Background: Caspase gene expression has previously been reported in terminal Alzheimer disease (AD) brain, but, currently, little is known about the temporal pattern of caspase gene expression relative to the onset and clinical progression of AD.

Objective: To derive a profile of caspase gene expression and proapoptotic indexes as a function of the clinical and neuropathologic progression of AD dementia.

Setting and Patients: Postmortem survey of nursing home patients characterized clinically by Clinical Dementia Rating (CDR) and neuropathologically by Consortium to Establish a Registry for Alzheimer’s Disease criteria.

Design and Outcome Measures: To assess messenger RNA expression of caspase-1, -2L, -2S, -3, -5, -6, -7, -8, and -9; apoptotic cell death by TUNEL assay; and poly (ADP-ribose) polymerase cleavage in postmortem brain tissue samples from cognitively normal (CDR 0), high risk of developing AD dementia (CDR 0.5), and severe dementia (CDR 5) cases.

Results: Compared with CDR 0 cases, elevated messenger RNA expression of caspase-1 and caspase-7 in the entorhinal cortex of CDR 0.5 cases coincided with increased poly (ADP-ribose) polymerase cleavage but not apoptotic cell injury. In the entorhinal cortex of CDR 5 cases, we found elevation of caspase-1, -2L, -3, -5, -6, -7, -8, and -9 and a greater than 4-fold increase in TUNEL-positive cells. Caspase messenger RNA expression was closely associated with neurofibrillary tangle and, to a lesser extent, neuritic plaque density.

Conclusions: Proapoptotic mechanisms may be at play early in the onset of AD (before overt signs of apoptosis) and may be a conditional factor for later apoptotic cell injury or death. These data have relevance to potential therapeutic interventions for AD using selective caspase inhibitors.

Arch Neurol. 2003;60:369-376
oxyuridine triphosphate–mediated nick end labeling) assay and DNA fragmentation.\(^5,7,24-35\)

However, much of the available information on proapoptotic caspase expression in AD comes from studies of cases of severe AD dementia and includes relatively few of the known caspases. To clarify the potential role of caspase activation with respect to proapoptotic events in AD onset and progression, we assessed the expression of initiator and executioner caspases in the EC (Brodmann area [BM] 36/38), a region highly affected in AD, and in the occipital cortex (OC) (BM17), an unaffected region in AD, as a function of AD dementia progression. All samples were obtained from patients characterized by Clinical Dementia Rating (CDR) and include cognitively normal (CDR 0), at high risk to develop AD (CDR 0.5), and late-stage AD (CDR 5) cases.

We report an early and select induction of proapoptotic caspases in the EC-BM36/38 of CDR 0.5 cases that coincided with increased cleavage of the DNA repair molecule poly (ADP-ribose) polymerase (PARP). Furthermore, whereas the disease progressed from CDR 0.5 to severe terminal CDR 5, comprehensive recruitment and elevation of caspase messenger RNA (mRNA) expression was associated with TUNEL-positive apoptotic cells in the EC-BM36/38 and density of NFTs. The studies suggest that induction of caspase expression may be an early event in AD dementia and may play an important role in the clinical and neuropathologic progression of this disease.

**METHODS**

**PATIENT SELECTION CRITERIA**

Human postmortem brain samples from AD and age-matched non-AD cases were obtained from the Alzheimer’s Disease Brain Bank of the Mount Sinai School of Medicine. The cases selected had either no significant neuropathological features or only neuropathological features associated with AD.\(^36,37\) A multistep approach based on cognitive and functional status during the last 6 months of life was applied to the assignment of CDRs\(^38\) as previously reported.\(^36,37\) Samples of OC and EC were divided into groups on the basis of their CDRs as follows (OC/EC): CDR 0, nondemented (n=9/9); CDR 0.5, at high risk of developing AD dementia (n=14/9); and CDR 5, severe dementia (n=9/9).

The extent of NFT and β-amyloid NP staining in the EC was assessed in accord with the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) neuropathologic battery.\(^1\) Multiple (∼5) high-power (×200, 0.5-mm\(^2\)) fields were examined in each histologic slide from multiple regions according to the CERAD regional sampling scheme. The density of NFTs and NPs was rated on a 4-point scale as follows: 0, absent; 1, sparse; 3, moderate; and 5, severe. Plaques were visualized after either Bielschowsky silver or thioflavin-S staining.\(^39\) Three of us (P.N.P., S.Y., and Z.X.) were masked to visualized after either Bielschowsky silver or thioflavin-S staining.\(^39\) Three of us (P.N.P., S.Y., and Z.X.) were masked to

**RIBONUCLEASE PROTECTION ASSAY**

Total RNA was prepared using an RNA isolation system (Ultraspex; BIOTECX Laboratories Inc, Houston, Tex), based on the acid guanidinium thiocyanate-phenol-chloroform method.\(^30,41\) RNA was assayed using a multiprobe ribonuclease protection assay system (BD RiboQuant; BD PharMingen, San Diego, Calif). A custom probe set containing complementary DNA templates for the following human caspase genes was used: caspase-1, -2L, -2S, -3, -5, -6, -7, -8, and -9. The probe set also included the housekeeping gene products L32 and glyceraldehyde phosphate dehydrogenase as internal controls for normalization of assay conditions. Details on the generation of (α-\(^32\)P) uridine-5′-triphosphate antisense RNA probes and conditions of the ribonuclease protection assay...
were in accord with the manufacturer’s instructions. The radioactively labeled ribonuclease protection fragments were quantitated using a Storm 860 Phosphor Screen Scanner with the ImageQuant software package (Molecular Dynamics, Piscataway, NJ). Each ribonuclease protection assay analysis was conducted with 10 µg of total RNA, according to 260 nm absorbance values. Data are expressed as a ratio of the specific mRNA of interest normalized to the constitutively expressed glyceraldehyde phosphate dehydrogenase mRNA. Normalization of caspase mRNA signals to L32 did not change the outcome of the results (data not shown). The validity of the RNA assay (BD PharMingen) for assessing changes in caspase gene expression throughout a variety of independent experiments was recently reported. For information regarding RNA sample quality and exclusion criteria, see Figure 1.

TUNEL APOPTOTIC ASSAY

The TUNEL method was used to identify apoptotic cell injury according to the protocol of an apoptosis detection kit (ApopTag; Intergen, Purchase, NY). Paraffin brain tissue sections (10µM) encompassing the dorsoventral extent of the EC-BM36/38 (n=5-6 cases for each CDR group, tissue from the contralateral hemisphere of that used for mRNA extraction) were deparaffinized in a clearing agent (Histoclear; National Diagnostics, Atlanta, Ga), rehydrated in descending concentrations of ethanol, and rinsed in phosphate-buffered saline solution. After pretreatment with protease K (20 µg/mL) for 15 minutes, the sections were incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C for 1 hour and then incubated with antidigoxigenin conjugate for 30 minutes. The TUNEL-labeled cells were then visualized using a DAB substrate kit (Vector Laboratories, Burlingame, Calif). The slides were counterstained with methyl green and mounted for microscopy. Control tissue sections incubated in the absence of terminal deoxynucleotidyl transferase enzyme had negative findings from staining.

For quantification of the number of TUNEL-positive cells, images were digitized using a high-resolution charged-coupled device camera (Sony, Tokyo, Japan) and quantified using image analysis software (Bioquant; R&M Biometrics Inc, Nashville, Tenn) as previously described.

WESTERN BLOT ANALYSIS OF DEATH SUBSTRATE PARP CLEAVAGE

Human brain tissue lysates were prepared by sonicating 20 mg of tissue powder suspended in lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1% Triton X-100) for 20 seconds. The HEPES, pH 7.4, 0.1% CHAPS, 1mM dithiothreitol (DTT), 140mM sodium chloride, and 0.1% Tween 20 (TBST) was performed using quantitation software (Quantity-One; Bio-Rad Laboratories); PARP cleavage was expressed as a ratio of intact 116-kd PARP, and the cleaved 85-kd fragment as a percentage of CDR 0 values.

STATISTICAL ANALYSIS

Statistical analysis was performed using a software package (StatSoft; StatSoft, Tulsa, Okla). Analysis of variance was used to evaluate differences in mean values among 3 or more groups, and a 2-tailed t test was used to test the significance between differences in mean values. For all analyses, the null hypothesis was rejected at P<.05.

RESULTS

PATIENT POPULATION

Patient information for samples of the EC-BM36/38 included in this study is summarized in Table 1. Analysis of variance indicated that there were no significant differences among the different CDR groups with respect to postmortem interval and age at death. Cause of death was reviewed for all the patients to rule out the possibility that intercurrent infection or other events would affect study measures. Of 25 patients included in the study, 21 died of acute cardiac failure (including cardiopulmonary arrest, ventricular fibrillation, cardiacopulmonary arrest, myocardial infarct, and congestive heart failure), 1 of cancer, 1 of pneumonia, and 2 of unknown causes.

REGIONAL DISTRIBUTION OF CASPASE mRNA EXPRESSION IN THE BRAIN OF COGNITIVELY NORMAL CASES

Caspase mRNA expression in the EC-BM36/38 and the OC-BM17 was first determined by ribonuclease protection assay analysis of cognitively normal cases (CDR 0) and was expressed as a ratio to the housekeeping gene glyceraldehyde phosphate dehydrogenase (Figure 1). The mean levels of mRNA expression of individual caspases (caspase-1, -2L, -2S, -3, -5, -6, -7, -8, and -9) were consistent between the EC-BM36/38 and the OC-BM17 (Figure 1). However, among the caspases, differences in baseline mRNA expression levels were evident. Caspase-8 had the lowest level of expression in the EC and the OC.

CASPASE mRNA EXPRESSION AS A FUNCTION OF THE CDR

Caspase mRNA expression was measured in the EC-BM36/38 and the OC-BM17 of normal cognitive control cases (CDR 0), cases at high risk of developing AD (CDR 0.5), and late-stage AD dementia cases (CDR 5). Evaluation of CDR 0.5 cases indicated a greater than 1.5-fold elevation of mRNA expression of caspase-1 and -7 in the EC-BM36/38 at this early CDR stage relative to the CDR
When CDR 5 cases were examined, we found that in addition to elevated caspase-1 and caspase-7 mRNA expression, caspase-2S, -3, -5, -8, and -9 levels exhibited greater than 1.5- to 2-fold increases in expression relative to the CDR 0 control group (Figure 2).

No significant difference in any caspase mRNA expression was observed in the OC-BM17 at any of the CDRs examined (Table 2). However, although not statistically significant, we observed a trend for decreased caspase expression in the OC-BM17 of cases at high risk of developing AD dementia (CDR 0.5).

### Table 1. Patient Demographics and Neuropathologic Data

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<th>Sex</th>
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<th>Cause of Death</th>
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<td>CPA</td>
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Abbreviations: AMI, acute myocardial infarction; BM (36—entorhinal cortex), Brodmann area; BP, bronchopneumonia; CA, cardiac arrest; CE, cerebral embolus; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; CF, cardiac failure; CHF, congestive heart failure; CPA, cardiopulmonary arrest; CRA, cardiorespiratory arrest; CRF, cardiorespiratory failure; MI, myocardial infarction; MPC, metastatic prostate cancer; and VF, ventricular fibrillation.

Figure 2. Ribonuclease protection assay profile of mean caspase messenger RNA (mRNA) expression (expressed as a ratio to the housekeeping gene glyceraldehyde phosphate dehydrogenase [GAPDH]) in the entorhinal cortex (Brodmann area 36/38) as a function of Clinical Dementia Rating (CDR) (CDR 0=cognitively normal, CDR 0.5=questionable dementia, and CDR 5=severe dementia); caspase/GAPDH mRNA levels are expressed as percentages of the CDR 0 group. Asterisk indicates $P<.05$; dagger, $P<.001$ vs CDR 0 by 2-tailed t test. Error bars represent SEM.
Next we explored caspase expression as a function of NFT and NP density. We found that caspase mRNA expression is elevated in the EC-BM36/38 as a function of NFT staining (Figure 3). No apparent elevation of caspase mRNA expression was found as a function of NP staining (Figure 4). Compared with cases with absent or sparse NFTs (CERAD 0-1), those with moderate NFT staining (CERAD 3) exhibited a select increase in mRNA expression of caspase-5 and caspase-6 only. No significant difference in caspase mRNA expression was observed in the OC of any of the CDRs examined (data not shown).

PARP CLEAVAGE AND APOPTOSIS
AS A FUNCTION OF CDR

Because the DNA repair enzyme PARP is a preferential substrate for caspase-1 and caspase-7 (and caspase-3), we next explored the potential functional relationship between elevated caspase-1 and caspase-7 mRNA expression and PARP cleavage in the EC-BM36/38. As assessed by Western blot immunoreactivity, we found a greater than 1.5-fold decrease in the ratio of the intact 116-kd native PARP to cleaved 85-kd processed PARP in CDR 0.5 cases relative to CDR 0 controls, suggesting increased processing of PARP in these cases. No change in the PARP ratio was found in the EC-BM36/38 of cases characterized by severe dementia (CDR 5) (Figure 5B). No differences in TUNEL-positive cell counts were observed in the OC of any of the CDR stages examined (data not shown).

The mRNA expression of caspase-1, -2L, -2S, -3, -5, -6, -7, -8, and -9 was assessed as a function of NFT density; elevation of caspase-9 expression was seen only in cases exhibiting severe NFT density. Error bars represent SEM. Asterisk indicates P<.05; dagger, P<.001; and double dagger, P<.005 by 2-tailed t test (caspase-2S data omitted).

The mRNA expression of caspase-1, -2L, -2S, -3, -5, -6, -7, -8, and -9 was assessed as a function of the progression of AD clinical dementia in samples of the EC-BM36/38 and OC-BM17 of patients characterized by CDR and CERAD criteria. Caspase expression was then compared with PARP cleavage activity, apoptotic cell counts by TUNEL assay, and the degree of NP and NFT staining (assessed by CERAD criteria). We found an early select up-regulation of caspase-1 (also known as interleukin 1β converting enzyme) and caspase-7 expression and increased PARP cleavage in the EC-BM36/38 of cases at high risk of developing AD (CDR 0.5) in the absence of apoptotic cell injury assessed by TUNEL. In the EC-BM36/38 of severe or terminal clinical dementia cases.

**Table 2. Caspase Expression in the Occipital Cortex (BM17)**

<table>
<thead>
<tr>
<th>Clinical Dementia Rating</th>
<th>Caspase-1</th>
<th>Caspase-2L</th>
<th>Caspase-2S</th>
<th>Caspase-3</th>
<th>Caspase-5</th>
<th>Caspase-6</th>
<th>Caspase-7</th>
<th>Caspase-8</th>
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<tbody>
<tr>
<td>0</td>
<td>100.0 ± 15.5</td>
<td>74.7 ± 13.3</td>
<td>116.8 ± 20.5</td>
<td>73.3 ± 9.9</td>
<td>114.7 ± 28.3</td>
<td>74.7 ± 13.3</td>
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<td>113.8 ± 15.6</td>
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<td>100.0 ± 12.9</td>
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<tr>
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<td>100.0 ± 15.5</td>
<td>74.7 ± 13.3</td>
<td>116.8 ± 20.5</td>
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<td>116.6 ± 20.5</td>
<td>80.4 ± 11.2</td>
<td>127.9 ± 28.0</td>
</tr>
</tbody>
</table>

Abbreviation: BM, Brodmann area.

*Values are expressed as mean ± SEM percentage of Clinical Dementia Rating 0; no significant differences were observed.

**COMMENT**

The mRNA expression of caspase-1, -2L, -2S, -3, -5, -6, -7, -8, and -9 was assessed as a function of the progression of AD clinical dementia in samples of the EC-BM36/38 and OC-BM17 of patients characterized by CDR and CERAD criteria. Caspase expression was then compared with PARP cleavage activity, apoptotic cell counts by TUNEL assay, and the degree of NP and NFT staining (assessed by CERAD criteria). We found an early select up-regulation of caspase-1 (also known as interleukin 1β converting enzyme) and caspase-7 expression and increased PARP cleavage in the EC-BM36/38 of cases at high risk of developing AD (CDR 0.5) in the absence of apoptotic cell injury assessed by TUNEL. In the EC-BM36/38 of severe or terminal clinical dementia cases.

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terleukin 1 expression is consistent with previous studies showing increased levels of the caspase-1 cleavage product in AD dementia. This evidence of elevated caspase-1 expression supports the notion that caspase-1 may be a risk factor for AD dementia, as it is elevated in cases at high risk of developing AD dementia. This finding suggests that caspases may be risk factors for AD dementia, and this is supported by previous studies.

The reported elevation of caspase-1 and caspase-7 expression in cases at high risk of developing AD dementia is consistent with previous studies. In support of this theory, in the EC-BM36/38 of CDR 5 cases we found an induction of the full spectrum of caspases assessed (caspase-1, -2, -3, -5, -6, -7, -8, and -9), except caspase 2S (an antagonist of apoptosis). This massive recruitment of caspases in terminal dementia coincided with a 5-fold increase in TUNEL-positive cells and a decrease in the overall level of PARP protein, with a shift toward decreased PARP cleavage. In view of recent evidence that PARP expression and activity are down-regulated during apoptotic cell death in vitro, our data suggest that caspase expression in terminal AD may sufficiently overcome antiapoptotic mechanisms and cause DNA damage sufficient enough to induce actual apoptotic cell death and, eventually, cell death.

However, the evidence supporting definitive apoptotic cell death in AD is equivocal. For example, we note that the TUNEL technique is limited to identifying only proapoptotic changes and not definitive apoptotic cell death (e.g., chromatin condensation and apoptotic bodies). Thus, it remains to be determined whether apoptosis is indeed a pathologic end point in AD and the role of caspases in the pathogenesis of AD dementia.
whether perhaps other nonapoptotic features of caspases may promote neurodegeneration and the progression of AD clinical dementia.

For example, caspases have been shown to cleave the amyloid precursor protein in vitro. As previously reported by Raina et al., mRNA expression of caspase-6 and caspase-5 coincided well with severe plaque staining. Also consistent with earlier studies, we report a strong positive association of caspase-1, -3, -5, -6, -7, -8, and, to a lesser extent, -9 with NFT staining. Thus, it may be the case that in addition to proapoptotic activity, caspase expression involves a pathologic interplay with cytoskeletal components during the clinical progression of AD dementia and impacts neuropathological features of AD.

Currently, it is difficult to offer a definitive resolution to the questions of caspase-related neurodegeneration in AD, but our data underscore the need for further characterization of proapoptotic and antiapoptotic cascades in AD. Cotman suggests that there is stringent competition between proapoptotic and antiapoptotic signals before commitment to apoptosis. Further understanding of the mechanisms related to caspase induction under normal and pathologic conditions will yield significant insight about the relevance of these events to the onset or progression of neuropathological features and will potentially provide a rationale for the therapeutic regulation of caspases in AD and similar neurodegenerative disorders.

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Author contributions: Study concept and design (Mr Pompl and Drs Ho, Mohs, and Pasinetti); acquisition of data (Drs Yemul, Xiang, Haroutunian, Purohit, and Mohs); analysis and interpretation of data (Mr Pompl and Dr Pasinetti); drafting of the manuscript (Mr Pompl and Dr Pasinetti); critical revision of the manuscript for important intellectual content (Mr Pompl and Drs Yemul, Xiang, Ho, Haroutunian, Purohit, Mohs, and Pasinetti); statistical expertise (Dr Yemul); obtained funding (Drs Mohs and Pasinetti); administrative, technical, and material support (Mr Pompl and Drs Xiang, Haroutunian, Purohit, Mohs, and Pasinetti); study supervision (Drs Ho and Pasinetti).

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