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The mechanisms of cell death in focal cerebral ischemia highlight neuroprotective perspectives by anti-caspase therapy

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Abstract

A number of studies have validated the importance of caspase activation in ischemia-induced brain damage. Caspases participate in both the initiation and execution phases of apoptosis, and play a central role in neuronal death after global cerebral ischemia. In focal ischemia, apoptosis occurs in the penumbra during the secondary phase of expansion of the lesion. However, ultrastructural and biochemical analysis have also shown signs of apoptosis in the initial lesion, or infarct core, which is traditionally considered necrotic. Specific caspase pathways are activated in the core and in the penumbra, and participate in both cytoplasmic and nuclear apoptotic events, notwithstanding their initial classification as activator or initiator caspases. This confirms previous suggestions that caspase inhibition holds tremendous neuroprotective potential in stroke and other apoptosis-related degenerative diseases. Consequently, two new approaches, aimed at treating stroke-induced brain damage by anti-apoptotic molecules, are being developed in academic and industrial laboratories. These are based, respectively, on the use of small peptide sequences corresponding to the preferred cleavage site of a caspase, and on genomic constructions derived from the fusion of endogenous anti-caspase molecules with a protein transduction domain from the human immunodeficiency virus-1. Fusion proteins containing endogenous caspases inhibitors efficiently counteract apoptosis *in vitro*. In *in vivo* models of focal cerebral ischemia, fusion proteins successfully cross the blood brain barrier and protect cells from ischemic death. This new approach by protein therapy could prove to be an interesting alternative for the reduction of the dramatic consequences of stroke, provided that the long-term efficiency of this protection in terms of functional recovery is demonstrated.

Keywords: Apoptosis; TAT domain; Middle cerebral artery; Necrosis; Protein therapy; Stroke

1. Introduction: apoptosis in acute stroke—more than the penumbra

Apoptosis is critical for the development and maintenance of healthy tissues. Deregulation of apoptotic cell death pathways is central to cancer or autoimmune diseases, and there is much evidence to support the active participation of apoptotic processes in progressive neurodegenerative disorders, such as amyotrophic lateral sclerosis, Parkinson's and Alzheimer's diseases, as well as in acute ischemic shock and in trauma [1,2]. Focal ischemic lesions are the result of a sudden decrease or arrest of the regional cerebral blood flow (rCBF), mainly due to arterial occlusion, and proceed in two steps [1,3]. The first step occurs in the area proximal to the occlusion site, the infarct core, where the rCBF drops below 10% of control values. As a consequence, ATP levels decrease dramatically, and the rapidity of pan-cellular death and histochemical reactivity have led to the idea that cell death in the infarct core is necrotic, i.e. out of therapeutic reach. In the remaining arterial territory, or penumbra, rCBF levels are kept at up to 40% of control values due to retrograde perfusion by anastomosis from adjacent arteries. In this area, ATP levels remain high enough to allow apoptosis [4,5], an energy-dependent mechanism, and cell death is delayed by hours or days. Neuroprotective strategies have thus focused on molecules able to interfere with apoptotic mechanisms in the penumbra.

For more than a decade, a series of controversial findings have progressively challenged this strict dichotomy

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Abbreviations: Flip, FLICE-inhibitory protein; HIV1, human immunodeficiency virus-1; IAP, inhibitors of apoptosis; PTD, protein transduction domain; MCAO, middle cerebral artery occlusion; rCBF, regional cerebral blood flow; TAT, transactivator domain.

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between cell death types in the core and the penumbra, leading to confusing scientific and semantic debates. The controversy was mainly based on the fact that ultrastructural observations of dying neurons in the core did not correlate with the original descriptions of necrosis [6]. During development, necrosis is characterized by cell swelling, rupture of protoplasmic and nuclear membranes, and of the membranes of organelles. The necrotic process is correlated with a strong inflammatory reaction due to the spilling of cell content into the extracellular space. However, in the infarct core, neurons never display the full panel of necrotic features, whereas apoptotic characteristics such as cytoplasmic and nuclear condensation with preserved membranes are frequent [6]. According to our observations in the cortex, the only dying cells with clear necrotic features after distal occlusion of the middle cerebral artery (MCAO) are protoplasmic astrocytes [7]. Protoplasmic or type 1 astrocytes represent the main astrocytic population of the cortical grey matter, while fibrous or type 2 astrocytes are preponderant in the hippocampus and striatum. In the latter structures, glial reactivity is triggered by neuronal death rather than being a direct response to ischemia [8,9]. In contrast, astrocytes in the cortical core die rapidly, without any expression of the biological markers of apoptosis, and show typical cell swelling and rupture of membranes within minutes following MCAO. Neurons never show extensive swelling, even during early stages of degeneration, except for swelling of the endoplasmic reticulum, which has been described as one of the first signs of apoptosis failure (see [10]). In addition, neurons of the core contain activated forms of a number of caspases [4,11]. Taken together, these observations suggest that apoptosis is also involved in the initial phase of cerebral infarction.

In addition to acute brain lesions, the therapeutic use of caspase inhibitors could concern a variety of neurological and non-neurological diseases associated with excessive apoptosis. Behind the theory, though, the concept of inhibiting apoptosis to limit cell death is still a controversial issue that requires a careful examination of a number of issues. First, inhibition of caspase activity must rescue the cell in terms of function, and avoid inducing a shift from apoptosis to necrosis, as reported *in vitro* and in a model of Parkinson's disease [12]. Second, the anti-caspase agent used must be devoid of any peripheral effects, which mainly means being able to preferentially penetrate brain tissues. Third, the use of anti-caspases to limit ischemic damage in the core requires that the administration be performed early enough to patients, a condition which necessitates dramatic modifications in medical care for stroke in most countries.

2. More roles for caspases in focal ischemia

Occlusion of brain arteries, or focal ischemia, is the major cause of ischemic cerebro-vascular diseases. In focal ischemia, caspases were initially thought to participate in the secondary step of the infarction process, i.e. the expansion of the lesion into the penumbral area. A closer investigation of the time-course of caspase activation in a murine model of focal ischemia induced by permanent MCAO revealed that caspases also participate in the initial phase of cell death in the core [13]. Several caspases, including caspase-1 and -8, are strongly activated in the core within a half-hour post-occlusion, when neurons of the penumbra have no structural abnormalities. In contrast, some neurons in the core already display a number of morphological features of early apoptosis, such as cytoplasmic condensation or dilatation of the endoplasmic reticulum. This indicates that apoptosis is actually triggered in the core, and may well be the default mechanism for cell death following acute cerebral ischemia. As demonstrated *in vitro* [14], and as suggested by a number of authors, the final mixed morphology very likely results from the abortion of the apoptotic process and a shift towards necrosis due to severe energy depletion in the core.

Interestingly, cell death in the core and in the penumbra involves distinct caspase activation cascades, in close correlation with energy levels [13]. Neuronal degeneration in the core is related to the activation of energy-independent pathways that include the "death-receptors" and the caspase-1 pathways. The mitochondrial pathway and its energy-consuming apoptosome is not involved at this stage. In contrast, pathways activated during the secondary expansion of the lesion into the penumbral area include the mitochondrial pathway [11], in agreement with the energy requirements of the apoptosome [15].

Caspases are divided into two functional groups based on their perceived role in apoptosis [16]. Initiator caspases are responsible for the activation of caspase cascades after receipt of an apoptotic signal, such as (i) stimulation of death receptors of the tumor necrosis factor-receptor (TNF-R) superfamily, i.e. caspases-2, -8, -10, (ii) mitochondrial dysfunction, i.e. caspase-9, (iii) lysosomal dysfunction, i.e. caspase-11, or (iv) endoplasmic reticulum stress, i.e. caspase-7 and -12. Downstream of these are the executor caspases (mainly caspase-3, -6), responsible for cell destruction by degradation of many structural, metabolic and repair proteins essential for cellular homeostasis [17–19]. Both initiator and executor caspases are activated during the neurodegenerative process following cerebral ischemia [4,11,20].

Again, the strict dichotomy suggested by this classification is no longer valid. In addition to their participation in cell degradation by the destruction of cytoplasmic targets, caspases act at nuclear levels to interfere with putative anti-apoptotic mechanisms. Nuclear roles have been demonstrated for the effector caspases-3 [21,22], caspase-7 [23,24], caspase-6 [25], and caspase-8 [13,26] through cleavage of poly(ADP-ribose)polymerases, and for caspase-2 [27]. The multiplicity of caspases targets renders the effects of anti-caspase strategies even more

unpredictable, which points to the crucial need for analyzing the final result of caspase inhibition *in vivo*.

3. Caspase inhibition as a therapeutic target in stroke

Ischemic vascular disease represents the third leading cause of death, and the first cause of disability and morbidity in industrialized countries. Stroke is also the second most common cause of dementia. In correlation with the socio-economic importance of stroke, an impressive number of neuroprotective compounds have been developed for clinical trial in the last decades. However, although these compounds have yielded very promising results in animal research, they have proven to be inefficient in clinical settings. The only effective therapy currently available is thrombolysis, which is restricted to a small proportion of stroke patients. Neuroprotective agents with different modes of action and the ability to be used during an extended time window are therefore urgently required.

As stated above, the effectiveness of blocking caspase activity is still largely debated (see [28]). First, it is still undetermined whether caspase inhibitors truly rescue cells, i.e. restore cell viability and function, instead of simply blocking the apoptotic molecular cascade. As a matter of fact, caspase inhibition by synthetic peptides in rodent models of global ischemia, or in *in vitro* models of Parkinson's disease, reduces neuronal loss but is not sufficient to maintain the functional integrity of rescued neurons [12,29,30]. On the other hand, successful limitation of the extent of neurological failure has been obtained in the case of mild ischemic insults [31–33]. Secondly, broad-range inhibitors may interact with vital cysteine proteases, which may only result in the deregulation of apoptosis. To illustrate this point, recent studies have shown that caspase inhibition can actually promote cell death by switching the outcome from apoptosis to necrosis [12,14,33]. This switch appears to occur in states of intracellular energy depletion, common to many acute and chronic neurodegenerative diseases, and particularly crucial in cerebral ischemia. Thus, the use of caspase inhibitors in acute or progressive neurodegenerative conditions linked to intracellular energy depletion should be cautiously evaluated, and has become a key question to answer before caspase inhibitors are considered for therapeutic purposes.

4. Peptides or proteins, which suits the most?

Caspase inhibitors include peptides, peptidomimetics and various small molecules, and proteins. Peptide-based inhibitors have low penetration into the brain, are not very selective and are rapidly cleared [34]. Peptidomimetics and small molecules are also associated with problems of toxicity [35], and further efforts are needed to improve their bioavailability, stability, and selectivity. Inhibitory proteins are represented by endogenous molecules initially identified in viruses, and adopted by the cell to control apoptosis.

Several homologues of viral antiapoptotic proteins have been evidenced in mammalian cells, including CrmA from cowpox virus [36], p35 and the inhibitors of apoptosis (IAPs; [37]) from baculovirus, and the Fas-associated death domain-like interleukin-1-β-converting enzyme-inhibitory proteins from herpes virus (FLICE-inhibitory protein, Flips; [38]). The X-chromosome-linked IAP (XIAP) is the most potent inhibitor among IAP family members. XIAP inhibits caspase-3, -7 and -9, and can in turn be cleaved by caspase-3. This cleavage ensures maximal efficacy of the proteolytic process induced by activation of the caspase cascades [39,40]. Neuronal loss following transient forebrain ischemia is reduced by adenoviral vector-mediated overexpression of XIAP *in vivo* [41]. Importantly, XIAP has a greater caspase inhibitory activity than synthetic peptides [37] and, in contrast to synthetic inhibitors [29], XIAP maintains the homeostasis of surviving cells, as demonstrated in a model of transient ischemia. In a study by Xu *et al.*, the overexpression of XIAP not only rescued CA1 hippocampal neurons but also reduced the functional impairment evaluated in terms of spatial memory loss [41].

c-Flip is the mammalian homologue of the equine herpes virus protein E8. The full-length protein variant, c-FlipL, is the most potent known inhibitor of apoptotic ''death receptors pathways'' [38], and targets pro-caspase-8, inhibiting its cleavage into active protease. The development of Flip-deficient mice provides evidence for an anti-apoptotic role of Flip in death receptor-induced apoptosis, as well as during embryonic development [42]. However, although Flip has mostly been endowed with an anti-apoptotic role, it is noteworthy that transiently produced Flips may result in cell death [43,44]. Recent investigations provide a putative explanation for this observation. During Fas-induced cell death, FlipL and FlipS associate with and induce clustering of pro-caspase-8 molecules [45]. Formation of increased pro-caspase-8 concentration can lead to auto-activation and initiate cleavage pathways. No data about the neuroprotective or deleterious actions of Flip in models of cerebral ischemia are currently available, but observations in cardiac tissue have revealed decreased Flip levels in cells undergoing apoptosis after ischemic injury [46], suggesting a down-regulation of the molecule, as observed for XIAP. Lesion-induced cleavage of XIAP has also been shown during motoneuronal death in a murine model of amyotrophic lateral sclerosis [39].

In pMCAO, however, we have observed contradictory responses of caspase inhibitors during infarction. The progression of the lesion in this model is correlated with a progressive increase in FlipL levels and decrease in XIAP levels (Fig. 1). This can be interpreted as a synergic

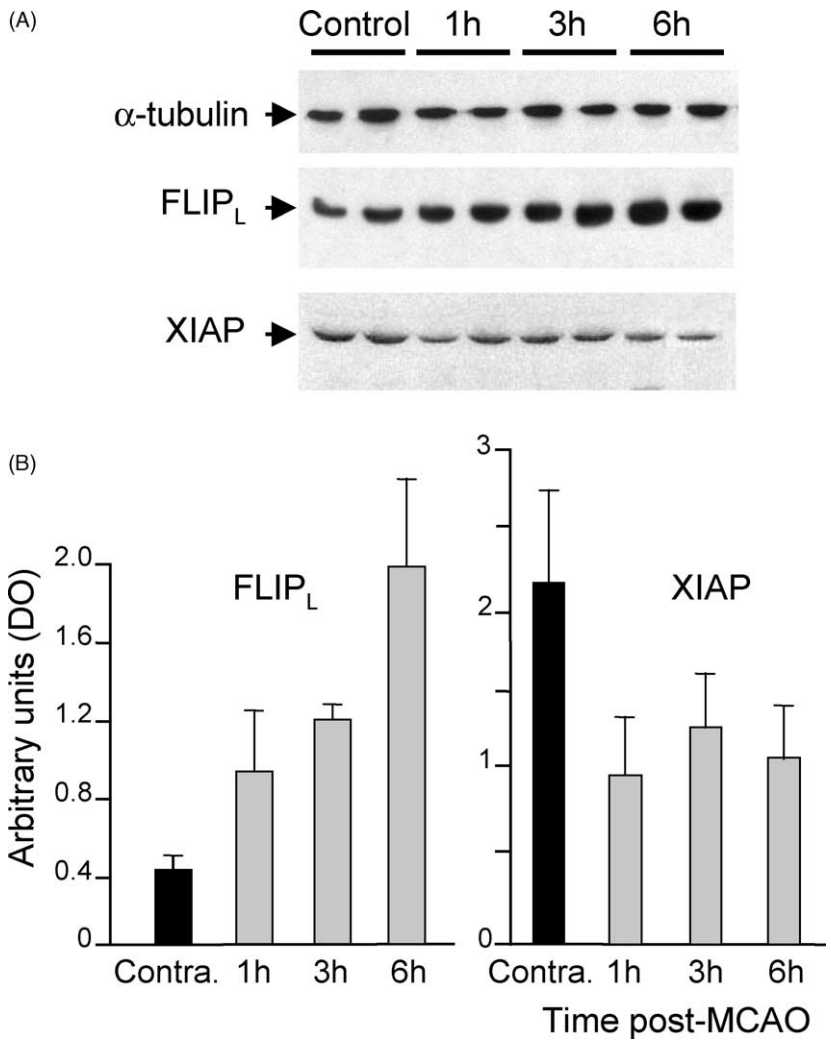

Fig. 1. Western blot analysis of FlipL and XIAP. (A) Protein extracts from cortices contralateral (''Contra.'') or ipsilateral to the occluded artery were obtained from mice sacrificed 1, 3, 6 hr post-occlusion. The expression of FlipL and XIAP was performed using monoclonal anti-Flip and rabbit polyclonal anti-XIAP antibodies. Each lane corresponds to a different animal and is representative of four to six animals per group. (B) Results of the densitometric analysis of Flip and XIAP contents corrected for alpha-tubulin contents for each lane.

pro-apoptotic response, if we suppose that FlipL contributes to the damage process by increasing caspase-8 activation, while anti-apoptotic XIAP declines due to its cleavage by caspase-3. However, Western blotting was performed on extracts from cortical tissue that included the whole arterial territory, i.e. both the core and the penumbra. A more specific investigation is currently being performed to distinguish events that occur in both areas.

The administration of substances to the brain has long been one of the major technical challenges in treating neurological injuries and disorders. Several methods for the delivery of large proteins to the central nervous system have been developed, with the majority consisting of invasive strategies that involve intracerebral administration of devices or cells. Although intracerebral implantation of fetal tissues have interesting therapeutic value in the treatment of Parkinson's and Huntington's diseases [47,48], brain surgery in stroke patients cannot be envisaged before a long post-stroke delay. To develop a non-invasive approach for *in vivo* studies, we have chosen a new delivery system, the transactivator domain (TAT)-fusion proteins. The fusion of a protein with the protein transduction domain (PTD) of human immunodeficiency virus (HIV) transactivator of TAT [49] confers transmembrane passage abilities. This technology was originally described in 1988 [50,51] and has several advantages. For example, it circumvents the problems inherent in the use of viral vectors. In addition, due to their ability to cross lipid membranes, TAT-fusion proteins can be administered systemically [52,53]. A number of fusion proteins with neuroprotective properties have been raised to date, including members of the Bcl2 family, and endogenous inhibitors of caspases. For instance, the intraperitoneal (i.p.) injection of a fusion protein containing the anti-apoptotic molecule Bcl-xL in mice results in robust protein transduction into neurons [54], and consequently, a strong reduction in cerebral infarction following focal transient ischemia [55–57]. In keeping with these

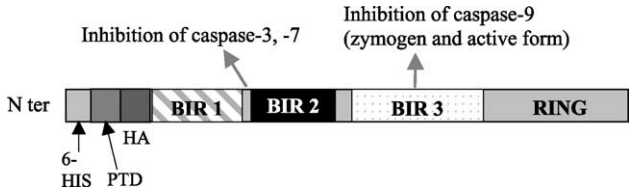

Fig. 2. Structures of the TAT-XIAP and TAT-FlipL fusion proteins. Both proteins contain an hemagglutinin (HA) tag, a 6-histidines (6-HIS) tag, and the PTD sequence (YGRKKRRQRRR).

results, we have recently created a XIAP fusion protein with similar properties (Fig. 2). PTD-XIAP was administered to mice in the form of a piece of Gelfoam® (UpJohn Comp.) impregnated with the protein placed over the cortex 30 min before MCAO. This treatment results in 50% lower infarct volumes at 24 hr when compared to mice that received only the vehicle solution. The infarct volume is still lower by 25% after 1 week, which is quite significant in the most severe animal model of cerebral infarction. These data encourage the development of a ''protein therapy'' approach with anti-apoptotic molecules to reduce ischemic brain damage.

Besides its deleterious function in neurological diseases, apoptosis is also of fundamental importance to the physiology of living organisms. Apoptosis regulates various biological processes, including normal cell turnover to maintain tissue homeostasis and to regulate the immune system [58]. Dysregulation of apoptosis through several pathways involving caspases, such as the Fas pathway, is relevant to the onset of a number of diseases, including autoimmune diseases [59] and malignancy [60]. Survivin, an inhibitor of caspase-1, promotes cell proliferation *in vitro*. Therefore, the systemic delivery of an anti-apoptotic protein able to penetrate any cell type raises justified concerns about the risk of cancer induction. Although no prominent side effects have been reported after i.p. delivery of the anti-apoptotic Bcl-xL TAT-fusion [56,57] protein, a specific investigation of the risk of cancer or dysregulation of the immune system has not so far been carried out. This crucial issue has to be carefully investigated before the use of anti-caspase agents is considered for clinical purposes.

5. Conclusion

As a result of repeated failure of therapeutic strategies designed for limiting stroke-induced neuronal death, new approaches are currently developed, that include gene-based therapies, administration of growth factors conjugated to peptidomimetic receptor ligands, transplantation of stem cells and modulation of the immune system. Protein therapy with PTD vectors is an interesting alternative since it allows non traumatic administration of large molecules linked to a small sequence that confers them the ability to penetrate any cerebral cell type. This approach may have great therapeutic potential if we link it to new targets identified by the recent improvements of our knowledge on the molecular mechanisms involved in ischemic cell death.

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