Chapter 6

# Caspases in Alzheimer's Disease

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## 1. Introduction

In AD, a significant synaptic loss ranging from 20% to 50% is reported. Biochemistry, electron microscopy and immunocytochemistry have shown a decrease in synaptic density, presynaptic terminals, synaptic vesicle and synaptic protein markers in AD brains compared with the normal aged controls (Terry et al., 1991; Geula, 1998; Larson et al., 1999; Yao et al., 1999; Ashe, 2000; Baloyannis et al., 2000; Terry, 2000; Masliah, 2001; Masliah et al., 2001; Price et al., 2001; Scheff and Price, 2001; Scheff et al., 2001; Stephan et al., 2001; Callahan et al., 2002; Chan et al., 2002; Dodd, 2002). Although synaptic loss is remarkable in AD, it is not specific to AD. Reduction in synaptic density is also found in Pick's disease, Huntington's disease, Parkinson's disease as well as in vascular dementia (Geula, 1998; Larson et al., 1999; Yao et al., 1999; Ashe, 2000; Baloyannis et al., 2000; Terry, 2000; Masliah, 2001; Masliah et al., 2001; Price et al., 2001; Scheff and Price, 2001; Scheff et al., 2001; Stephan et al., 2001; Callahan et al., 2002; Chan et al., 2002; Dodd, 2002).

Since one of the most important physiological functions of synapses is to release and accept neurotransmitters, the changes of activity of these neurotransmitters in neurodegenerative diseases have also been intensively studied (Terry, 2000). In AD, most significant lesions happen in the cholinergic, adrenergic and serotoninergic systems (Davies and Maloney, 1976; Geula, 1998; Larson et al., 1999; Yao et al., 1999; Ashe, 2000; Baloyannis et al., 2000; Terry, 2000; Masliah, 2001; Masliah et al., 2001; Price et al., 2001; Scheff and Price, 2001; Scheff et al., 2001; Stephan et al., 2001; Callahan et al., 2002; Chan et al., 2002; Dodd, 2002). Some other peptidergic neurotransmitters also decrease in AD, such as somatostatin, neuropeptide Y and substance P (Terry, 2000).

Synaptic loss might be one of the first events in AD development (Terry et al., 1991; Terry, 2000; Selkoe, 2002). Decrease in presynaptic terminals, synaptic vesicle and synaptic protein markers occur in very early stage of AD (Ashe, 2000; Terry, 2000; Masliah et al.,

2001; Price et al., 2001; Scheff et al., 2001; Callahan et al., 2002; Chan et al., 2002; Dodd, 2002). In the transgenic mice with FAD mutations, synaptophysin, marker for presynaptic protein, decreases before the appearance of Aβ deposits and formation of plaques (Hamos et al., 1989; Masliah et al., 1989; Selkoe, 2002). Most important, the decline of function of synaptic transmission occurs even before synaptic structural changes (Masliah, 2001; Scheff and Price, 2001; Chan et al., 2002; Selkoe, 2002). Long-term potentiation (LTP) is commonly accepted as a measurement for capacity of synaptic plasticity, which is the basis of learning, memory and complex information processing. The incidence and duration of LTP formation are used as an indication for formation and maintenance of working memory. Several lines of FAD mutant transgenic mice show a decline in the formation of LTP and synaptic excitation before the appearance of synaptic loss, plaques and other AD pathology (Geula, 1998; Ashe, 2000; Masliah, 2001; Masliah et al., 2001; Scheff and Price, 2001; Callahan et al., 2002; Chan et al., 2002; Selkoe, 2002). In summary, synaptic loss seems to appear earlier than all other pathological markers and the functional loss of synapses may be responsible for the initiation of cognitive decline in AD patients.

## 2. Neuronal loss in AD

Synaptic loss and degeneration induce neuronal dysfunction and cell body loss. Neuronal loss in the cerebral cortex and the hippocampus is a hallmark feature of AD. Some of AD patients at late stage of the disease can have a severe decrease in brain volume and weight due to either neuronal loss or atrophy (Smale et al., 1995; Cotman and Su, 1996; Gomez-Isla et al., 1996; Gomez-Isla et al., 1997; Li et al., 1997; Su et al., 1997; Gomez-Isla et al., 1999). Assumption-based and design-based unbiased stereological cell counting show decreased density of neurons in the cerebral cortex, the entorhinal cortex, the association cortex, the basal nucleus of Meynert, the locus coeruleus and the dorsal raphe of AD brains (Bondareff et al., 1982; Lippa et al., 1992; Gomez-Isla et al., 1996; Gomez-Isla et al., 1997; Gomez-Isla et al., 1999; Colle et al., 2000). Profound neuronal loss is especially observed in the entorhinal cortex in the mild AD brains (Gomez-Isla et al., 1996; Gomez-Isla et al., 1997; Gomez-Isla et al., 1999). Besides AD, significant neuronal loss is also observed in the entorhinal cortex in very mild cognitive impairment patient brains (Gomez-Isla et al., 1996; Gomez-Isla et al., 1997; Gomez-Isla et al., 1999). These data suggest that neuronal loss may be one of the early events before formation of SPs and NFTs in AD development.

The loss of cholinergic neurons in AD is widely studied. The hippocampus and cortex receive major cholinergic input from the basal forebrain nuclei (Hohmann et al., 1987). Decrease of choline acetyltransferase activity and acetylcholine synthesis correlate well with the degree of cognitive impairment in AD patients (Mesulam, 1986; Hohmann et al., 1987; Pearson and Powell, 1987). Cholinergic neuronal lesion can be detected in the patients that have showed clinical memory loss symptoms for less than 1 year (Whitehouse et al., 1981; Whitehouse et al., 1982; Francis et al., 1993; Weinstock, 1997). However, markers for dopamine, γ-aminobutyric acid (GABA), or somatostatin are not altered (Whitehouse et al., 1981; Whitehouse et al., 1982; Francis et al., 1993). These results suggest that cholinergic neuronal loss is probably one

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of the early events in AD. Besides the main pathology discussed above, some other pathologies of AD include granulovacuolar degeneration, cerebral amyloid angiopathy, blood-brain barrier disorder, white matter lesions, neuropil thread and gliosis (Jellinger, 2002a; Jellinger, 2002b, c; Jellinger and Attems, 2003).

As discussed above, stereological cell counting shows that densities of neurons in the AD cerebral cortex, the entorhinal cortex, the association cortex, the basal nucleus of Meynert, the locus coeruleus and the dorsal raphe decrease significantly compared to the age-matched non-AD controls (Bondareff et al., 1982; Lippa et al., 1992; Gomez-Isla et al., 1996; Gomez-Isla et al., 1997; Gomez-Isla et al., 1999; Colle et al., 2000). Neuronal cell loss is one of the first events during AD development. In the mild AD patient brains, remarkable neuronal cell loss of more than 40% is seen in the entorhinal cortex (Gomez-Isla et al., 1996; Gomez-Isla et al., 1997). Even in the mild cognitive impairment patient brains, significant neuronal loss is also observed in the entorhinal cortex (Gomez-Isla et al., 1996). Furthermore, the degree of neuronal loss correlates better with the clinical dementia level in AD than other pathology.

It was thought until recently that neuronal loss is mainly due to passive neurotrophic factor withdrawal. In 1988, Martin et al. (1988) showed that sympathetic neuronal death could be prevented by inhibiting RNA and protein synthesis, indicating that some of the neuronal death might be actively programmed (apoptotic) instead of passive (necrotic).

## 3. Apoptosis

Apoptosis, or programmed cell death (PCD), is a term proposed by Kerr, Wyllie and Currie in 1972 (Kerr et al., 1972) to describe a common type of cell death characterized by membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of corpses by surrounding cells (Kerr et al., 1972). It rapidly cleans out dysfunctional cells, limits toxic effects, saves energy and recycles molecules for future *de novo* synthesis.

Two major apoptotic pathways in mammalian cells are mediated through either death receptors or mitochondria (Figure 1). The so-called extrinsic pathway is initiated by death receptor CD95 (Apo-1/Fas), or other death receptors, such as tumor necrosis factor (TNF) receptor and tumor necrosis factor related apoptosis inducing ligand (TRAIL) receptor. CD95 is linked to pro-caspase-8 by an adapter factor FADD. Pro-caspase-8 has a death effecter domain (DED) which binds to the death domain (DD) on death receptor adapter FADD, and a caspase activation and recruitment domain (CARD), which binds to other downstream caspases and further processes the downstream caspases. Ligand binding signals the activation of pro-caspase-8 to form a tetramer of active caspase-8. Caspase-8 then mediates the cleavage of pro-caspase-3 and initiates the caspase activation cascade, which leads to protein and DNA cleavage and final termination of the cells. In this pathway, caspase-8 activation can be prevented by its natural inhibitors, such as cellular FADD-like interlukin-1-β-converting enzyme (FLICE/caspase-8)-inhibitory proteins (c-FLIPs) (Irmler et

al., 1997; Thome et al., 1997) and apoptosis repressor with caspase recruitment domain (ARC) (Koseki et al., 1998) (Figure 1).

The intrinsic pathway is triggered mainly by internal insults such as DNA damage. Damaging insults such as UV irradiation activate tumor suppression gene product p53, a transcriptional factor. There are several hypotheses for p53 activation mechanism. First, stress-activated protein kinases, such as DNA-dependent protein kinase (DNA-PK), phosphorylates p53. This phosphorylation prevents p53 from degradation. For example, DNA-PK can be activated by DNA damage, and then phosphorylate p53 at Ser-15 in the N terminal, which prevents the interaction between p53 and its inhibitory protein MDM-2. In addition to phosphorylation, dephosphorylation, such as the one caused by 14-3-3 at Ser-376, can also enable DNA binding of p53 and activate its function. The second model states that p53 is constitutively active and is regulated by the negative regulator MDM-2. MDM-2 protein can bind to p53 and send it out of the nucleus for degradation. Interestingly, the MDM-2 gene can be activated by p53, therefore, p53 is negatively self-regulated. After activation, p53 acts as a transcriptional factor controlling the expression of certain genes. These genes are involved in cell cycle control (eg. p21, GADD45, 14-3-3, CyclinD1, CyclinG), DNA repair (eg. GADD45, p21), apoptosis (eg. Bax, Bcl-2, FASL, DR5), angiogenesis (eg. TSP-1, BAI1) and cellular stress response (eg. TP53TG1, CSR, PIG3).

During apoptosis, p53 activates transcription of the pro-apoptotic Bax and, at the same time, transcriptionally represses the anti-apoptotic Bcl-2. A family of Bcl-2 proteins is implicated in apoptosis. This family contains three subgroups of proteins, some of them are pro-apoptotic while some are anti-apoptotic. The Group I proteins, such as Bcl-2, have a transmembrane domain and conserved Bcl-2 homology (BH) 1-4 domains. The Group II lacks the BH4 domain, such as Bax, while in the Group III, only the BH3 domain is in common, such as Bid and Bik (Hengartner, 2000). Bax is a pro-apoptotic protein causing the depolarization of mitochondrial membrane and release of cytochrome c from the mitochondria to the cytosol. The detailed mechanism of Bax leading to cytochrome c release is unknown. Bax is located in the cytosol as monomers and upon the apoptotic stimulation, Bax oligomerizes and translocates to the mitochondria. There are several models suggested to explain how Bax oligomers cause intermembrane protein cytochrome c release (Degli Esposti and Dive, 2003). First, Bax oligomers may form a pore structure on the mitochondria outer membrane leading to cytochrome c release. Second, interaction between Bax and other mitochondrial proteins, such as voltage dependent anion selective channel (VDAC) and adenosine nucleotide transporter (ANT) may induce pore formation by VDAC and ANT, through which cytochrome c is released. Third, the pore may form on the membrane by low-selective ion channels and induce cytochrome c release through these channels (Degli Esposti and Dive, 2003). Cytochrome c, together with the adapter molecule Apaf-1 and pro-caspase-9, forms an apoptosome that cleaves pro-caspase-3 into its active form and initiates apoptosis. Bcl-2, the other member of Bcl-2 family in the Group I, is an anti-apoptotic protein preventing Bax-mediated cytochrome c release efficiently.

The two apoptotic pathways cross at Bid, a pro-apoptotic protein from Bcl-2 family Group III. Bid can be cleaved by active caspase-8 and the truncated Bid translocates to the mito-

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chondrial membrane, increases mitochondrial outer membrane permeability, facilitates pore formation and potentiates cytochrome c release on the mitochondrial outer membrane (Figure 1) (Hengartner, 2000).

This apoptosis machinery is self-amplifying. For example, second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI (Smac/DIABLO) and apoptosis-inducing factor (AIF) proteins (Hengartner, 2000; Cregan et al., 2002) are released from the mitochondria with cytochrome c to facilitate apoptosis. In addition, recent studies showed that caspase-3, -6, -7 and –8 can initiate cytochrome c release by activating cytosolic factors (Figure 1). Since cytochrome c acts as an initiator for the caspase activation cascade, this self-amplified loop facilitates cellular apoptosis (Figure 1) (Bossy-Wetzel and Green, 1999).

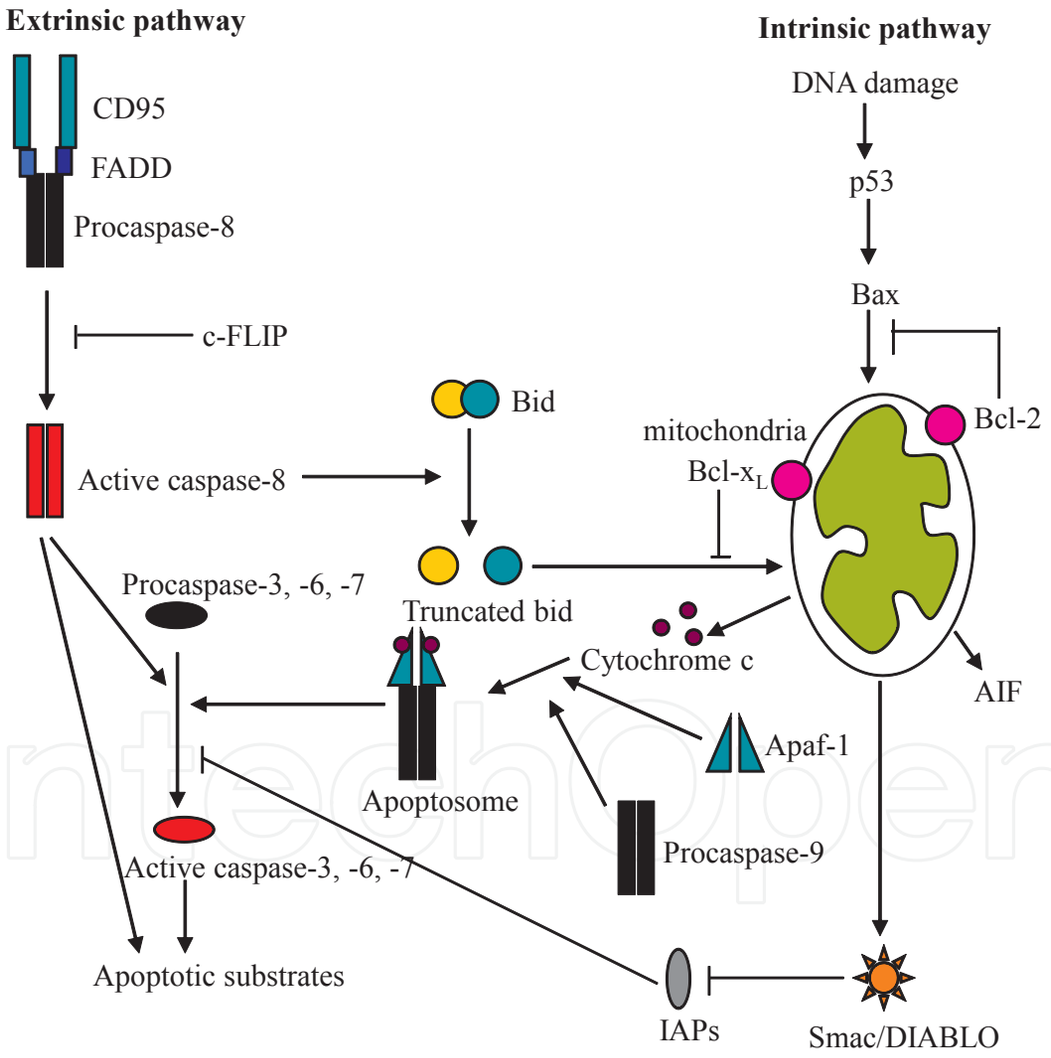


**Figure 1.** Schematic drawing of two major pathways involved in apoptosis. Schematic digram showing the two major pathways mediating apoptosis. The extrinsic pathway is mediated by death receptors, such as FADD, and caspase-8 activation. The intrinsic pathway is induced by DNA damage and mediated by caspase-9. IAP: inhibitor of apoptosis protein, Smac/DIABLO: second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI, AIF: apoptosis inducing factor.

## 4. Apoptosis involvement in neuronal cell loss of AD

There is evidence for apoptosis involvement in neuronal loss in AD, but the evidence so far is not sufficient to support a significant role for apoptosis in the neuronal cell loss in AD. Evidence of apoptosis in AD is as follows. First, overexpression of FAD-related mutations causes apoptosis in the transfected cell lines, cultured neurons and transgenic mice. For example, overexpression of FAD mutations of $APP^{V642I}$, $APP^{V642F}$ and $APP^{V642G}$ in COS or F11 cells increases number of apoptotic cells as determined by DNA fragmentation and terminal dUTP nick end labeling (TUNEL) staining assay, which can be inhibited by anti-apoptotic protein Bcl-2 overexpression (Yamatsuji et al., 1996). These results support the role of FAD mutations in inducing apoptosis. Similarly, the data from transgenic mice confirm the above observations. Transgenic mice overexpressing FAD mutant $APP^{V717F}$ develop neuritic dystrophy similar to some pathological features in AD patients (Games et al., 1995; Holcomb et al., 1998). The degenerating neurons in these mice also show typical apoptotic features, such as chromatin segmentation and condensation, and positive TUNEL staining (Nijhawan et al., 2000). However, in these studies, the FAD mutant proteins are normally overexpressed far beyond the physiological levels. However, FAD neurons do not necessarily undergo apoptosis. In the mutant PS1 expressing neurons, increased apoptosis is not reported (Bursztajn et al., 1998). Also, there is no neuronal loss found in mutant PS1 transgenic mice (Takeuchi et al., 2000).

Second, some studies indicate apoptosis in AD patient brains using *in situ* detection of DNA fragmentation by TUNEL staining (Su et al., 1994; Dragunow et al., 1995; Lassmann et al., 1995; Smale et al., 1995; Anderson et al., 1996; Su et al., 1996; Troncoso et al., 1996; Gervais et al., 1999; Anderson et al., 2000). However, in the AD brain tissues, some TUNEL-positive neurons show typical apoptotic morphology whereas some do not, suggesting degenerating neurons in AD may undergo both apoptosis and passive cell death, necrosis (Su et al., 1994; Troncoso et al., 1996; Lucassen et al., 1997; Yuan and Yankner, 2000). It is now commonly accepted that TUNEL staining sometimes is not specific to apoptosis (Stadelmann et al., 1998). The staining of TUNEL may indicate increased vulnerability of cells to a secondary insult, not necessarily undergoing apoptosis. On the other hand, the number of apoptotic neurons is difficult to measure precisely due to the chronic nature, relatively long progress of the disease and rapid clearance mechanism of dead cells.

Third, there are reports of changes of expression of apoptosis related proteins, such as p53, Bcl-2 and Bcl-$x_L$, in AD brains (Paradis et al., 1996; Kitamura et al., 1997; MacGibbon et al., 1997; Su et al., 1997; Cotman, 1998; Torp et al., 1998; Tortosa et al., 1998). For example, pro-apoptotic protein p53 is increased in AD brains (Kitamura et al., 1997), while anti-apoptotic proteins Bcl-2 and Bcl-$x_L$ are decreased in AD brains (Kitamura et al., 1998; Tortosa et al., 1998). Also, another pro-apoptotic protein Bax is increased in AD brains (Paradis et al., 1996; Kitamura et al., 1997; MacGibbon et al., 1997; Su et al., 1997; Cotman, 1998; Torp et al., 1998; Tortosa et al., 1998). However, the regulation of these pro- or anti-apoptotic proteins could be primary or secondary response to insults. On the other hand, the upregulation of either pro-

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or anti-apoptotic proteins could be explained by either the neurons undergoing apoptosis or neurons responding against apoptotic insults to prevent initiation of apoptosis.

Therefore, in summary, to date, there is no strong evidence showing significant involvement of apoptosis in AD neuronal loss. Since caspases, a family of cysteinyl proteases, play an important role in cell death, especially in apoptosis as discussed in the previous section (Thompson, 1995; Strasser et al., 2000; Yuan and Yankner, 2000), it is of interest to identify which caspase is responsible for human neuronal cell loss, how it is regulated, and whether it can be inhibited. In addition, caspase activation may be easier to use for identification of apoptosis since it is an upstream event of DNA fragmentation. Therefore, the apoptotic cells have not been cleared yet.

## 5. Caspase involvement in apoptosis and APP metabolism in AD

### 5.1. Caspases

Caspases (cysteinyl aspartate-specific proteases) belong to a cysteinyl protease family that cleaves specifically after an aspartic acid. To date, 14 caspases (11 of them are found in human) have been identified in mammals. The connection between apoptosis and caspases was first reported by Yuan et al. (1993) that caspase-1 is a homolog to CED-3, a gene regulating cell death in *Caenorhaditis elegans* (Yuan et al., 1993). The important role of caspases in apoptosis is also supported by the strong correlation between caspase activity and apoptosis in various cell types. Furthermore, caspase inhibition prevents apoptosis both *in vitro* and *in vivo* (Yuan et al., 1993; Kuida et al., 1995; Schwartz and Milligan, 1996; Alnemri, 1997; Thornberry, 1997; Li and Yuan, 1999; Yuan and Yankner, 2000).

Inactive caspases contain a pro-domain, a large subunit and a small subunit. According to their pro-domain structure and function, caspases are divided into "inflammatory", "initiator" and "effecter" caspases (Figure 2A) (Nicholson, 1999; Hengartner, 2000). Caspases are normally present as inactive precursors in cells. After receiving apoptotic signals, caspase pro-enzymes undergo proteolytic processing to remove the N terminal pro-domain and cleave between large and small subunits to produce the active form of a tetrameric enzyme formed by 2 copies of the large subunit and 2 copies of small subunit. To date, three caspase activation pathways are known in mammalian cells: recruitment-activation, trans-activation and autoactivation (Nicholson, 1999). Recruitment-activation is triggered by death receptors of the tumor necrosis factor receptor family. The so-called initiator caspases, namely caspase-2, -8, -9 and –10 (Figure 3A), are thought to be directly activated through the signals from death receptor (Boldin et al., 1996; Muzio et al., 1996). In addition, more recent data show that the initiator caspase-8, -9 and –10 can be activated by homodimerization of their monomeric zymogens, a so called "proximity-induced activation" (Boatright et al., 2003). Trans-activation occurs through downstream or executioner caspases, caspase-3, -6 and –7 that can be activated by direct proteolysis by their upstream initiator caspases. By such trans-activation cascade, apoptosis is well controlled and regulated (Darmon et al., 1995; Martin et al., 1996; Andrade et

al., 1998). Caspase activation can also occur by activation of the dormant enzyme molecule by the already active one (autoactivation). The supporting evidence of this mechanism comes from the observation that arginine-glycine-aspartate (RGD) motif-containing peptides can induce caspase-3 autoactivation by triggering conformational changes of pro-caspase-3 (Buckley et al., 1999). A similar mechanism has been suggested for pro-caspase-8 and –2 activation (Hengartner, 2000).

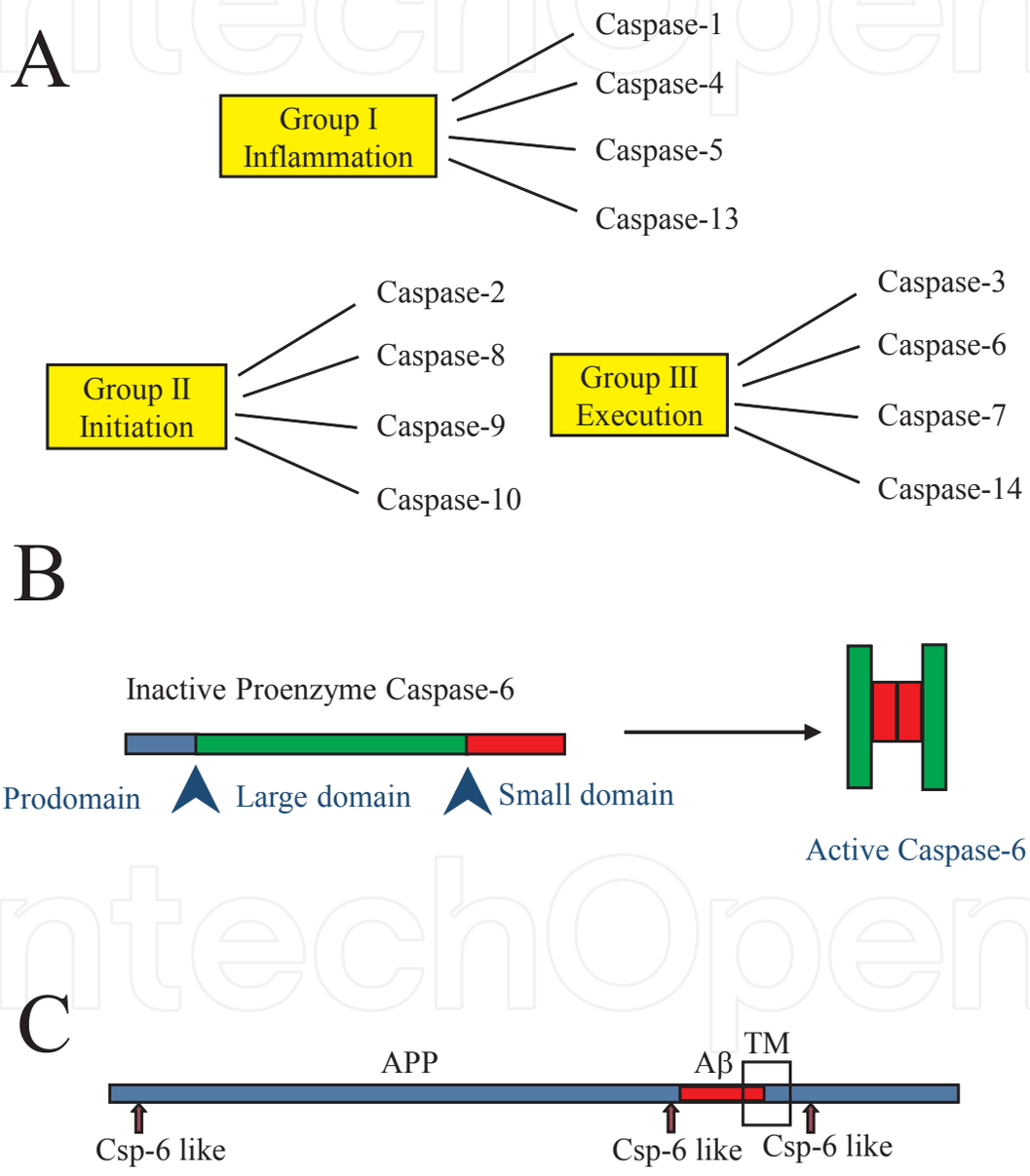


**Figure 2.** Caspases are cysteinyl proteases responsible for protein degradation during apoptosis. A. Schematic diagram of caspase category according to function. There are three groups of caspases according to their basic functions: inflammation, initiation and execution. B. Schematic drawing of pro-caspase-6 activation into active caspase-6. Caspase-6 activation by cleavage at the sites between pro-domain and large subunit (p20) as well as large subunit and small subunit (p10). Arrow head: cleavage. C. Schematic drawing of caspase-6-like cleavage sites on APP. There are three caspase-6-like cleavage sites on APP. Arrow: cleavage. TM: transmembrane domain.

After activation, caspases recognize four amino acid substrate sites as their targets and cleave the C terminal to an obligatory aspartic acid (XXXD). According to their specific substrates, caspases are divided into 3 groups: caspase-1, -4, -5 and –13 (substrates: WEHD and YVAD); caspase-2, -3 and -7 (substrate: DEXD) and caspase-6, -8, –9 and -10 (substrates: (I, V, L)EXD) (reviewed by (Thornberry, 1997)). Once activated, caspases cleave downstream substrates in a highly specific and rapid manner. More than 250 substrates are found including downstream caspases or apoptosis-related proteins (e.g. Bid, Bcl-2), structural proteins (e.g. lamins, actin, fodrin, gelsolin), DNA repair proteins (e.g. PARP, p21) and some other proteins involved in neurodegenerative diseases (eg. APP, tau, PSs, Huntingtin) (reviewed by (Bounhar et al., 2002).

Most of the morphological changes in apoptosis described by Kerr et al. (1972) are caused by caspases that are activated specifically in PCD. Caspase cleavage of nuclear lamins results in nuclear shrinking and blebbing (McCarthy et al., 1997; Sakahira et al., 1998). Loss of cell structure may be due to caspase cleavage of cytoskeletal proteins, such as fodrin (Vanags et al., 1996; Janicke et al., 1998) and gelsolin (Kothakota et al., 1997). DNA fragmentation is also caused by caspase-activated DNase (CAD), which is activated by caspases through removing the inhibitory subunit (ICAD) from the inactive CAD enzyme complex (Hengartner, 2000).

### 5.2. Involvement of caspases in neuronal loss in AD

In general, the evidence supporting the involvement of caspases in neuronal loss in AD is still not conclusive. The involvement of caspases in AD is first suggested by the finding that caspases, acting as proteases, are involved in PSs and APP metabolism and Aβ peptide generation in AD. Caspase-3 directly cleaves PSs during apoptosis (Kim et al., 1997). Caspase-3, -6, -7, -8 and –9 can cleave APP and generate Aβ or Aβ-containing fragments, therefore, giving a possibility of Aβ accumulation in AD (Barnes et al., 1998; Gervais et al., 1999; LeBlanc et al., 1999; Pellegrini et al., 1999; Weidemann et al., 1999). In chick motor neurons, caspase-3 cleaves APP and generates Aβ products (Barnes et al., 1998). In human 293 cells, caspase-9 cleaves APP at the C terminal and produces a "C31" peptide, which is cytotoxic to cells (Lu et al., 2000). Caspase-3 cleaves APP in NT-2 cells and is involved in Aβ formation (Gervais et al., 1999). APP is a caspase substrate in staurosporine-treated NT-2 cells and Fas-treated human Jurkat cells (Pellegrini et al., 1999). In addition, caspase inhibitors can prevent the formation of Aβ (Barnes et al., 1998; Gervais et al., 1999; LeBlanc et al., 1999).

Second, more direct proof of caspase involvement in AD comes from immuno-detection of active caspases in AD brain tissues, although the evidence is not sufficient enough to be conclusive. Some studies have shown the activation of caspase-8 in AD brains (Rohn et al., 2001; Rohn et al., 2002), while others found decreased level of caspase-8 in AD brains (Engidawork et al., 2001a). In yet another study, both inactive and active caspase-8 immunoreactivity are not changed in AD compared to control brains (Engidawork et al., 2001b). The cleavage fragment of caspase-9 is also detected in AD but not in the control brains (Lu et al., 2000; Goyal, 2001; Rohn et al., 2002), but the alteration of two caspase-9 activation co-factors, Apaf-1 and cytochrome c, is not detected (Engidawork et al., 2001b). Furthermore, there is recent evidence showing that pro-caspase-9 is activated through dimerization, but not cleavage (Boatright et al., 2003). Among all caspases, caspase-3 is the most intensively studied

since the caspase-3 knockouts develop abnormal brains with significantly excessive numbers of neurons (Cregan et al., 1999; Keramaris et al., 2000; Simpson et al., 2001; Fernando et al., 2002). Some studies report increased caspase-3-like immunoreactivity in AD brains (Masliah et al., 1998; Gervais et al., 1999). However, the protein and mRNA levels of caspase-3 do not appear altered in AD compared to control brains (Desjardins and Ledoux, 1998; LeBlanc et al., 1999). Active caspase-3 is detected in granulovacuolar degeneration, an aging associated pathology that is not necessarily specific to AD (Stadelmann et al., 1999; Roth, 2001). Given that apoptosis results in a rapid clearance of dysfunctional cells, only small amount of caspase activation can be detected at a certain time window. Therefore, the extensive detection of active caspase-3 in some studies may be due to lack of immunospecificity of the caspase-3 antibody. Furthermore, although caspase-3 is critical for apoptosis in many cell types, it does not have significant role in the human neuronal loss in AD (Desjardins and Ledoux, 1998; LeBlanc et al., 1999; Selznick et al., 1999; Stadelmann et al., 1999).

Interestingly, LeBlanc et al. have shown that caspase-6 is activated in serum deprivation induced cell death of human neurons in primary cultured (LeBlanc et al., 1999). Therefore, could caspase-6 be the responsible caspase in human neuronal cell death in AD?

### 5.3. Caspase-6 is activated during human neuronal cell death

#### *5.3.1. Introduction to caspase-6*

Caspase-6 (Mch-2), located on human chromosome 4q25, is an "effecter" caspase with a short pro-domain. It recognizes VEID substrates and cleaves after the amino acid D. Its common substrates include APP (LeBlanc et al., 1999), cytoskeleton proteins, such as keratin 18 (Caulin et al., 1997), focal adhesion kinase (Gervais et al., 1998), tau (LeBlanc et al., 1999), β-catenin (Van de Craen et al., 1999), vimentin (Byun et al., 2001) and desmin (Chen et al., 2003), nuclear proteins, such as lamin A (Ruchaud et al., 2002), lamin B (Slee et al., 2001), PARP (Fernandes-Alnemri et al., 1995), DNA topoisomerase I (Samejima et al., 1999) and emerin (Columbaro et al., 2001), several transcriptional factors, such as SATB1, and AP-2$\alpha$ (Galande et al., 2001; Nyormoi et al., 2001). It is highly expressed in the heart, lung, liver, kidney and testis in murine tissues. There is immunodetectable caspase-6 in human brain and neurons (LeBlanc et al., 1999; Zheng et al., 1999; Harrison et al., 2001). Caspase-6 can be activated by caspase-1, -3, -7, -8 and –11 (Fernandes-Alnemri et al., 1995; Chinnaiyan et al., 1996; Orth et al., 1996). Pro-caspase-6 is a ~ 34 kDa protein, that can be cleaved into ~20 kDa (p20) and ~10 kDa (p10) fragments. The p20 and p10 fragments form a tetramer, which is the active form of the enzyme (Figure 3C) (Fernandes-Alnemri et al., 1995). Caspase-6 knockout mice do not show abnormal phenotype during development, which does not rule out the role of caspase-6 in cell death in later stage of life or under stress conditions (Zheng et al., 1999).

#### *5.3.2. Caspase-6 involvement in human neuronal cell death and APP processing*

Pro-caspase-6 decreases during apoptosis induced by serum deprivation in human neurons in primary cultures (LeBlanc et al., 1999). Moreover, caspase-6 active 10 kDa fragments is detected only in AD brains, and not in the normal aging control brains (LeBlanc et al., 1999),

although thus increase is not dramatic. Given that AD is a long progressive disease, at a certain postmortem time window, there is only limited amount of cell death where caspase activation can be detected. Meanwhile, in contrast to caspase-6, changes in the levels of pro- and active caspase-3 levels are not detectable in AD brains, suggesting that caspase-6, but not caspase-3, may be involved in human neuronal cell death (LeBlanc et al., 1999). Caspase-6 can also alter APP metabolism to generate Aβ-containing fragments. There are several caspase-6-like sites on $APP_{695}$ (Figure 2C). However, incubating recombinant caspase-6 and $APP_{695}$-containing neuronal extract does not show APP cleavage. One of these cleavages at $^{591}$VKMD$^{594}$ generates a 6.5 kDa fragment denoted "Capp6.5" containing the Aβ sequence (Figure 2C) (LeBlanc et al., 1999). Although caspase-6 does not directly induce 4 kDa Aβ, pulse-chase experiments showed that this Capp6.5 fragment is able to generate 4 kDa Aβ in human neurons (Figure 2C) (LeBlanc et al., 1999). Caspase-6 can cleave APP close to β-secretase site at $D^{653}$ to further generate $Aβ_{2-40}$ or $Aβ_{2-42}$ (Gervais et al., 1999). In the Swedish mutation of APP, which changes the VKMD$^{653}$ sequence at the β-secretase site to VNLD$^{653}$, the caspase-6 cleavage of VNLD-AMC is 6 fold higher than the VKMD-AMC fluorogenic peptide *in vitro* (Gervais et al., 1999). Also, caspase-6 can cleave APP after the γ-secretase site at VEVD$^{720}$/A (Gervais et al., 1999). Therefore, caspase-6 may process APP similar to β- and γ-secretase activity. Direct microinjecting caspase-6 into human neurons induces dramatic cell death (Zhang et al., 2000). Taken together, the above evidence suggests that caspase-6 plays important role in human neuronal cell death, Aβ formation, and may be responsible for neuronal loss in AD (LeBlanc et al., 1999).

### 5.4. Inhibition of active caspases

#### *5.4.1. Natural inhibitors to caspases*

The natural inhibitors to caspases include Cowpox virus product cytokine response modifier A (crmA), FLIPs, protease inhibitor 9 (PI-9), p35, ARC and inhibitor of apoptosis proteins (IAPs). CrmA is a member of serpin family, a group of serine protease inhibitors. CrmA inhibits caspases by acting as a pseudosubstrate that binds to active caspases, such as caspase-1, -4, -5, -8 and –9 (Ray et al., 1992; Komiyana et al., 1994). Besides crmA, FLIPs inhibits caspase-8 by acting as the dominant negative form to suppress caspase-8 mRNA expression (Thornberry, 1997). The mammalian homolog of crmA is PI-9. PI-9 mRNA expression can be rapidly induced by estrogen in human liver (Kanamori et al., 2000). PI-9 is a granzyme B inhibitor (GBI). Granzyme B is a 30 to 32 kDa serine protease, which cleaves peptides at aspartyl residue in the killer T thymocytes and natural killer cells. Granzyme B is involved in the perforation of target cells and then initiation of proteolysis that leads to apoptosis. Although PI-9 can inhibit granzyme B and granzyme B-mediated apoptosis, *In vitro* experiments do not show that PI-9 inhibits active caspases (Bird et al., 1998; Bird, 1999).

p35 is a baculoviral protein that can block the defensive apoptotic response of insect cells to viral infection (Clem et al., 1991; Ekert et al., 1999). p35 inhibits CED-3 in *C. elegans* and mammalian caspase-1, -3, -6, -7, -8 and -10 (Ekert et al., 1999). P35 is cleaved at its P1 residue by caspases, and the cleaved fragment forms an inhibitory complex to block caspase activation (Zhou et al., 1998). ARC interacts with caspase-2, -8 and CED-3, but not cas-

pase-1, -3, or –9. ARC inhibits caspase-8 enzyme activity in 293 cells, and further attenuates apoptosis induced by FADD through stimulation of death receptors coupled with pro-caspase-8 (Koseki et al., 1998).

IAPs were found in a search for viral genes with a similar function to p35. A group of cellular IAP homologs are found in yeast, *C. elegans*, *Drosophila* and vertebrates (Ekert et al., 1999). To date, the IAP family contains about a dozen proteins from viruses, *Drosophila*, mice and humans. All known IAPs share a baculovirus inhibitory repeat (BIR) domain, which contains a number of conserved residues including a zinc-binding region. Most of the IAPs also contain a RING zinc-binding finger motif at the C terminal. Both BIR motifs and RING finger are important for IAP function (Ekert et al., 1999). The neuronal apoptosis inhibitory protein (NAIP) in human was found by searching for mutations in spinal muscular atrophy (SMA), which is characterized by degeneration of the anterior horn cells in the spinal cord. The NAIP gene is deleted in the SMA patients. During development, excessive neurons are ultimately needed to send out axons, the neurons do not target properly undergo apoptosis. It is suggested that NAIP is involved by preventing apoptosis in the "successful" cells (Miller, 1997).

The most studied IAP is the X-linked IAP (XIAP). XIAP binds to active caspase-9 small subunit through its BIR3 domain and cleaves the small subunit to inactivate caspase-9 (Srinivasula et al., 2001). On the other hand, XIAP binds to active caspase-3 or –7 through BIR2 domain and mask the active site of these caspases (Huang et al., 2001; Riedl et al., 2001; Stennicke et al., 2002). In addition, evidence shows that in insects, some IAPs interact with apoptosis-related proteins, such as Grim, Reaper and Hid (Hay et al., 1995; Miller, 1997; Vucic et al., 1997; Vucic et al., 1998). IAPs can also inhibit caspase-3 directly and cytochrome c-induced caspase-9 (Deveraux et al., 1998). However, up to now, although IAPs may inhibit caspase-6 activation by blocking upstream caspase-9 or –3 activation (Deveraux et al., 1998), there is no evidence showing that mammalian IAPs directly inhibit active caspase-6 enzyme activity.

### *5.4.2. Synthetic inhibitors to active caspases*

Synthetic caspase inhibitors function as pseudosubstrates for active caspases and therefore, they are competitive inhibitors of active caspases (Ekert et al., 1999). The N terminal blocking groups of the pseudosubstrate peptides are usually acetyl- (Ac-) or benzocarbonyl (Z-). The biochemical property of these synthetic inhibitors depends on the chemical group linked to pseudosubstrate peptides. Aldehyde (CHO-) group inhibitors are reversible since there is no covalent bond formed between these inhibitors and caspases. These CHO inhibitors have poor cell membrane permeability, which limits largely the use of these inhibitors on live cells and animals (Schotte et al., 1999; Bounhar et al., 2002). The inhibitors linked to methylketone (chloromethylketone, cmk or fluoromethylketone, fmk) irreversibly inhibit caspases due to the formation of thyomethylketone bonds with the cysteine in the active site of caspases (Bounhar et al., 2002). These inhibitors are membrane permeable, but have less specificity of inhibitory action to caspases (Schotte et al., 1999; Bounhar et al., 2002).

Although synthetic caspase inhibitors are widely used in research, they may not be the ideal candidates for disease treatment since these inhibitors cannot inhibit caspase activation in specific certain cell types. For example, in AD, caspase-6 is the key caspase responsible for

neuronal loss. If the synthetic caspase-6 inhibitor is applied and it would inhibit active caspase-6 activity in all types of cells, which has a large potential to develop cancer in cells that proliferate. If a natural inhibitor to active caspase-6 can be activated somehow specifically in human neurons, the risk of oncogenesis in the brain would be greatly reduced.

Since neuronal loss is a striking feature of AD, decreasing or suppressing neuronal cell loss may benefit early AD patients in retaining cognitive capacities and prevent or delay disease progression. Caspases seem to play a significant role in human neuronal cell loss in AD, thus it is intriguing to determine if caspase activity can be inhibited after activation by neuroprotective agents.

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