents are released, causing an inflammatory response in the surrounding tissue. It should be noted, however, that depending on the nature and strength of a death stimulus, and the type of the affected tissue, not all classical hallmarks of necrosis or apoptosis are displayed in a given cell. It has also been hypothesized that apoptosis and necrosis might represent the two opposing ends of a whole spectrum of intermediate forms of cell death[5].

Activation of proteases of the caspase family appears to be one of the common mechanisms that is irreversibly connected to apoptotic cell death[6–10]. Caspases belong to the cysteine protease family and specifically cleave their substrates immediately after aspartic acid residues. Caspases are normally expressed as proenzymes that are proteolytically processed to their active form upon appropriate stimulation. So far, 14 members of the caspase family are known, and they can roughly be classified as initiator and executioner caspases according to their function[8,11–13]. Caspase-3, which can be activated by the initiator caspases-8 and -9, as well as by the serine protease granzyme B, is a well-characterized typical executioner caspase involved in many proteolytic processes in apoptotic cells[12,13]. In particular, caspase-3 mediates the cleavage of proteins that are essential for cell stability, DNA repair and activation of DNases[14–16]. An initial event in triggering the caspase cascade is the translocation of cytochrome *c* from the mitochondria to the cytoplasm, where it interacts with apaf-1 and procaspase-9 in the apoptosome complex, resulting in the (auto)activation of procaspase-9 and the subsequent activation of procaspase-3 (Refs 17–19). Once cytosolic caspase-3 is activated and functions as a proteolytic enzyme, a 'point of no return' is reached and most cells are destined to die primarily by an apoptotic phenotype. However, blockade of caspase-3 does not always prevent cell death and additional roles of activated caspases outside of apoptosis have recently been discussed[20].

Caspases and cell death in cultured neurons

In cultures of primary neurons derived from embryonic or neonatal animals, stimulation of the NMDA receptor by exogenous glutamate leads to acute excitotoxic cell death involving Ca^{2+} and Na^{+} influx and K^{+} efflux. After weak NMDA receptor stimulation, apoptotic death is characterized by caspase-3-dependent nuclear factor (NF)-κB activation[21] and DNA fragmentation[22]. Inhibition of the NMDA-receptor-mediated K^{+} efflux attenuated apoptosis[23], and positive staining with an anti-caspase-3 antibody or labeled tetrapeptide aldehyde inhibitor (DEVD-CHO) confirmed activation of procaspase-3 after mild NMDA stimulation[24]. There is some evidence that the mode of cell death after glutamate exposure is dependent on mitochondrial functionality and cellular energy levels: high energy levels favor apoptosis, whereas low energy levels mainly result in necrosis[25]. To further add to the complexity, it has recently been shown that, following activation of calpain I by increasing intracellular Ca^{2+} levels, the excitotoxic neuronal death is converted into a caspase-independent death by inhibiting activation of caspase-9 and -3 (Ref. 26). It is also interesting to note that calpain I and caspase-3 share several substrates and both enzymes can mediate neuronal cell loss under certain circumstances[27].

Both a fast form of cell death with typical signs of necrosis and a more delayed form of cell death have been observed in an alternative *in vitro* model induced by removal of glucose and oxygen from the culture medium[28]. Given that the delayed cell death was reduced to some extent either by endonuclease inhibition, exposure to low K^{+} concentration or inhibition of RNA synthesis, it was hypothesized that this cell death was apoptotic in nature. Recent experiments using primary cultures of hippocampal neurons of postnatal day-18 rats revealed induction of DEVDase activity after hypoxic conditions[29]. In addition, inhibition of caspases with a caspase-1 prototype inhibitor (AcYVADcmk) reduced cell death (induced by oxygen and glucose deprivation) in hippocampal slice cultures of postnatal day-8–10 rat brain[30]. Taken together, the data from cultured neurons provide circumstantial evidence for a potential role of activated caspases in excitotoxicity contributing to certain aspects of the cell death process. It has to be kept in mind that the neurons used in these cultures are mostly derived from embryonic or postnatal tissue and contain substantial amounts of constitutively expressed procaspase-3. However, additional mechanisms, such as profound disturbances in ion homeostasis, mitochondrial dysfunction, reactive oxygen species production and/or

calpain activation, represent severe insults by themselves that might lead to cell death in a caspase-independent fashion.

Caspases in the developing and adult brain

The significance of caspase-3 in mediating developmentally regulated neuronal cell death has been demonstrated *in vivo* using gene-knockout technologies. Caspase-3-deficient mice have various hyperplasias and disorganized cell deployment in the developing brain as a consequence of decreased apoptosis[31]. Mice with targeted disruption of the *Casp9* or apoptotic protease-activating factor (*Apaf1)* gene have a similar phenotype, with an excess of cells in the CNS and decreased cell death of neuroepithelial cells[32–35]. These results strongly suggest that caspase-3, caspase-9 and apaf-1 are clustered in an apoptotic pathway that is fundamental to the regulation of tissue homeostasis in the developing mammalian brain[36].

There is also evidence that caspase expression is developmentally regulated. For instance, caspase-14 mRNA is undetectable in adult mouse tissues although it is abundantly expressed in mouse embryos[37,38]. Likewise, caspase-8 expression appears to be highest in day-seven embryos, but it is also detected during later stages of development and in adult mouse tissues[39]. Interestingly, a profound downregulation of caspase-3 transcription has been reported during rat brain development[40,41], and this developmental regulation of procaspase-3 (but not of procaspase-2) expression has been confirmed in immunoblotting experiments[42]. There is a good correlation between the downregulation of procaspase-3 expression and a decline of procaspase-3 activation in the maturating rat brain[43,44] (Niederhauser *et al.*, unpublished) and the functional role of caspase-3 in the adult brain needs to be carefully evaluated. Although caspase-3-mediated cleavage of cellular proteins such as amyloid precursor protein[45], presenilins[46] and huntingtin[47] has clearly been demonstrated in cultured cells, the evidence for the occurrence of such proteolytic events in the adult brain is largely lacking.

Neuronal cell death in cerebral ischemia: is caspase-3 involved?

Under ischemic conditions, brain cells mainly die from a lack of oxygen and glucose supply, which is caused either by rupture of a blood vessel, formation of a clot in one of the major vessels or cardiac failure. The outcome of an ischemic insult is different in the ischemic core and in the so-called penumbra. (The penumbra refers to the tissue adjacent to the ischemic core area where sufficient blood flow is maintained to preserve functional and structural integrity for a certain time.) It is well accepted that cells in the ischemic core usually die with typical signs of necrosis[48–50]. Early membrane damage leads to fundamental disturbances of the ion homeostasis and is accompanied by a massive release of glutamate. NMDA receptors become chronically activated and extracellular Ca^{2+} enters the cells in an uncontrolled fashion. It is this Ca^{2+} influx that is assumed to play a key role in the fatal outcome of an ischemic insult. Ca^{2+} overload might trigger permanent opening of the mitochondrial transition pore, resulting in production of reactive oxygen species, uncoupling of mitochondrial respiration and subsequent failure of the energy supply[51]. In some models, an inflammatory response involving increased levels of interleukin-1 (IL-1) has been reported, further demonstrating the primarily necrotic nature of this form of cell death[52,53].

The penumbra represents tissue that is at risk but that can be salvaged with appropriate intervention. The biochemical events that lead to neuronal cell death in the penumbra are generally more subtle and, to date, there is much debate as to whether this type of cell death represents a classical form of caspase-3-mediated apoptosis. The remainder of this article will critically review experimental data and interpretations that have been derived by many groups and have been taken as evidence to support a major role of caspase-3 in ischemia-induced neurodegeneration. The list compiled in Table 1 serves as a guide in this evaluation by summarizing the major findings obtained in various ischemia models.

Animal models of ischemia

Various animal models have been developed to study cerebral ischemia and they can be broadly classified as either focal or global. Focal ischemia models try to mimic a human stroke situation, whereas global models try to replicate the consequences of global ischemia following situations such as cardiac arrest or drowning. There is no doubt that such animal studies represent only certain aspects of the complex human disease of cerebral ischemia.

The most stringent model of stroke or focal ischemia is permanent occlusion of the middle cerebral artery (MCA) via coagulation or using a suture threaded into the MCA via the internal carotid artery. Permanent MCA occlusion using the coagulation method is used mostly in rats, whereas permanent or transient MCA occlusion using the suture method can be used in mice and rats. Focal ischemia results in a necrotic core that is surrounded by a salvageable penumbra.

The global models of cerebral ischemia consist of a brief period of ischemia (5–15 min), during which complete arrest of cerebral blood flow to the brain is achieved, followed by reperfusion. The common models used are

Table 1. Summary of experimental data related to ischemia-induced neuronal damage and the functional role of caspase-3 in animal models of brain ischemia.

Ischemia model	Parameter assessed											Refs
	HP/HC	TUNEL staining	DNA laddering	PARP cleavage	Cyt *c* release	Casp-3 ISH	Casp-3 northern	Casp-3 IHC	Casp-3 western	DEVDase activity	Caspase inhibitor	
Adult animals												
Transient												
Rat: CCAO	✓	✓	–	✓	–	✓	✓	✓	✓	✓	✓	75
Rat: CCAO	✓	✓	✓	–	✓	–	–	–	–	–	–	72
Rat: CCAO	✓	–	–	–	–	–	–	–	–	×	–	73
Rat: 4VO	✓	–	–	–	–	–	–	–	–	–	×	95
Rat: 4VO	✓	✓	–	–	–	–	–	✓	–	–	✓[a]	96
Rat: 4VO	✓	✓	–	–	–	✓	–	–	–	–	–	103
Rat: 4VO	✓	✓[b]	–	–	–	–	–	✓[c]	–	–	–	104
Rat: MCAO	✓	–	–	–	–	–	–	–	–	–	✓	95
Rat: MCAO	✓	✓	–	–	–	–	–	✓	–	–	–	105
Rat: MCAO	–	–	–	–	–	✓	–	–	–	–	–	77
Rat: MCAO	✓	✓	–	–	✓	–	–	–	–	–	–	71
Rat, mouse: MCAO	✓	–	–	–	–	–	–	–	–	–	✓	93
Rat: CA	✓	✓	–	–	–	✓	✓	–	–	✓	–	76
Mouse: MCAO	✓	–	–	–	✓	–	–	–	–	×	–	73
Mouse: MCAO	✓	✓	✓	–	–	–	–	✓	✓	✓	–	81
Mouse: CCAO	✓[d]	✓[d]	–	–	–	–	–	×[e]	–	–	–	106
Gerbil: CCAO	✓	–	–	–	–	–	–	–	✓	–	–	82
Gerbil: CCAO	✓	✓	–	✓	–	–	–	–	–	–	✓	98
Permanent												
Rat: MCAO	✓	✓	–	✓	–	–	–	✓	–	–	✓	97
Rat: MCAO	✓	✓	✓	–	–	–	–	✓	×	×	–	83
Rat: MCAO	✓	–	–	–	×	–	–	–	–	×	×	73
Rat: MCAO	✓	✓	–	–	–	–	–	✓	–	✓	✓	90
Rat: MCAO	✓	–	–	–	–	–	–	–	–	–	✓	60
Mouse: MCAO	✓	✓	–	–	–	–	–	✓[f]	–	–	–	107
Mouse: MCAO	✓	–	✓	✓	✓	–	–	✓	–	–	–	70
Mouse: MCAO	✓	–	✓	–	✓[g]	–	–	–	–	–	–	69
Hypoxic ischemia												
Rat (PND60): CCAO	✓	–	–	–	–	–	–	×[h]	×	–	–	44
Neonatal animals												
Hypoxic ischemia												
Rat (PND7): CCAO	✓	–	–	–	–	–	–	✓	✓	–	–	44
Rat (PND7): CCAO	✓	✓	✓	✓	–	–	–	–	–	✓	✓	91
Rat (PND7): CCAO	✓	✓	–	–	–	–	–	✓	✓	–	–	88
Rat (PND7): CCAO	✓	✓	–	–	–	–	–	✓	–	–	–	86
Rat (PND7): CCAO	–	–	–	✓	–	–	–	–	✓	✓	–	87
Rat (PND7): CCAO	–	–	–	✓	–	–	✓[i]	✓	✓	✓	–	43
Rat (PND7): CCAO	–	–	–	–	–	–	–	–	–	✓	–	[j]

✓, indicates that the experiment has been performed and a positive result was obtained; ×, indicates that the outcome or read-out of the experiment was reported to be negative.

Abbreviations: 4VO, four-vessel occlusion, global ischemia; caspase inhibitors, effect of peptidic caspase inhibitors on ischemia-induced brain damage; CA, cardiac arrest, global ischemia; CCAO, common carotid artery occlusion with concomitant hypotension (rats) or hypoxia (mice), transient forebrain ischemia; HP/HC, histopathology/histochemistry; IHC, immunohistochemistry; ISH, *in situ* hybridization; MCAO, middle cerebral artery occlusion, focal ischemia; PND, postnatal delivery; PARP, poly-ADP ribose polymerase; TUNEL, transferase-mediated uridine 5′-triphosphate-biotin nick end-labeling

[a]zDEVDfmk treatment has no effect on hippocampal long term potentiation (LTP); [b]TUNEL staining in CA1 neurons reduced by overexpression of X-chromosome-linked inhibitor of apoptosis protein (XIAP); [c]Reduced staining of caspase-3 $(p17/p12)_2$ conformer in CA1 neurons by adenovirus-mediated overexpression of XIAP; [d]Delayed cell death in CA1 neurons in Bcl-2 transgenic mice; [e]No caspase-3 p17 staining in CA1 neurons (occasional staining in dentate granule cells); [f]Less caspase-3 p17 subunit staining in caspase-11-deficient mice; [g]More cytochrome *c* release in superoxide dismutase (SOD) –/+ heterozygous mice after MCAO; [h]<4% of damaged neurons in striatum and <1% of damaged neurons in neocortex show positive staining for caspase-3; [i]No increase after hypoxic ischemia; [j]Gill, R. *et al*., unpublished.

bilateral occlusion of the carotid arteries in gerbils, occlusion of vertebral arteries and carotid arteries (four-vessel occlusion; 4VO) for 10–20 min in rats, and occlusion of the carotid arteries (two-vessel occlusion; 2VO) with concomitant hypotension to 50 mm Hg for 5–15 min in rats. Global ischemia tends to result in delayed neuronal degeneration in the hippocampus, cortex and striatum; in general, this delayed degeneration is also believed to be necrotic[54]. There are also animal models of perinatal hypoxia that involve occlusion of one carotid artery and a period of hypoxia, and this model results in a mixture of focal and global ischemia in the brain.

Assessment of ischemic damage

Ischemic brain damage and infarct size are routinely analyzed on histological preparations of brain tissue by visualization under a light microscope using various fixation and staining methods. Such histopathological examination usually reveals macroscopic tissue damage and cell death on the ipsilateral side, but not on the contralateral side, in focal ischemia models. However, detailed analysis of single-cell morphology can only be obtained by electron microscopy, which is an extremely reliable technique to assess structural integrity and is considered to be the gold standard in the analysis of apoptotic versus necrotic morphology[4]. Such studies do not support the concept of apoptotic neuronal cell death in an animal model of focal cerebral ischemia after permanent occlusion of the MCA (Ref. 48), and detailed electron microscopy also failed to reveal typical signs of apoptosis in models of transient forebrain ischemia[54,55]. Rather, necrotic phenotypes with swollen cells and organelles, disrupted membranes and electron-dense inclusions, most probably representing secondary lysosomes closely associated with normal and degenerate mitochondria, were seen in these studies. A recent analysis of post-mortem samples of human stroke patients also failed to reveal an apoptotic morphology and indicated an increase of the necrotic core area and a shrinkage of the penumbra over time[52]. In summary, electron microscopic inspection clearly confirms necrotic neuronal cell death, rather than classical apoptosis, subsequent to an ischemic insult in animal models of ischemia and in stroke patients.

TUNEL staining

A widely used technique to assess cell death after ischemia is terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end-labeling (TUNEL) staining, which reveals DNA strandbreaks induced by activated endonuclease(s) during apoptosis. Cells in the ischemic regions in the brain usually show positive TUNEL staining and, previously, this has been taken to indicate apoptotic cell death[56,57]. However, there is now substantial evidence that TUNEL can stain not only apoptotic but also necrotic cells[48,58–60]. TUNEL staining is also known to be susceptible to preparational artefacts, which might result in false-positive signals[61]. Furthermore, it has recently been demonstrated that TUNEL labeling can be mediated by endogenous endonucleases that are activated and/or released during protease or microwave pretreatment of the damaged tissue[52]. These findings clearly demonstrate that TUNEL positivity cannot be taken as a true parameter for distinguishing between apoptotic and necrotic cell death.

DNA fragmentation

Caspase-3-mediated fragmentation of DNA into oligomers of 180 bp is a recognized feature of apoptosis and can be visualized on agarose gels as a characteristic band pattern. The appearance of such DNA ladders has been interpreted to indicate caspase-3-mediated apoptotic processes, and it has also been observed in various models of ischemia (Table 1). However, fragmentation of DNA is also induced during necrosis and is indistinguishable from that seen in apoptosis; this necrotic DNA fragmentation can be inhibited by a serine-protease inhibitor but not by the pan-caspase inhibitor zVADfmk (Ref. 62). It has also been shown that an increase of intracellular Ca^{2+} in chemically hypoxic cells undergoing necrosis resulted in nuclear condensation, hydrolysis of lamin A and C (which was thought to be specific for apoptosis) and cleavage of DNA into large 150 kb fragments, similar to those seen in the early stage of apoptosis[63]. These data suggest that DNA laddering itself is not sufficient to demonstrate a caspase-3-mediated apoptotic process unequivocally.

Cleavage of PARP

Poly-ADP ribose polymerase (PARP) is a nuclear protein used for DNA repair. It is one of the prototype substrates for caspase-3-mediated cleavage and has been used in many systems to indicate the presence of activated caspase-3. However, PARP was also recognized as a substrate for proteases other than caspase-3, such as calpain[27] and granzyme B (Ref. 64); thus, disappearance of full-length PARP might not necessarily represent a unique marker for caspase-3-mediated apoptosis. Nevertheless, PARP is a suitable parameter to study cell death, particularly if the cleavage products are analyzed by size or antibodies specific for cleavage fragments, allowing identification of the protease(s) involved[65,66]. It is interesting to note that the susceptibility of hepatocytes, thymocytes and primary neurons to various inducers of apoptosis was unaltered in PARP-deficient mice, suggesting that neither activation

nor cleavage of PARP has a causal role in apoptotic cell death[67], although a different conclusion was reached in a later study[68].

Release of mitochondrial cytochrome c *during ischemia*

It is now generally accepted that mitochondria release cytochrome *c* into the cytoplasm in response to an apoptotic stimulus (reviewed in Ref. 17). Most of these studies have been performed with cultured cells, but an increase of soluble (i.e. cytosolic) cytochrome *c* has also been reported in ischemic brain areas after permanent[69,70] or transient[71] occlusion of the MCA, or transient occlusion of both common carotid arteries[72]. It has been claimed that redistribution of mitochondrial cytochrome *c* is one of the early and essential steps after an ischemic insult, necessary to activate the proteolytic cascade and to mobilize the intracellular apoptotic machinery. Although a regulatory role of manganese superoxide dismutase in early mitochondrial cytochrome *c* release has been implicated[69], evidence for such a function was not obtained in a later study[70].

Our own data, regarding cytochrome *c* distribution in ischemic brain regions after permanent occlusion of the MCA in rats, do not support the view of cytochrome *c*-mediated degenerative processes. Although we occasionally detected cytosolic cytochrome *c* in the brain samples taken at various times post-ischemia, there was no clear correlation of mitochondrial cytochrome *c* release and the extent of ischemic damage[73]. In addition, this released cytochrome *c* represented only a minor fraction of cytochrome *c* still retained in mitochondria, which is consistent with earlier findings[70,71]. In a mouse model of transient focal ischemia, we observed a small amount of cytochrome *c* in the cytosolic fraction of the ipsilateral hemisphere but, again, the majority of the cytochrome *c* remained in the mitochondria.

Whether the difference in cytosolic cytochrome *c* levels between ipsi- and contralateral sides reflects a specific mitochondrial cytochrome *c* release, or whether the ischemic conditions render mitochondria more vulnerable to subsequent tissue fractionation with a minor loss of cytochrome *c*, is currently not known. In addition, it cannot be excluded that an ischemic insult affects the efficiency of the mitochondrial protein-import machinery and, as a consequence, mitochondrially targeted, newly synthesized proteins accumulate in the cytosol. In conclusion, the appearance of cytosolic cytochrome *c* in ischemic brain areas is likely to be a consequence of the general tissue damage rather than a specific mechanism or event in an early apoptotic response. This is particularly likely to be the case in the adult brain, where expression of procaspase-3, the target of cytochrome *c*/apaf-1-activated caspase-9, is downregulated (discussed later).

Is procaspase-3 expression upregulated during brain ischemia?

There is ample evidence for the developmental regulation of procaspase-3 transcription and translation such that low or even undetectable levels occur in the adult brain[40,41,44,74]. Using mostly *in situ* hybridization techniques, it has been claimed that ischemic conditions re-induce procaspase-3 expression in adult animals[75–78]; however, these data have not been confirmed in DNA array hybridization experiments (Gill *et al.*, unpublished)[79]. It is known that, although a variety of genes are induced at the transcriptional level following ischemia[57], the translational machinery is severely affected and protein synthesis rapidly decreases[80]. Therefore, it is important to confirm the presence of any upregulated gene at the protein level and, so far, the data for procaspase-3 have not been conclusive. Immunoblot analysis did not show a clear upregulation of procaspase-3 expression in the adult brain after an ischemic insult[75,81,82] and conflicting data with respect to the presence of activated procaspase-3 under these conditions have been reported[75,81–83].

A commonly used technique to visualize proteins is immunohistochemistry (IHC) and, as indicated in Table 1, many groups have used anti-caspase-3 antibodies in IHC of fixed brain-tissue sections. Although this is a powerful and sensitive method, special care has to be taken to rule out any unspecific interaction of the antibodies with undefined structures in degenerating tissue. In general, the antigenicity of damaged neurons is increased and the risk of monitoring unspecific antibody binding in dying neurons is substantial. Furthermore, crossreactivity of antibodies with proteins other than the antigen used for immunization could lead to false-positive staining signals in tissue sections, and it is an absolute requirement to confirm the selectivity of the antibody by careful analysis of the same tissue in immunoblotting experiments. A recent study has revealed a strong crossreactivity of the CM1 antibody, a widely used antibody that recognizes active caspase-3, with an unknown ~20 kDa protein and with caspase-6 (Ref. 84); such unexpected antibody (cross)reactivities have to be considered in the interpretation of tissue-staining experiments. To illustrate this issue further, staining of mouse brain tissue with antibodies specific for the pro-apoptotic protein Bax yielded similar staining patterns in both wild-type and *bax*$^{-/-}$ mice, clearly demonstrating that, in contrast to observations with antibody pre-absorption experiments, the Bax-like immunoreactivity was not specific[85].

In summary, although several groups have found an apparent upregulation of procaspase-3 expression by immunohistochemical examination of ischemic brain regions in adult animals, the correlation with western blot data is

largely missing and, thus, firm conclusions on increasing procaspase-3 levels in ischemic areas cannot be drawn at the present time. However, it should also be noted that small subpopulations of neurons might exist that express procaspase-3 but escape detection by immunoblot analysis of whole brain homogenates because of the limited sensitivity of the assay method.

A different situation is encountered in neonatal animals, where procaspase-3 is constitutively expressed and ischemic conditions undoubtedly lead to activation of procaspase-3, as shown by IHC, immunoblot analysis and enzymatic activity assays[43,86–88]. In this model, a good correlation between activated caspase-3 and markers of DNA damage and apoptotic morphology was found, clearly indicating a functional role of active caspase-3 in neonatal neuronal cell death. It is interesting to note that, in contrast to the observed increase of caspase-3 immunostaining in tissue sections, no significant time-dependent changes of procaspase-3 levels were detected by densitometric analysis of immunoblots, further arguing against an ischemia-induced upregulation of procaspase-3 expression[88].

DEVDase activity in ischemic brain regions

Any postulated upregulation of procaspase-3 expression must translate into a measurable enzymatic activity to confirm its biological relevance in driving the cell-death process. Fluorescent coumarin derivatives of tetrapeptides have widely been used to probe caspase activity in tissue or cell homogenates, and the sequence DEVD has been identified as the prototype recognition sequence for caspase-3-mediated cleavage[89]. However, other caspases, such as caspase-2 and -7, might also recognize the DEVD sequence and, thus, DEVDase activity does not necessarily represent a specific caspase-3 activity.

Using the substrate DEVD-7-amino-4-methylcoumarin (DEVD–AMC), we have been unable to demonstrate induction of DEVDase activity in adult rat or mouse brain following focal or global ischemia[73]. A similar lack of DEVDase detection has been reported in rat brain samples after permanent MCA occlusion[83]. By contrast, a peak of increased DEVDase activity at 30 min after reperfusion has been seen in a mouse model of transient ischemia[81], and DEVDase activity has been detected at 8–24 h after reperfusion in a similar model in rats[75]. Recently, a massive induction of DEVDase (and YVADase) activity has been described in a permanent rat MCA occlusion model[90], a finding that is in sharp contrast to the absence or low levels of induced DEVDase activity in similar ischemia models mentioned earlier.

Although the reasons for the apparent discrepancy in DEVDase activity are currently not known, the lack of constitutive procaspase-3 expression in the adult brain and the inconclusive data on ischemia-induced upregulation of procaspase-3 argue against substantial caspase-3 activation. In further support of this notion, a robust DEVDase activity peaking at around 24 h after the ischemic insult has been found in a hypoxic ischemia model in neonatal rats[43,87,91], correlating well with proteolytic processing of procaspase-3 as shown on western blots[44]. The activation of procaspase-3 in this model also translated into an apoptotic morphology as shown by electron microscopic analysis[91], and these data further underscore the fundamental differences of neuronal cell death pathways in neonatal and adult brain.

Effect of caspase inhibitors in animal models of ischemia

Historically, the involvement of caspases in ischemia-induced cell death was inferred from studies demonstrating that zVADfmk (*N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone), BAF (butyloxycarbonyl-Asp(OMe)-fluoromethylketone), or zDEVDfmk (*N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone), which are broad-spectrum caspase-inhibitors, were neuroprotective in animal models of focal and global ischemia[75,92–94]. However, the efficacy of these inhibitors in focal ischemia was less clear in a later study[95]. zDEVDfmk has also been tested in a transient ischemia model in rats and, although some reduction in neuronal cell death was observed, impairment of motor-function and long-term potentiation was not affected[96]. A cocktail of three caspase inhibitors [YVADcmk (acetyl-Tyr-Val-Ala-Asp-chloromethylketone), DEVDfmk (acetyl-Asp-Glu-Val-Asp-fluoromethylketone)and IETDfmk (acetyl-Ile-Glu-Thr-Asp-fluoromethylketone)] was found to reduce the number of TUNEL-positive cells at 12 h post-permanent MCA occlusion in the rat[97]. Another pan-caspase inhibitor, zD (benzyloxycarbonyl-Asp-CH_2-dichlorobenzene), was also shown to have neuroprotective effects in a transient ischemia model in gerbils[98].

In general, peptide-based inhibitors have low brain penetration and are usually given by intracerebroventricular injections to overcome this limitation. In most cases, the inhibitors have been given before or at the onset of ischemia and the relevance of such animal data for a human stroke situation, where a potential treatment occurs substantially later than the primary ischemic insult, remains to be seen. In our own studies, zVADfmk, when administered at 5, 60 and 120 min after permanent MCA occlusion, did not yield significant neuroprotection as measured at 48 h; by contrast, in the same study, the NMDA antagonist MK801 was clearly neuroprotective[73]. It has also been claimed that zVADfmk or other pan-caspase inhibitors might act as

anti-inflammatory agents by reducing the production of the proinflammatory cytokine interleukin (IL)-1 via caspase-1 inhibition and thereby attenuating the ischemic brain damage[93,99]. However, there is still an ongoing debate as to what extent inflammatory reactions, which certainly occur as a consequence of the general ischemic damage, contribute to the severity of the disease.

The ketone or aldehyde derivatives of peptidic caspase inhibitors can potentially interact with other cysteine or aspartyl proteases, and the relative unselectivity of this compound class could contribute to the outcome of a complex biological system, such as ischemia-induced neurodegeneration[43,100]. In recent years, several peptidomimetic caspase inhibitors have been developed that inhibit caspases in the nanomolar and subnanomolar range. One of these inhibitors (IDN5370), an irreversible broad-spectrum caspase inhibitor, potently inhibits caspase-3 with an IC_{50} value of 15 nM, but its neuroprotective effect in cultured neurons after an apoptotic challenge is in the micromolar range (i.e. >100-fold above the concentration required for caspase-3 inhibition)[101,102]. These data clearly demonstrate that inhibition of caspase-3 alone is not sufficient for survival and that inhibition of other events, conferred by the caspase-3 inhibitor, might be more crucial for successful survival promotion. A similar phenomenon could be responsible for the partial neuroprotective effects observed in the ischemic brain with some peptidic caspase inhibitors, as described above. The downregulation of procaspase-3 expression in the adult brain and the lack of unambiguous evidence for the re-induction and activation of procaspase-3 during ischemia also favor this interpretation, and suggest that these caspase inhibitors are not targeting caspase-3 but instead target other mechanisms or pathways that are crucial for neuronal survival.

Conclusion

Any therapeutic target relevant for the treatment of human stroke must be expressed in affected brain regions and, if it is important in disease progression, changes in expression and/or activity have to be evident. Furthermore, its contribution to the disease and its role in mediating the ischemic damage need to be confirmed in animal models of stroke, for instance by using specific antagonists or inhibitors. The animal model that best replicates the human pathological consequences of stroke is permanent occlusion of the MCA in rats. In this model, the damage evolves over 24 h, although 80% of the damage has already occurred by 4 h. For caspase-3, there is no evidence for upregulation and/or activation at relevant time points after stroke in this experimental model and, therefore, it does not appear to be a prime target for the treatment of cerebral ischemia in the adult brain.

Considerable controversy exists as to the relative roles of apoptosis and necrosis in the ongoing neuronal degeneration during ischemia. Although the existence of a functional pro-apoptotic pathway involving procaspase-3 activation and classical apoptotic morphology can easily be demonstrated in neonatal hypoxic ischemia models, the phenotype of ischemia-induced neuronal degeneration in the adult brain is different and shows typical signs of necrosis. The downregulation of procaspase-3 expression (and perhaps other components of the apoptotic machinery) at the late stages of brain development certainly contributes to the observed change in neuronal cell death characteristics, and might reflect a strategy of the organism to ensure protection of post-mitotic neurons from accidental pro-apoptotic insults. It also explains the failure to detect enzymatically active caspase-3 (measured as DEVDase activity) in various models of ischemia in adult animals, as observed in some of the studies. There is evidence that DNA fragmentation and PARP cleavage, events that are associated with ischemia-induced neurodegeneration, can occur independently of active caspase-3 and are, therefore, not in contradiction to the developmental regulation of procaspase-3 expression.

In summary, the data claiming caspase-3 as a clearly identified and validated target in experimental models of ischemia are not convincing, and the development of highly selective, non-peptidic caspase inhibitors for the treatment of stroke must be viewed as high risk, even if a possible combination with other therapeutic strategies is considered. Future studies will have to reveal whether such caspase inhibitors, after *in vitro* optimization for selectivity and potency, will indeed yield neuroprotective agents with efficacies similar to their caspase inhibitor profiles.

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