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EXPRESS COMMUNICATION

**Arun K. Raina · Ayala Hochman · Xiongwei Zhu
Catherine A. Rottkamp · Akihiko Nunomura ·
Sandra L. Siedlak · Heather Boux
Rudolph J. Castellani · George Perry · Mark A. Smith**

Abortive apoptosis in Alzheimer's disease

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Abstract Multiple studies suggest that neuronal death in Alzheimer's disease (AD) is the result of an apoptotic mechanism. However, the stereotypical manifestations that define the terminal phases of apoptosis, such as chromatin condensation, apoptotic bodies, and blebbing, are not seen in AD. In this study, we show that the caspases, such as caspase 6, which cleave amyloid-β protein precursor (AβPP) and presenilins, are localized to the pathological lesions associated with AD. However, while upstream caspases such as 8 and 9 are clearly found in association with the intraneuronal pathology in AD, downstream caspases such as 3, 6 and 7 are present only at control levels. Given that execution of apoptosis requires amplification of the caspase-mediated apoptotic signal, our results indicate that in AD there is a lack of effective apoptotic signal propagation to downstream caspase effectors. Therefore, while the presence of caspases, especially caspase 6, in association with extracellular deposits of amyloid-β, could obviously have important ramifications on the proteolytic processing of AβPP and, thereby, on disease pathogenesis, it seems that AD represents the first in vivo situation reported in which the initiation of apoptosis does not proceed to caspase-dependent cell death. This novel phenomenon of apoptotic avoidance, which we term abortive apoptosis, or abortosis, may represent an exit from the caspase-induced apoptotic program that leads to neuronal survival in AD.

Keywords Alzheimer disease · Apoptosis · Caspases · Neurofibrillary pathology · Neuronal survival

The first two authors contributed equally to this study

A.K. Raina · X. Zhu · C.A. Rottkamp · S.L. Siedlak
R.J. Castellani · G. Perry · M.A. Smith (✉)
Institute of Pathology, Case Western Reserve University,
2085 Adelbert Road, Cleveland, OH 44106, USA
e-mail: mas21@po.cwru.edu,
Tel.: +1-216-3683670, Fax: +1-216-3688964

A. Hochman
Department of Biochemistry,
George S. Wise Faculty of Life Sciences, Tel Aviv University,
Tel Aviv, Israel

A. Nunomura
Department of Psychiatry and Neurology,
Asahikawa Medical College, Asahikawa 078-8510, Japan

Heather Boux
StressGen Biotechnologies Corporation, Inc.,
350–4243 Glanford Avenue, Victoria,
British Columbia V8Z 4B9, Canada

Introduction

The nature and time course of neuronal cell death in Alzheimer's disease (AD) is controversial [2, 7]. Evidence for and against neuronal death mediated by apoptosis has further complicated the mechanisms underlying cell death in AD. For example, there are a vast array of putative apoptogenic stimuli present in neurons in AD that can act alone and/or synergistically with reactive oxygen species [26], amyloid-β [39], energy failure [38] and 4-hydroxynonenal (HNE) oxidants [13, 22]. Added to this is the apoptotic risk posed in familial AD [5]. However, while internucleosomal DNA fragmentation and end-labeling techniques have been used as absolute indices of apoptotic cell death in AD [1, 4, 34], it is now apparent that DNA fragmentation, in addition to being inferentially related to apoptosis, is also seen secondary to oxidative stress [28, 34, 37] and postmortem autolysis [30]. Indeed, unlike DNA fragmentation, the hallmark end-stage signs of apoptosis such as nuclear chromatin condensation and apoptotic bodies are absent in AD [17]. Added to the mechanistic and biochemical ambiguity of neuronal apoptotic mechanisms in AD is the temporal dichotomy between the acuteness of apoptosis and the chronicity of AD [18]. To address this issue further, we undertook a systematic study of apoptotic cascade proteins in AD by the systematic evaluation of initiator (caspases 8 and 9) as well as the executioner proteins (caspases 3, 6 and 7) of apoptosis. Our results show that, while upstream caspases are present in association with the pathological lesions in all cases of AD, downstream caspases including 3, 6 and 7 that signal completion of the apoptosis pathway remain unchanged in

such neurons. Therefore, the neurons that are present and viable at latter stages in AD must have employed survival compensations to the presumptive pro-apoptotic environment encountered in AD. This phenomenon appears to equate to apoptotic avoidance, or abortosis, a pathway that likely leads to neuronal survival rather than caspase-induced apoptosis.

Materials and methods

Tissue section preparation

Temporal lobe samples, including hippocampal formation, subiculum, entorhinal cortex and neocortex, were obtained postmortem from patients (n=10, ages 72–95 years) with histopathologically confirmed AD [8, 15], as well as from younger (ages 31 and 46 years) and aged-matched controls (n=8, ages 56–81 years). No significant differences in agonal status between the groups were apparent from the available medical records. Tissue was fixed in methacarn (methanol:chloroform:acetic acid in a 6:3:1 v/v/v) or buffered formalin by immersion for 16 h at 4°C. Tissue was subsequently dehydrated through graded ethanol and xylene solutions and embedded in paraffin. Microtome sections (6 μm thick) were prepared and placed on silane-coated slides. Tissue removed at biopsy from a diffuse large B cell lymphoma of humans was obtained to serve as a positive control and was fixed and processed in parallel.

Immunocytochemistry

Rabbit antisera to caspases 3, 6, 7, 8, and 9 were obtained from StressGen Biotechnologies Corporation, Inc. (Victoria, B.C., Canada). These antisera recognize both active and zymogen forms of the enzymes. Following hydration and incubation of the tissue sections with the antisera, sections were immunostained by the peroxidase-antiperoxidase procedure with 3,3'-diaminobenzidine as chromogen [33]. Adjacent serial sections were also immunostained with antibodies to tau, rabbit antiserum and monoclonal antibody AT8 (Innogenetics, Zwijndrecht, Belgium), to locate pathological changes. To verify the specificity of immunolabeling, adsorption experiments were performed by incubating the antibodies of the various caspases with their specific immunogen (StressGen Biotechnologies) at 1 μg/ml at 4°C for 16 h. Sections were pretreated with 70% formic acid to retrieve antigen.

Results

Sections of medial temporal lobe from patients with AD demonstrated numerous neuritic plaques and neurofibrillary tangles throughout the temporal neo- and allocortices. The younger controls showed no pathology, while the age-matched controls showed only isolated neurofibrillary tangles in the entorhinal pre-α layer in one of the cases.

Upstream caspases

Caspase 8 immunohistochemistry demonstrated strong immunolabeling in nearly all of the NFT in each of the AD cases, with no differences in regional distribution within the medial temporal lobe (Fig. 1A). Non-NFT-containing neurons in AD cases (Fig. 1A) and neurons in control cases (Fig. 1B) showed only weak cytoplasmic staining that was similar between AD cases and controls. Double-immunostaining using antibodies to caspase 8 and tau (AT8) further demonstrated caspase 8 labeling of a subset of neuropil threads, while dystrophic neurites showed caspase 8 staining only rarely. No staining of amyloid cores of cored plaques, or blood vessels involved by amyloid angiopathy could be discerned.

NFT-containing neurons also demonstrated caspase 9 immunoreactivity (Fig. 1C), while, again, the level of caspases in neurons not containing NFT was similar between AD and control cases (Fig. 1D). Caspase 6 was found in association with senile plaques in almost all cases of AD (Fig. 1E) and was present at low levels in neurons in both AD and control cases (Fig. 1F).

Downstream caspases

Downstream caspases 3 and 7 showed weak, uniform immunolabeling of neuronal cytoplasm in both AD and control cases (Fig. 2A–D). Two out of ten AD cases also showed caspase 7 immunolabeling of isolated neurofibrillary tangles and neuritic plaques (less than 1% of total tangles and plaques).

Specificity and B cell lymphoma positive control

The specificity of upstream markers (i.e., caspases 6, 8 and 9) was verified by adsorption of the antisera with immunizing antigen with commensurate diminution of signal. In addition to these absorption experiments for those upstream markers that elicited positive immunorecognition, we also determined the recognition ability of all antisera used in this study. To accomplish this, we examined tissue from diffuse large B cell lymphoma tissue, where apoptosis is prevalent, and found specific recognition of apoptotic cells with all caspase antibodies in this study (data not shown).

Discussion

In an attempt to resolve the issue of programmed cell death and apoptosis in AD, in this study we systematically investigated the entire apoptotic cascade. Our results indicate that, while upstream caspases including 8 and 9 are clearly elevated in NFT-containing neurons in AD, downstream caspases, or so called effector caspases, such as 3 and 7, remain at control levels. While caspase 6, which likewise has been described as an effector caspase, is also found in AD neurons, its localization differs significantly from the upstream caspases. Indeed, the restriction of caspase 6 to senile plaques, and not neurons, suggests that extracellular pathophysiology (i.e., amyloid-β deposition as senile plaques) differs from the neuronal pathophysiology in AD. Although all of the antibodies used in our study recognize both zymogen and cleaved caspase forms, the results presented are significant given that the upstream caspases are

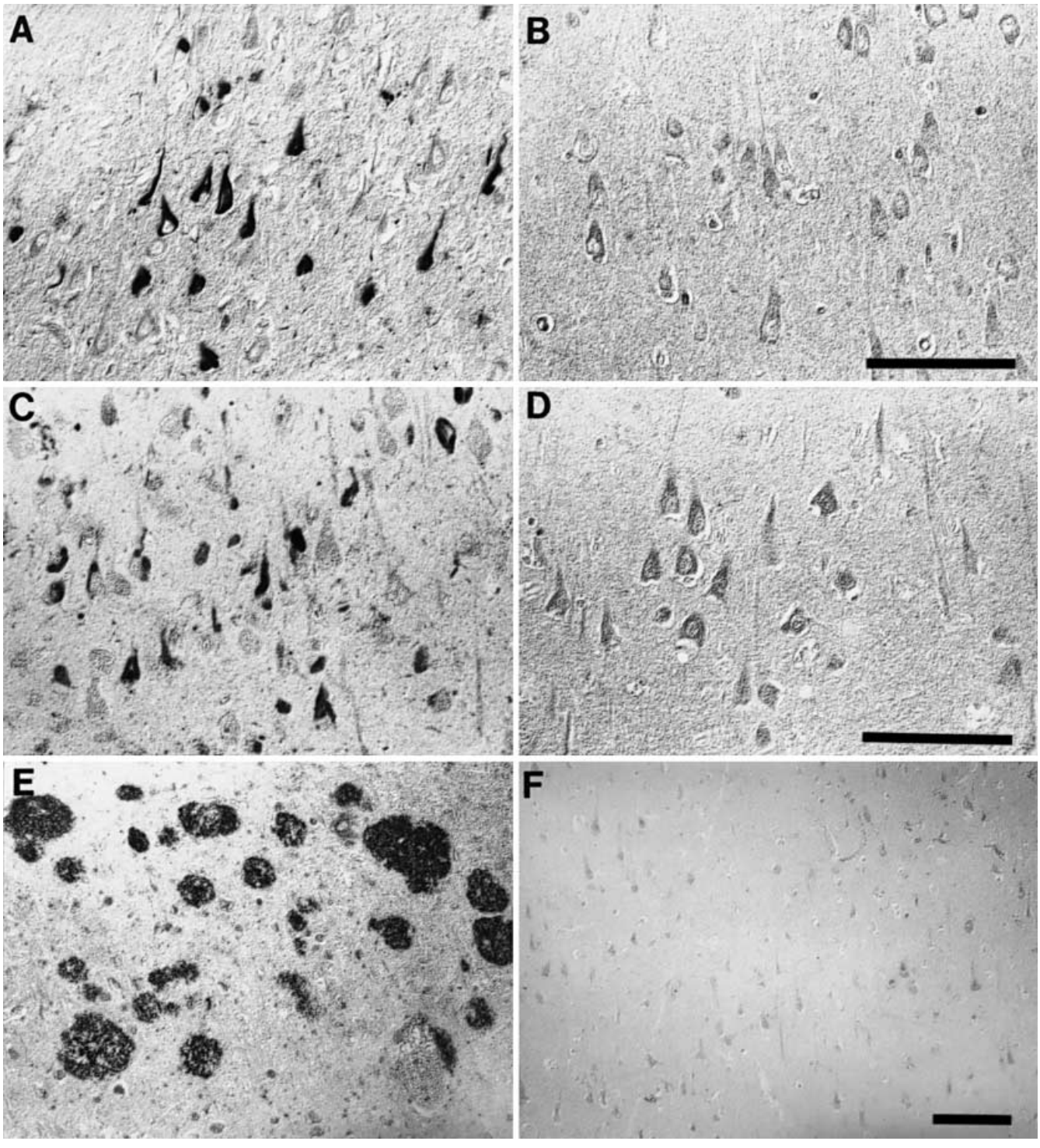

Fig. 1 Upstream caspases 8 (**A**) and 9 (**C**) are associated with NFT in Sommer's sector (CA-1) neurons of AD cases. Control cases showed no histopathological abnormalities and no induction of caspases 8 (**B**) and 9 (**D**) in such neurons. Caspase 6 associates to neocortical plaques in AD (**E**), but showed no increase in control cases (**F**) (*NFT* neurofibrillary tangles, *AD* Alzheimer's disease). *Bars* 100 μm

localized specifically to susceptible neurons in AD. Additionally, given that clustering of zymogen caspase is sufficient for substrate proteolysis [21], the condensed localization we observe here may be an indicator of active enzymes. However, the lack of effector caspases clearly indicates a lack of propagation of the initial apoptotic signal.

The presence of caspase 8, a downstream component of the Fas-FADD pathway and a specific activator of caspase 3 [32], along with caspase 9, which through the down-

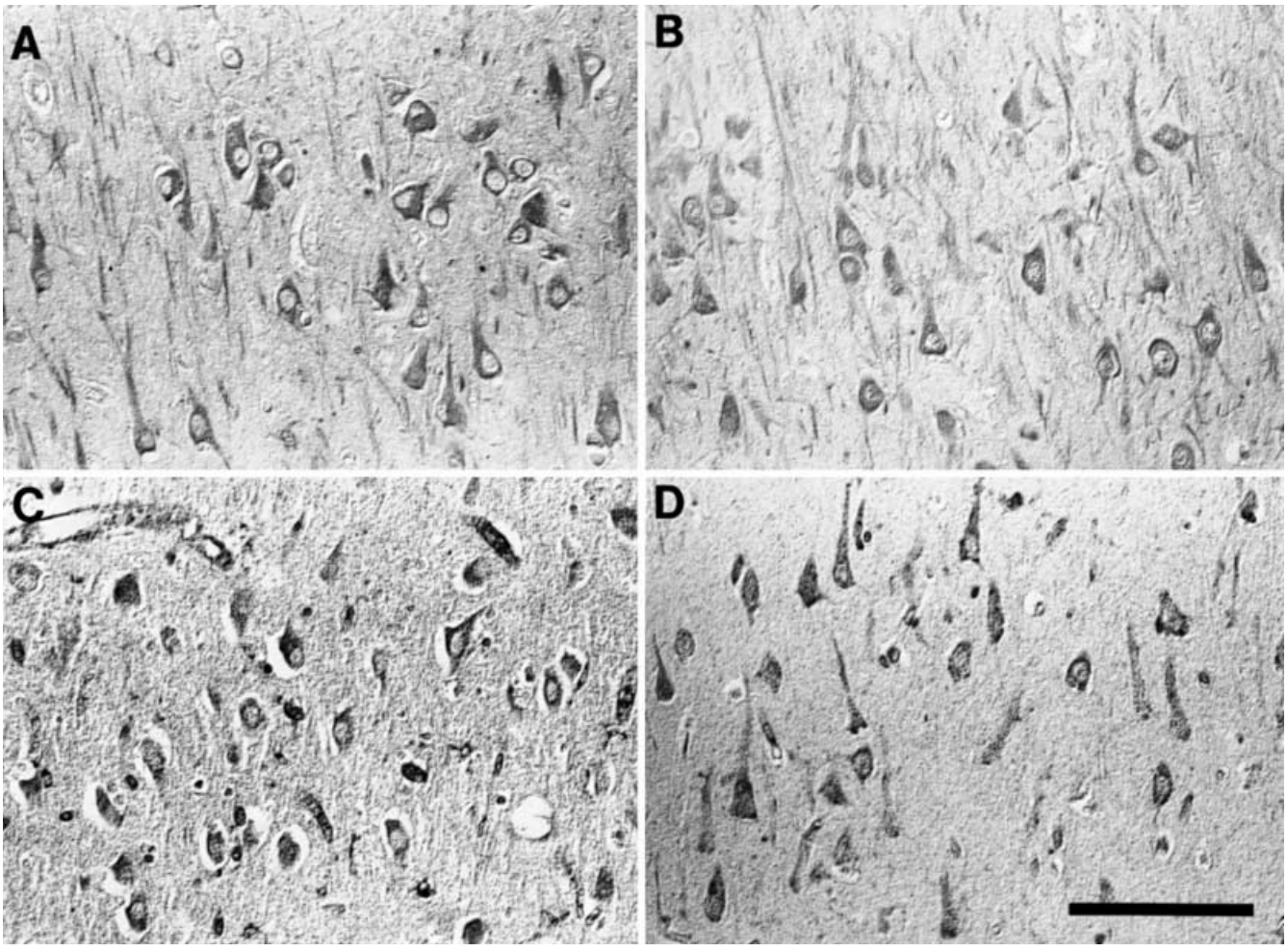

Fig. 2 Downstream caspases 3 (**A**, **B**) and 7 (**C**, **D**) are not increased in AD cases (**A, C**) in comparison to controls (**B, D**). **A**–**D** Sommer's sector (CA-1) of hippocampus are depicted. *Bar* 100 μm

stream mitochondrial pathway also leads to caspase 3 activation, argues for the recruitment of the initiator components of the caspase pathway in neurons of AD. However, the lack of increased caspase 3 and 7 in the neuropathology of AD [23] and the sporadic incidence of other crucial downstream events of the caspase cascade [3] indicates incomplete or effectively absent amplification of the upstream apoptotic signal. Indeed, since such downstream caspases and their proteolytic products are recognized as markers of apoptotic irreversibility [36], their avoidance or sporadic appearance in AD indicates an absence of effective distal propagation of the caspase-mediated apoptotic signal(s). Indeed, caspase 3 and 9 activity are important pro-apoptotic regulators of postmitotic neuronal homeostasis [20].

Recently, activated caspase 3 has been shown to be present within autophagic granules and rarely within neurons in AD and Down syndrome cases [23, 31]. However, the localization of caspase 3 activity, like that of caspase 6 in our study, within a select subcellular compartment, i.e., autophagic granules, does not necessarily indicate a global, effective caspase amplification. Moreover, modulation of distal substrates of activated caspase 3 may lead to further modification of this cell death pathway and may explain the lack of an apoptotic death pathway. This may explain the lack of evidence demonstrating the acute end stages, including nuclear compensation and blebbing, in AD susceptible neurons [17, 18].

Apoptosis can be prevented by anti-apoptotic members of the Bcl-2 family, many of which have been reported in AD, including Bcl-xL and Bcl-w (reviewed in [19]; Zhu et al., unpublished data). Additional evidence for the anti-apoptotic nature of the intraneuronal environment in AD includes the expression of GADD 45, a growth arrest DNA damage-inducible protein in select neurons in AD where its early expression is associated with the expression of Bcl-2. Thus, this may in turn confer survival advantages in AD [35]. Furthermore, the hyperphosphorylated intraneuronal state that exists in AD acts to inhibit downstream substrate proteolysis and thus can promote neuronal survival. Indeed, accumulating evidence points to dephosphorylation being associated with increased capability to cleave PARP [14]. Additionally, chronic oxidative stress, a major component of early AD pathophysiology [27], would further inhibit downstream propagation of caspase-mediated apoptotic signals [6].

The up-regulation of individual caspases, while seemingly not leading to apoptosis, could have great significance related to the pathogenesis of AD. For example, caspase 6, found here in association with amyloid-β senile plaques, cleaves amyloid-β protein precursor (AβPP) resulting in a 6.5-kDa amyloid fragment and is proposed as an alternate AβPP processing pathway [10]. The lack of caspase 6, a downstream effector caspase, in neuronal pathology, however, argues against a specific role in the apoptotic cascade.

Given that execution of apoptosis requires amplification of the caspase-mediated apoptotic signal, our results indicate that in AD there is a lack of effective apoptotic signal propagation to downstream caspase effectors. AD represents that first in vivo situation reported in which the initiation of apoptosis does not directly lead to apoptotic cell death, an interpretation consistent with earlier reports [9, 11, 12, 24, 25]. Obviously, in certain brain areas in AD, while many neurons do degenerate, it is unclear whether this is through successful propagation of the apoptotic cascade or by another pathway such as paratosis [29]. However, in those surviving neurons, it is clear that neuronal viability in AD is, in part, maintained by the lack of distal transmission of the caspase-mediated apoptotic signal(s). This novel phenomena, which we term apoptotic avoidance or abortosis, represents an exit from the caspase-induced apoptotic program that, given the robust survival of abortotic neurons with neurofibrillary tangles [16], leads to prolonged neuronal survival in AD.

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