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Alteration of Caspases and Apoptosis-Related Proteins in Brains of Patients with Alzheimer's Disease

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Dysregulated programmed cell death or apoptosis is suggested to be involved in the pathogenesis of Alzheimer's disease (AD). Caspases, the major effectors of apoptosis, are cysteine proteases that cleave crucial substrate proteins exclusively after aspartate residues. The activity of caspases are delicately regulated by a variety of proteins that possess distinct domains for protein–protein interaction. To further substantiate the role of apoptosis in AD, we investigated the levels of nine different proteins involved in apoptosis by Western blot technique in frontal cortex and cerebellum of control and AD subjects. The protein levels of caspase-3, -8, and -9, DFF45 (DNA fragmentation factor 45), and FLIP (Fas associated death domain (FADD)-like interleukin-1β-converting enzyme inhibitory proteins) were decreased, whereas those of ARC (apoptosis repressor with caspase recruitment domain) and RICK (Receptor interacting protein (RIP)-like interacting CLARP kinase) increased in AD. In contrast, cytochrome *c* and Apaf-1 (apoptosis protease activating factor-1) were unchanged. Regression analysis revealed no correlation between levels of protein and postmortem interval. However, inconsistent correlation was found between age and levels of proteins as well as among the levels of individual proteins. The current findings showed that dysregulation of apoptotic proteins indeed exists in AD brain and support the notion that it may contribute to neuropathology of AD. The study further hints that apoptosis in AD may occur via the death receptor pathway independent of cytochrome *c*. Hence, therapeutic strategies that ablate caspase activation may be of some benefit for AD sufferers.

Key Words: **caspases; apoptosis; Alzheimer's disease; Western blot; cytochrome *c;* frontal cortex; cerebellum.**

Apoptosis or cellular suicide is a morphologically and biochemically distinct form of programmed cell death that plays an essential role in development, homeostasis, and defense in multicellular organisms (1). The cell death machinery is conserved throughout evolution and is composed of activators, inhibitors, and effectors (2). The effector arm of the cell death pathway is composed of a novel family of cysteinyl aspartate specific proteases related to the *Caenorhabditis elegans* (*C. elegans*) cell death product CED-3, termed caspases (3). Thus far, at least 14 family members have been identified and can be divided into two groups based on the length of the prodomain (4, 5). Several of these caspases, notably caspase-2, -3, -4, -6, -7, -8, -9, and -10 have been implicated in induction of apoptosis (6). Caspases with large prodomains are known as upstream caspases as they are thought to be involved in the initiation of the apoptotic response and appear to be activated through interaction with specific adaptor proteins. Whereas those with short prodomains are called downstream or effector caspases and are probably activated predominantly, if not exclusively, through cleavage by upstream caspases (7, 8).

Caspases are synthesized as inactive precursors with an N-terminal prodomain, a large subunit, and small subunit. Proteolytic cleavage at specific aspartate residues produces two subunits of approximate molecular masses of 20 and 10 kDa, which on association form the active heterotetramer. Caspase activation may proceed by autoactivation, transactivation, or proteolysis by distinct proteases (9, 10). The proapoptotic function of the active enzymes is mediated by processing key intracellular substrates at a DXXD motif by the effector caspases (6, 7, 11).

Little is known about the regulation of caspase activity during apoptosis. In the nematode *C. elegans*, activation of the cell death protease CED-3 is positively regulated by CED-4 and inhibited by CED-9 through direct protein-protein interactions (12, 13). Likewise, Apaf-1 (apoptotic protease activating factor-1), a human protein that resembles *C. elegans* CED-4, inter-

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acts with caspase-9, a step that is required for the activation of the downstream protease caspase-3 (14). The cytochrome *c*:Apaf pathway in turn, is regulated by Bcl-2 family members (7) The prodomains of several apical caspases contain a protein module termed CARD (caspase activation and recruitment domain) or DED (death effector domain) that is conserved in several apoptosis regulatory molecules, including Apaf-1 and cellular inhibitors of apoptosis proteins (15, 16). The CARD has been proposed to play a regulatory role in apoptosis by allowing proteins such as Apaf-1 to associate with caspase-9 (17). A number of novel CARD or DED containing proteins that modulate apoptosis have been identified, including ARC (apoptosis repressor with CARD) (18), FLIP (FADD like interleukin-1β-converting enzyme inhibitory proteins) (19), and RICK (RIP-like interacting CLARP kinase) (20).

There is accumulating evidence that dysregulated apoptosis may lead to several pathologies including ischemic injury, cancer, and neurodegenerative disorders such as Alzheimer's disease (AD) (16, 21). The finding that Aβ (amyloid β) can cause apoptosis *in vitro* and APP (amyloid precursor protein) is a caspase substrate in neuronal and non-neuronal apoptotic death (22, 23) strengthened the notion that dysregulated apoptosis underlies the pathology of neurodegenerative disorders (16). These reports formed the Rationale for us to examine the levels of different proteins involved in apoptosis including caspase-3, caspase-8, caspase-9, Apaf-1, cytochrome *c*, RICK, ARC, FLIP, and DFF45 (DNA fragmentation factor 45) in brain of patients with AD. The findings demonstrate that apoptosis in AD may be mediated via cytochrome *c* independent pathway.

MATERIALS AND METHODS

Materials. Antibodies against caspase-9 (210-759-C100), FLIP (210-736-C100), DFF45 (210-737-C100), ARC (210-212-C100), Apaf-1, and RICK (210-213-C100) were purchased from Alexis Biochemicals (San Diego, CA). Rabbit polyclonal anti-caspase-8 (PC335) and anti-caspase-3 (H-277) were purchased from Oncogene Research Products (Boston, CA) and HybriDomus (Copenhagen, Denmark), respectively. Monoclonal mouse anti-cytochrome *c* (804-122-C100) was from PharMingen (San Diego, CA). Horseradish peroxidase-conjugated anti-mouse IgG (H + L) (1031–05) and anti-rabbit IgG (H + L) (4050–05) were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL). Enhanced chemiluminescence reagents were from NEN Life Science Products, Inc. (NEN Life Science Products, Inc., Boston, MA) and protein size markers (Rainbow) from Amersham (Uppsala, Sweden).

Human brain postmortem tissue Postmortem brain samples (frontal cortex, superior frontal gyrus and cerebellum) of patients with AD (n = 10; 4 females and 6 males; 60.8 ± 8.4 years old) and controls (n = 10; 3 females and 7 males; 62.6 ± 8.0 years old) were obtained from the Medical Research Council's, Brain Bank for Neurodegenerative Diseases, Department of Neuropathology, Institute of Psychiatry, London, UK. The major cause of death in AD was bronchopneumonia and heart disease in controls. The control brains were from individuals with no history of neurological or psychiatric illness. The AD patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria for probable AD (24). The histological diagnosis of AD was established and consistent with the consortium to establish a registry for Alzheimer disease (CERAD) criteria (25) for a definite diagnosis of AD. Postmortem interval of brain dissection was 25.7 ± 20.6 for AD; and 37.3 ± 18.4 h for controls. All samples were stored at −70°C and the freezing chain was never interrupted.

Sample preparation. Frozen brain samples were thawed on ice and homogenized in 4 volumes of TBS buffer (100 mM Tris, 150 mM NaCl, pH 7.5) containing protease inhibitors (1 μg/ml each of antipain, pepstatin A, and leupeptin and 10 mM of phenylmethylsulfonyl fluoride) and 0.05% Tween 20. Samples were homogenized on ice for 30 s (6–8 strokes) with a Potter–Elvehjem homogenizer. The brain homogenate then was centrifuged for 10 min at 10,000*g* and 4°C. To the supernatant, equal volume of sample buffer (125.5 mM Tris–HCl base, 70 mM sodium dodecyl sulfate, 0.001% Bromophenol blue, 20% glycerol, and 2% 2-mercaptoethanol, pH 6.8) was added and the mixture was heated at 95°C for 15 min. The concentration of protein was determined by the Bicinchoninic acid assay using bovine serum albumin as a standard (Pierce, U.S.A.).

Immunoblotting. Protein extracts containing either 100 μg (cytochrome *c*, RICK, DFF 45, ARC, FLIP), 50 μg (capase-3, -8, and -9) or 20 μg (Apaf-1) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (200 V for 3 h), and transferred to polyvinylidene fluoride membranes (Immobilon-P, MilliPore, Bedford, MA) (192 V for 1 h). The membranes were then washed two times for 5 min in Tris buffer (100 mM Tris–HCl, 150 mM NaCl, pH 7.5) and later blocked with blotto (1.5% nonfat dry milk in TBST (100 mM Tris–HCl, 150 mM NaCl, pH 7.5, 1 mM $MgCl_2$, 0.1% Tween 20)) overnight at 4°C followed by incubation with the respective primary antibodies in blotto (1:500 dilution for caspase-3, 2.5 μg/ml for caspase-8, 1:2000 for Apaf-1, and 1:1000 for the others) at room temperature for 3 h on a rocking shaker. The membranes were washed three times for 15 min with the blocking solution prior to incubation with respective horseradish peroxidase conjugated secondary antibody in blotto (1:1000 for caspase-3 and 1:2000 for the others) for one hour at room temperature on a rocking shaker. The membranes were washed again three times for 15 min followed by incubation with NEN chemiluminescence reagent for 1 min and subsequent exposure to films (Kodak Blue XB-1, Rochester, NY). Developed films were scanned and densities of the immunoreactive bands were calculated by RFLP Scan 2.1 software program (Scanalytics, Billerica, U.S.A.).

Data analysis. Values are expressed as mean ± standard deviation of optical density. Inter-group differences were analyzed by nonparametric Mann–Whitney U test and the significance was set at $P < 0.05$ for a two-tailed test. The relationship between age or postmortem interval and levels of the proteins as well as the relation amongst the levels of individual proteins was examined with linear regression analysis.

RESULTS

Proapoptotic Proteins

Caspases. Caspases are synthesized as inactive proforms which on receiving an apoptotic signal activated and subsequently cleaved into a prodomain and two subunits. The cleavage appears to occur in an ordered fashion with cleavage between the large and small subunits preceding removal of the prodomain. Caspase-3 is the major terminal effector or terminator caspase that acts on multiple death substrates to culminate in the classic morphologic features in cells un-

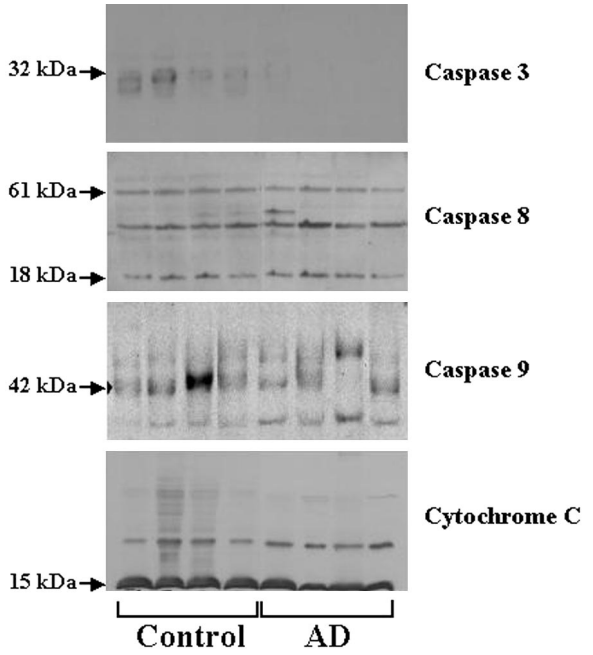

FIG. 1. Immunodetection of caspase-3, -8, -9, and cytochrome *c* in frontal cortex of control and AD. Brain homogenate containing 50 and 100 μg protein/lane from the frontal cortices of 10 controls and 10 AD brains were used for the immunodetection of caspases and cytochrome *c*, respectively. The immunoblots of 4 controls (lanes 1–4) and 4 AD (lanes 5–8) cases were shown in the upper panels.

dergoing apoptosis (26, 27). Initial cleavage of caspase-3 yields a P20 or P19 (depending on which aspartate site is cleaved) and P12 fragments and subsequent cleavage of P20 or P19 fragment give rises to the P17 protein (6). On immunoblots, a 32 kDa procaspase-3 protein was detected with anticaspase-3 antibody (Fig. 1). The antibody also reacted with two other proteins with apparent molecular size of 30 and 27 kDa that may correspond to caspase-3 lacking a proarm and/or heterodimer of the active subunits. Quantification of caspase-3 precursor immunoreactivity (IR) revealed an extremely significant reduction in cerebellum ($P < 0.001$) (Table 1A) as well as frontal cortex ($P < 0.001$) of AD patients (Table 1B). The decrement in total caspase-3 IR in AD showed more or less the same pattern. It was significantly reduced in both cerebellum ($P < 0.0001$) and frontal cortex ($P < 0.001$).

Caspase-8 is considered as the apical caspase and mediates signal transduction downstream of death receptors located on the plasma membrane (28). A 43 and 11 kDa proteins are obtained on first cleavage followed by the 18 kDa. Procaspase-8 ran on SDS–PAGE with an apparent molecular mass of 61 kDa accompanied by two other proteins with molecular mass of 43 and 18 kDa (Fig. 1). The two bands may represent the partially processed large subunit that results from initial cleavage and the fully processed P18 subunit, respectively, as the antibody reacts with the 18 kDa subunit. No significant difference was observed between controls and AD in the total caspase-8 as well as p18 and p45 IR in the regions examined. By contrast, the IR of caspase-8 precursor had shown a significant decrease in AD cerebellum ($P < 0.01$) as well as frontal cortex ($P < 0.01$) (Table 1A and 1B).

Caspase-9 also known as Apaf-3 mediates apoptotic signals after mitochondrial damage (17). A 37 kDa partially processed large subunit that later fully processed to an 18 kDa, and a 12 kDa fragments are obtained following cleavage. Three bands with molecular size of 56, 42, and 29 kDa were detected by making use of anti-caspase-9 antibody (Fig 1). The 42 kDa protein represents procaspase-9 and whereas the 27 kDa could be a split product of the 42 kDa that would not be processed to the active enzyme. The 56 kDa protein may be a splice variant, pathologically modified caspase or caspase complex. Both the 56 and 42 kDa proteins were not significantly altered in frontal cortex, although levels of the 42 kDa appeared to be lower in AD, whereas they were significantly decreased in AD cerebellum ($P < 0.0001$ and $P < 0.01$, for the 56 and 42 kDa, respectively). Likewise, the IR of the total caspase-9 and 29 kDa exhibited a significant decrease only in AD cerebellum ($P < 0.01$ and $P < 0.0001$, respectively) (Table 1A and 1B).

RICK, Apaf-1, and cytochrome c. RICK represents a novel kinase that regulate apoptosis mediated by the CD95 receptor pathway (20). It contains an N-terminal kinase catalytic domain and a C-terminal CARD domain. The protein migrated as a single band with an apparent molecular weight of ~40 kDa (Fig. 2). Densitometric analysis showed no significant change in RICK-IR of AD cerebellum (Table 1A) but a highly significant increase ($P < 0.01$) was noted in AD frontal cortex (Table 1B). Apaf-1 is the mammalian homologue of the key cell death gene CED-4 in *C. elegans* (14). Although Apaf-1 is not a caspase, its N-terminal region contains a CARD, suggesting that it may recruit caspase-9 through their respective CARDs (14). However, this domain is not exposed unless dATP or ATP is present (17). Anti-Apaf-1 antibody detected Apaf-1 that migrated as a 130 kDa and two other bands with molecular size of ~53 kDa and ~30 kDa (Fig. 2). The 53 kDa protein may correspond to a truncated form of Apaf-1 reported recently (29) and the third band could be a split product. Apaf-1 did not show any significant change in both regions of AD, so did the total IR (Table 1A and 1B).

Cytochrome *c* is normally present exclusively in mitochondria and thought to be released to the cytosol in response to a variety of apoptosis inducing agents. Release of cytochrome *c* has a pivotal role in binding Apaf-1 and causing caspase-9 activation that executes the death program (17). Based on its presumed role we analyzed its level and the Western blot pattern showed

TABLE 1

Levels of Proapoptotic Proteins in Cerebellum (A) and Frontal Cortex (B) of Controls and AD Cases

Protein	Control	AD
	A. Cerebellum	
(a) Caspase 3		
TIR	2.88 ± 1.31 (n = 9)	0.27 ± 0.11**** (n = 10)
Precursor (32 kDa)	1.81 ± 1.08 (n = 9)	0.20 ± 0.11*** (n = 10)
30 kDa	0.47 ± 0.28 (n = 9)	0.04 ± 0.02** (n = 10)
27 kDa	0.48 ± 0.18 (n = 9)	0.12 ± 0.10**** (n = 10)
(b) Caspase 8		
TIR	7.81 ± 2.29 (n = 9)	6.13 ± 1.92 (n = 9)
Precursor (61 kDa)	2.81 ± 0.84 (n = 9)	1.65 ± 0.68** (n = 9)
P18	2.29 ± 1.31 (n = 9)	1.95 ± 0.78 (n = 9)
P43	2.72 ± 0.90 (n = 9)	2.53 ± 1.36 (n = 9)
(c) Caspase 9		
TIR	3.31 ± 1.61 (n = 10)	1.26 ± 0.71** (n = 10)
Precursor (42 kDa)	1.35 ± 1.04 (n = 10)	0.35 ± 0.31** (n = 10)
56 kDa	0.53 ± 0.36 (n = 10)	0.08 ± 0.05**** (n = 10)
29 kDa	1.42 ± 0.96 (n = 10)	0.08 ± 0.05**** (n = 10)
(d) RICK	2.07 ± 1.07 (n = 9)	2.12 ± 1.44 (n = 10)
(e) Cytochrome *c*		
TIR	16.37 ± 5.77 (n = 9)	14.30 ± 6.52 (n = 9)
60 kDa	2.06 ± 1.25 (n = 9)	1.51 ± 1.33 (n = 9)
25 kDa	3.72 ± 2.85 (n = 9)	2.15 ± 1.41 (n = 9)
15 kDa	10.59 ± 2.05 (n = 9)	9.92 ± 2.60 (n = 9)
(f) Apaf-1		
TIR	9.88 ± 1.63 (n = 9)	8.72 ± 2.54 (n = 9)
130 kDa	0.51 ± 0.24 (n = 9)	0.52 ± 0.31 (n = 9)
	B. Frontal	
(a) Caspase 3		
TIR	2.44 ± 1.58 (n = 10)	0.06 ± 0.02*** (n = 10)
Precursor (32 kDa)	1.29 ± 1.08 (n = 10)	0.02 ± 0.01*** (n = 10)
30 kDa	1.29 ± 1.08 (n = 10)	0.03 ± 0.01*** (n = 10)
27 kDa	0.35 ± 0.27 (n = 10)	0.02 ± 0.01*** (n = 10)
(b) Caspase 8		
TIR	4.81 ± 1.62 (n = 10)	3.73 ± 1.85 (n = 6)
Precursor (61 kDa)	2.38 ± 0.86 (n = 10)	1.24 ± 0.40** (n = 6)
P18	2.22 ± 1.16 (n = 10)	1.84 ± 1.07 (n = 6)
P43	0.44 ± 0.17 (n = 10)	0.65 ± 0.43 (n = 6)
(c) Caspase 9		
TIR	3.82 ± 1.37 (n = 10)	3.67 ± 2.46 (n = 10)
Precursor (42 kDa)	2.15 ± 1.21 (n = 10)	1.89 ± 1.48 (n = 10)
56 kDa	0.37 ± 0.12 (n = 10)	0.74 ± 0.67 (n = 10)
29 kDa	1.45 ± 1.13 (n = 10)	1.15 ± 0.90 (n = 10)
(d) RICK	1.58 ± 0.73 (n = 10)	4.22 ± 3.20** (n = 10)
(e) Cytochrome *c*		
TIR	20.9 ± 4.68 (n = 10)	20.24 ± 5.16 (n = 8)
60 kDa	0.45 ± 0.25 (n = 10)	0.25 ± 0.13 (n = 8)
25 kDa	5.90 ± 3.25 (n = 10)	3.75 ± 1.39 (n = 8)
15 kDa	14.4 ± 3.02 (n = 10)	16.06 ± 3.77 (n = 8)
(f) Apaf-1		
TIR	0.59 ± 0.17 (n = 9)	0.60 ± 0.15 (n = 8)
130 kDa	0.12 ± 0.07 (n = 9)	0.07 ± 0.01 (n = 8)

Note. Following Western blot, immunoreactivity of 10 controls and 8–10 AD cases was quantified by scanning densitometry. Values are expressed as means ± standard deviation of arbitrary optical density and inter-group differences were analyzed by non-parametric Mann–Whitney U test and the significance was set at $P < 0.05$ for a two-tailed test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to controls. TIR, total immunoreactivity.

one minor and two major bands with molecular size of 52, 25, and 15 kDa, respectively with the 15 kDa protein representing cytochrome *c* (Fig. 1). Neither the total nor the 15 kDa cytochrome *c*-IR did exhibit any significant change in both cerebellum and frontal cortex of AD patients (Table 1A and 1B).

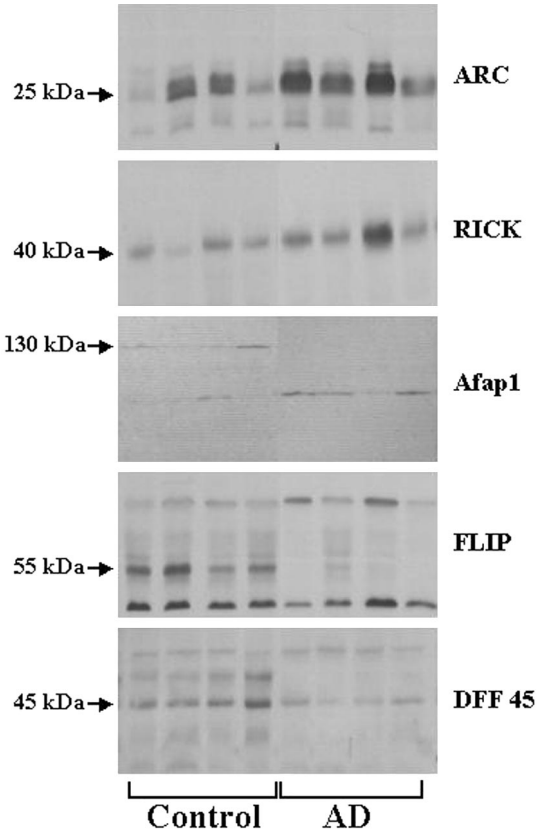

FIG. 2. Immunodetection of Apaf-1, RICK, ARC, FLIP, and DFF45 in frontal cortex of control and AD. Brain homogenate containing 20 μg (Apaf-1) and 100 μg (the others) protein/lane from the frontal cortices of 10 controls and 10 AD brains were used for the immunodetection. The immunoblots of 4 controls (lanes 1–4) and 4 AD (lanes 5–8) cases were shown in the upper panels.

Antiapoptotic Proteins

Among the proteins that negatively modulate apoptosis, DFF 45, ARC, and FLIP can be cited as an example. A total of 5 bands with three major bands ranging in molecular size from 25 to 60 kDa were detected using anti-DFF 45 antibody with DFF 45 migrating as a 45 kDa protein (Fig. 2). The two other major bands that lie above and below the 45 kDa protein could be a heterodimer of DFF40/DFF45 and cleavage product of DFF45, respectively as inactive DFF40 complexed with DFF45 was separated by gel filtration (30) and DFF45 was shown to be cleaved into smaller fragments (31). When the total IR for DFF 45 was calculated, frontal cortex of AD patients displayed a highly significant reduction ($P < 0.001$) (Table 2). Although the numerical mean in AD cerebellum appears to be lower than controls, a high degree of variation among the results precluded statistical significance. The intensity for the protein representing DFF 45, however, showed a highly significant reduction in both AD cerebellum ($P < 0.0001$) and frontal cortex ($P < 0.001$) (Table 2). FLIP also ran as a 55 kDa protein along with two other bands with apparent molecular mass of 37 and 80 kDa (Fig. 2). Since FLIP is a substrate for caspases (19) the 80 kDa and 37 kDa protein may represent a complex of caspase and FLIP and a cleavage product of FLIP, respectively. The IR for the 55 kDa protein displayed an extremely significant decrease in cerebellum ($P < 0.0001$) as well as frontal cortex ($P < 0.001$) of AD subjects. Neither AD cerebellum nor frontal cortex showed any significant difference in the IR of the 37 kDa protein. The total IR of the three bands, however, showed a significant change only in AD frontal cortex ($P < 0.05$) (Table 2). One major band at 25 kDa that represents ARC and two minor bands at 15 and 80 kDa were detected using anti-ARC antibody (Fig. 2). The 25 kDa ARC-IR significantly increased in AD frontal cortex ($P < 0.05$). In cerebellum, although it tended to increase the difference did not reach significance level. Total ARC-IR showed the same pattern of increase in the test subjects with a significant change only in AD cerebellum ($P < 0.01$) (Table 2).

Linear regression analysis revealed no significant correlation between post-mortem interval and expression levels of all the investigated proteins. However, inconsistent correlation was found between age and

TABLE 2

Levels of Antiapoptotic Proteins in Cerebellum and Frontal Cortex of Controls, DS, and AD Cases

Protein	Control	AD
Cerebellum		
(a) DFF 45		
TIR	11.75 ± 7.1 ($n = 8$)	5.46 ± 3.08 ($n = 10$)
DFF 45	2.65 ± 1.52 ($n = 8$)	0.26 ± 0.23**** ($n = 10$)
(b) ARC		
TIR	5.57 ± 3.92 ($n = 8$)	16.92 ± 9.40** ($n = 10$)
25 kDa	2.78 ± 1.50 ($n = 8$)	5.73 ± 3.09 ($n = 10$)
(c) FLIP		
TIR	5.81 ± 2.10 ($n = 8$)	7.10 ± 3.58 ($n = 10$)
55 kDa	2.03 ± 0.72 ($n = 8$)	0.08 ± 0.04**** ($n = 10$)
37 kDa	2.83 ± 1.11 ($n = 8$)	3.73 ± 1.46 ($n = 10$)
Frontal		
(a) DFF 45		
TIR	6.18 ± 1.92 ($n = 10$)	2.59 ± 1.09*** ($n = 5$)
DFF 45	1.83 ± 1.06 ($n = 10$)	0.19 ± 0.17*** ($n = 5$)
(b) ARC		
TIR	7.33 ± 4.91 ($n = 10$)	12.09 ± 6.81 ($n = 8$)
25 kDa	2.55 ± 1.19 ($n = 10$)	4.25 ± 1.72* ($n = 8$)
(c) FLIP		
TIR	10.65 ± 3.00 ($n = 10$)	6.00 ± 3.25* ($n = 6$)
55 kDa	4.04 ± 1.88 ($n = 10$)	0.26 ± 0.17*** ($n = 6$)
37 kDa	4.04 ± 1.88 ($n = 10$)	4.16 ± 2.41 ($n = 6$)

Note. Following Western blot, immunoreactivity of 10 controls and 8–10 AD cases were quantified by scanning densitometry. Values are expressed as means ± standard deviation of arbitrary optical density and inter-group differences were analyzed by non-parametric Mann–Whitney U test and the significance was set at $P < 0.05$ for a two-tailed test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to controls. TIR, total immunoreactivity.

levels of proteins as well as amongst the levels of individual proteins (data not shown).

DISCUSSION

The results presented in this study demonstrate alteration in different proteins involved in the apoptotic process, lending further support for the hypothesis that apoptosis may account for the neuronal loss observed in AD. Levels of all the procaspases examined in the present study were significantly decreased compared to controls, the decrease being similar in frontal cortex and cerebellum signifying that the cerebellum is equally affected in apoptosis.

The decrease in procaspases level may partly be explained by activation of precursors, as similar decrease was also observed in ceramide induced apoptosis in human neuroblastoma cell line (32). In addition, several of the known procaspases are found in multiple intracellular compartments and translocate from these compartments to others following apoptotic stimuli (5) which could also contribute to the observed reduction. Previous studies show inconsistent findings ranging from no change (33) to increased (34, 35) procaspase-3 protein levels in AD compared to controls. The discrepancy might have arisen either due to the difference in cellular compartments from which the sample is prepared or problems associated with the use of postmortem tissues. Although apoptosis may have a role in enhancing caspase expression, when we consider the fact that large amounts of caspases are synthesized as zymogens in advance and activated on demand (10), activation seems quite likely a logical explanation for the decrease in procaspases. Contrary to this notion, we did not find any change in the fully processed P18 large subunit of caspase-8 probably due to rapid clearance of cells containing activated caspases (35). It has recently been shown that caspases are substrates of calpains and cleavage by calpains yields inactive fragments (36). Furthermore, calpains were reported to be increased in AD (37). Thus, calpain cleavage of caspases may serve as an alternative explanation for the decrease in procaspases and production of different fragments.

At least two pathways of caspase activation have been described (Fig. 3). In the first, cytochrome *c* has a pivotal role in binding Apaf-1 and causing caspase-9 activation leading to the cascade of caspase activation that executes the death program (17). In the second, apoptosis is activated by cell surface death receptors and caspase-8 plays a central role. It is activated upstream of mitochondria and directly processes the effector caspases like caspase-3 (28). The receptor pathway involves death receptors like CD95(APO-1/Fas). CD95 belongs to the tumor necrosis factor/nerve growth factor receptor superfamily of cell surface proteins and has been shown to mediate receptor dependent programmed cell death. When engaged by CD95 ligand, CD95 oligomers are formed on the target cell and a signal to enter the cell's intrinsic apoptotic pathway is transmitted via the cytoplasmic so called "death domain," which in turn triggers the caspase cascade executing apoptosis (38).

The lack of detectable translocation of cytochrome *c* from mitochondria to cytosol is suggestive that caspase activation in AD may be effected by the receptor pathway. This is further supported by the unchanged levels of Apaf-1, a cytoplasmic protein that serves as a docking protein for cytochrome *c* and caspase-9 (17). Indeed, in many types of cells caspase activation and apoptosis were shown to be induced via cytochrome *c* independent pathway (39, 40). Hence, it appears likely that mitochondria participate in apoptosis in AD, not because of cytochrome *c* but rather due to oxidative stress as they are the major source of reactive oxygen species (ROS).

The unchanged levels of cytosolic cytochrome *c* leaves the receptor pathway to emerge as the likely pathway responsible for caspase activation. If this is the case what initiates caspase activation via this pathway? There appear to be some candidates which could be considered as a triggering factor. The first one is Aβ. Aβ, one of the hallmarks of AD, is considered as key etiological factor for neuronal loss mediated by apoptosis *in vitro* (41). Caspases are activated in neurons exposed to Aβ and caspase inhibitors block Aβ induced cell death (42). Thus, it is suggested that Aβ might initiate apoptosis by cross-linking death receptors (43). In addition, APP is a substrate of caspases, with caspase-6, and -8 displaying higher efficiency than caspase-3 and -9 (23) indicating a self-reinforcing mechanism that commits the cell to the death pathway. The second candidate is oxidative stress. ROS play an important role in cell death induced by diverse stimuli. There is fair evidence indicating the association between ROS and CD95. ROS were shown to increase CD95 mRNA as well as protein expression in microglial cells (44). Furthermore, 4-hydroxynonenal, a diffusible product of lipid peroxidation, was reported to cause Fas induced activation of caspase-8 (45). In AD brain, oxidative stress has been considered a possible contributor to neurodegeneration, associated with the formation of amyloid deposits and neurofibrillary tangles (33). Hence, it seems that ROS either directly or through Aβ can activate the receptor pathway. The third one is p53. p53 is a stress induced transcription factor that can be activated by a number of adverse stimuli including DNA damage, hypoxia, and ROS. Increased expression of p53 protein under these conditions causes growth arrest or apoptosis (46, 47). p53 and CD95 proteins may be inter-related because in non-neuronal cells, P53 activation was observed to induce CD95 expression (48, 49). A similar correlate was also reported in AD (50). The upregulation of p53 (51)

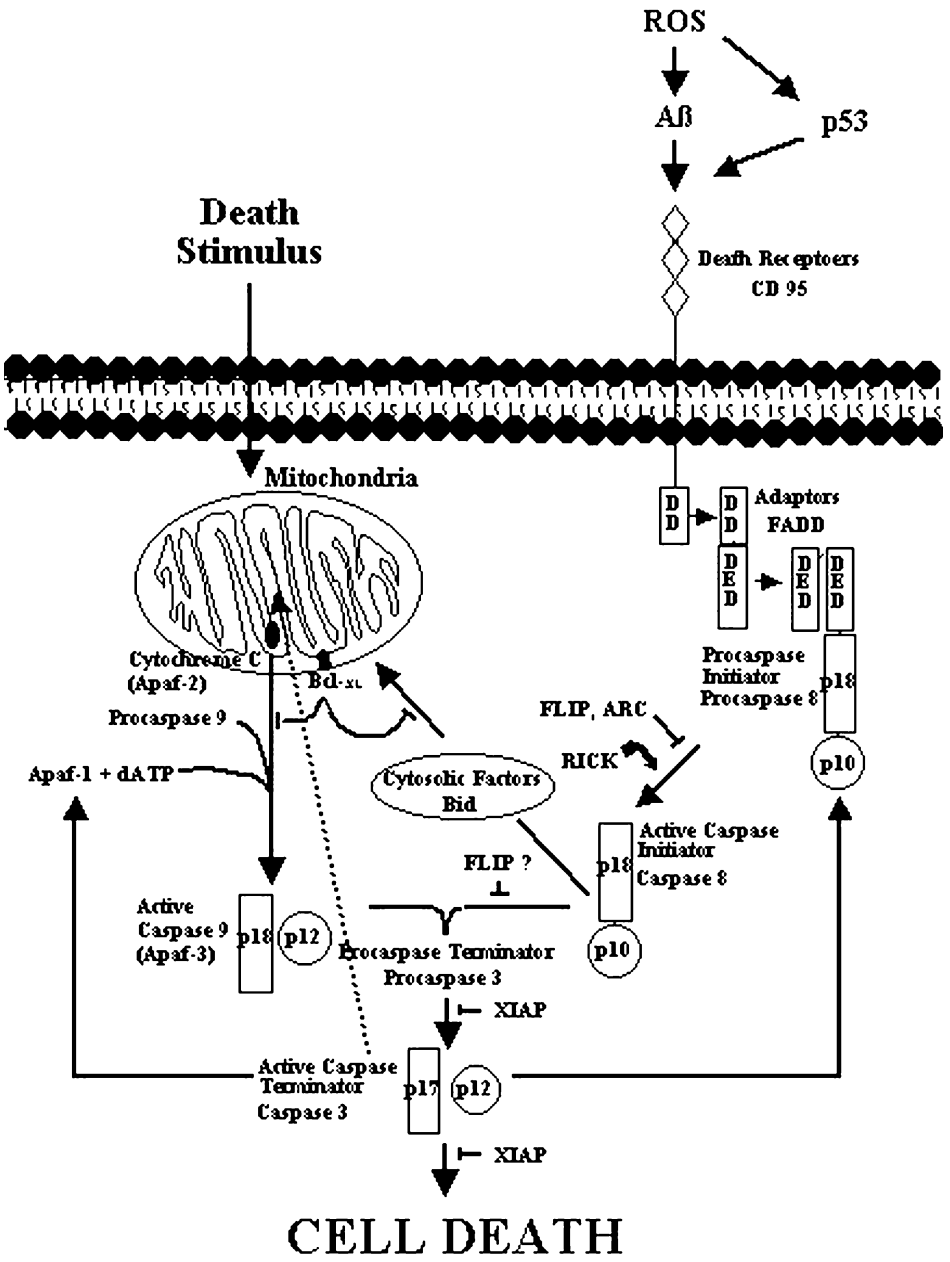

FIG. 3. Proposed pathway of apoptotic death activated by factors like Aβ in AD (adapted from Cryans and Yuan, 1998; Ivins *et al.*, 1999). Caspases can be activated by one of the two interacting pathways: a cytochrome *c* (left) and receptor (right) pathway. In the cytochrome *c* pathway, apoptotic stimuli such as DNA damaging agents (ROS?) trigger the release of cytochrome *c* from mitochondria into the cytosol a process that probably involves Bax redistribution in the reverse direction and is inhibitable by Bcl-2. Cytochrome *c* binds with Apaf-1 in the presence of dATP (ATP), a step that eventually exposes the CARD domain of Apaf-1 for binding and subsequent activation of procaspase-9. Active caspase-9 in turn, proteolytically activates the downstream terminator caspases such as caspase-3 that cause cell death by cleaving key intracellular proteins. In contrast, in the receptor pathway Aβ aggregation induced by ROS may either cross-link the death receptors or cause the release of death ligands that leads to the recruitment of a variety of DD bearing proteins such as FADD. FADD in turn recruits the initiator caspases such as caspase-8 to the complex through the DED module, an event that leads to the proteolytic cleavage of active caspase-8. This is further facilitated by p53 mediated upregulation of the death receptors. Active caspase-8 can initiate the caspase cascade by directly cleaving, bypassing mitochondria, the terminator caspases that results in the demise of the cell. Alternatively, active caspase-8 can activate the C-terminal fragment of the proapoptotic Bcl-2 family protein, Bid to cause the release of cytochrome *c*. This typically exemplifies the crosstalk between the two pathways. Activation of procaspase-8 and -9 and further release of cytochrome *c* by the active terminator caspases occur as a self-reinforcing mechanism to ensure death of the cell. The receptor pathway can be inhibited by preventing procaspase-8 recruitment and/or activation by FLIP and ARC whereas stimulated by RICK. Moreover, FLIP may also act as a persistent pseudosubstrate reminiscent of p35, a viral protein that inhibits caspases. The mammalian homologues of the baculovirus IAPs serve as inhibitors of the terminator caspases.

and CD95 (44) by ROS indicates the strong association between them and provides support for the involvement of p53 and ROS in activating the receptor pathway. Taken together, it is plausible that apoptosis in AD may be induced by factors, such as Aβ activation of caspase-8 in a cytochrome *c* independent manner.

The apoptotic regulatory proteins also showed altered levels like caspases. RICK contains an N-terminal serine-threonine kinase catalytic domain like RIP and a C-terminal that has significant similarity to the prodomain of caspases (20). Although the precise mechanism RICK promotes apoptosis is not known, its expression augments apoptosis promoted by caspase-8 and -10 (20). The increase in RICK observed in the frontal cortex of AD strengthens the proposed role of caspase-8 in mediating apoptotic death in this disorder. ARC is a novel CARD containing protein that inhibits CED-3, capase-2, and -8 through direct binding to death proteases (18). Though human ARC is mainly expressed in skeletal muscle and cardiac tissue (18), the Western blot pattern shows its expression in the brain as well (Fig. 2). ARC is significantly increased in AD frontal cortex consistent with the hypothesis that anti-apoptotic mechanisms that inhibit caspase activation may be upregulated with persistent apoptotic signal such as Aβ (41).

FLIP, another apoptotic regulating protein is also known by several names such as, Casper (52), FLAME-1 (4), CASH (53), and CLARP (54). It is expressed in mammalian cells as two alternatively spliced messages, as $FLIP_L$ and $FLIP_S$. The protein is composed of two N-terminal DEDs fused to a C-terminal caspase like domain devoid of protease activity, as it lacks key residues involved in catalysis (19). The role of FLIP in apoptosis is controversial, some studies reported its apoptosis inducing properties (52, 54), while others advocate for its antiapoptotic property (4, 19, 55, 56). Cleavage of FLIP by caspases *in vitro* results into two fragments topologically equivalent to the large and small subunits of caspases, the same is thought to occur *in vivo* (4). This cleavage may result in a strong FLIP-caspase-8 interaction, thereby blocking further processing and activation of caspase-8, reminiscent of p35 (19). The decrease in the 55 kDa FLIP levels obtained in AD in the present study supports such a scenario. Indeed, the 37 kDa protein, which is thought to be a split product of FLIP, constitutes 94.1% of the total pool of FLIP in AD frontal cortex compared to controls which is 57.4%.

DFF consists of two subunits of 45 kDa and 40 kDa, of which the 45 kDa subunit can be cleaved by caspase-3 into smaller peptides in cells undergoing apoptosis (30, 31). DFF45/ICAD is thought to serve as specific chaperone allowing the proper folding of the nascent DFF40/CAD polypeptide then it remains complexed with CAD to inhibit its DNase activity (30). Moreover, DFF45 is also suggested to inhibit DFF40 translocation into the nucleus (30). The reduction in DFF45 in AD is in line with its degradation by caspase-3 and further confirms the contribution of apoptosis in AD neuronal death.

Our data described here provide evidence for the alteration in levels of different pro-and anti-apoptotic proteins in AD brain. The study suggests that apoptosis triggered by factors, such as Aβ activation of caspases via cytochrome *c* independent pathway may account for the neuronal loss observed in this disorder. Therapeutic strategies aimed at caspase inhibition therefore might have some benefit in AD.

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