Cytokine Gene Expression as a Function of the Clinical Progression of Alzheimer Disease Dementia

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Background: Inflammatory cytokines have been linked to Alzheimer disease (AD) neurodegeneration, but little is known about the temporal control of their expression in relationship to clinical measurements of AD dementia progression.

Design and Main Outcome Measures: We measured inflammatory cytokine messenger RNA (mRNA) expression in postmortem brain specimens of elderly subjects at different clinical stages of dementia and neuropathological criteria, IL-6 mRNA expression in both the entorhinal cortex (P<.05) and superior temporal gyrus compared with the occipital cortex. Compared with age-matched controls, subjects with severe/terminal dementia, but not subjects at earlier disease stages, had higher IL-6 and TGF-β1 mRNA expression in the entorhinal cortex (P<.01) and superior temporal gyrus (P<.01). When stratified by the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) neuropathological criteria, IL-6 mRNA expression in both the entorhinal cortex (P<.05) and superior temporal gyrus (P<.01) correlated with the level of neurofibrillary tangles but not neuritic plaques. However, in the entorhinal cortex, TGF-β1 mRNA did not correlate with the level of either neurofibrillary tangles or neuritic plaques. Interestingly, in the superior temporal gyrus, TGF-β1 mRNA expression negatively correlated with neurofibrillary tangles (P<.01) and showed no relationship to the pathological features of neuritic plaques.

Setting and Patients: Postmortem study of nursing home patients.

Results: In brains of cognitively normal control subjects, higher interleukin 6 (IL-6) and transforming growth factor β1 (TGF-β1) mRNA expression was observed in the entorhinal cortex and superior temporal gyrus compared with the occipital cortex. Compared with age-matched controls, subjects with severe/terminal dementia, but not subjects at earlier disease stages, had higher IL-6 and TGF-β1 mRNA expression in the entorhinal cortex (P<.01) and superior temporal

Conclusions: The data are consistent with the hypothesis that cytokine expression may differentially contribute to the vulnerability of independent cortical regions during the clinical progression of AD and suggest that an inflammatory cytokine response to the pathological effects of AD does not occur until the late stages of the disease. These findings have implications for the design of anti-inflammatory treatment strategies.

Arch Neurol. 2000;57:1153-1160
METHODS

PATIENT SELECTION CRITERIA

Human postmortem brains from AD and age-matched non-AD cases were obtained from the Alzheimer’s Disease Research Center (ADRC) of the Mount Sinai School of Medicine, New York, NY. The cases selected had either no significant neuropathological features or only neuropathological features associated with AD.17,18 A multistep approach based on cognitive and functional status during the last 6 months of life was applied to the assignment of Clinical Dementia Rating (CDR) scores, as previously reported.17,18 Subjects were divided into groups on the basis of their CDR scores as follows: 0, nondemented; 0.5, questionable dementia; 1, mild dementia; 2, moderate dementia; and 4 to 5, very severe dementia.

The extent of neurofibrillary tangle and β-amyloid neuritic plaque deposition was assessed in accord with the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) neuropathological battery.17-19 Multiple (5 in general) high-power fields (×200, 0.5 mm²) were examined in each histological slide, containing specimens obtained from multiple brain regions, according to the CERAD regional sampling scheme. The density of neurofibrillary tangles and neuritic plaques was rated on a 4-point scale as follows: 0, absent; 1, sparse; 3, moderate; and 5, severe. Plaques were visualized following either Bielschowsky silver or thioflavine S staining.20,21 The investigators were blind to the clinical diagnosis of each case until all quantitative analyses were completed and values were assigned to each specimen.

RNA PREPARATION

Total RNA was prepared with the Ultraspec RNA Isolation System (Biotecx Laboratories, Houston, Tex), based on the acid guanidinium thiocyanate-phenol-chloroform method.22

RIBONUCLEASE PROTECTION ASSAY

Total RNA was assayed with the RiboQuant Multiprobe RNase (ribonuclease) Protection Assay System (PharMingen, San Diego, Calif). A custom probe set containing complementary DNA (cDNA) templates for the following human cytokine genes was used: IL-6, TGF-β1, IL-1β, TNF-β, TNF-α, IL-1α, IL-1 receptor antagonist (IL-1Ra), and interferon gamma (IFN-γ). The probe set also included the housekeeping genes L32 and glyceraldehyde-3’ phosphate dehydrogenase (GAPDH) for normalization of phosphatase P32-labeled antisense RNA probes and conditions of the RNase protection assay (RPA) are provided by the manufacturer. The quantity of radioactively labeled RNase protection fragments was determined using a Molecular Dynamics Storm 860 Phosphor Screen Scanner with the ImageQuant software package (Molecular Dynamics, Sunnyvale, Calif). Each RPA analysis was conducted with 10 µg of total RNA, according to A260 values. Data are expressed as a ratio of the specific mRNA of interest normalized to the constitutively expressed GAPDH mRNA. Normalization of cytokine mRNA signals to L32 mRNA did not change the outcome results (not shown).

RNA SAMPLE QUALITY AND EXCLUSION CRITERIA FOR RPA

Initial quality control consisted of agarose gel assessment of ethidium bromide staining of both 18–Svedberg flotation unit (SD) and 28-5f ribosomal RNA (rRNA) integrity as well as detection of low-molecular-weight “smearing” of degraded mRNA. All samples were subjected to RPA, regardless of initial quality control assessment. Only samples with nondegraded RNA determined by both ethidium bromide and RPA analysis were included in the study. For the entorhinal cortex, 19 (24%) of the 78 subjects were removed from the study because of poor RNA quality; for the superior temporal gyrus, 16 (20%) of 79 patients were removed; and for the occipital cortex, 9 (36%) of 25 patients were removed. In each brain region examined, RNA exclusion was similarly distributed among the different CDR categories.

STATISTICS

Data were analyzed by analysis of variance (ANOVA) combined with a post hoc Dunnett multiple comparison test to compare each experimental group with the control group. Correlation analysis was performed by calculating the Pearson R coefficient.

PATIENT POPULATION

Patient information for this study is summarized in Table 1. Extraneous factors that might influence cytokine gene expression were similar across all groups. Cytokine mRNA degradation is a concern with long postmortem delays. The ANOVA indicated that there were no significant differences (P>.05) among the different CDR groups with respect to postmortem intervals (data not shown). Furthermore, all groups had a statistically similar (P>.05) age at death (data not shown). Cytokine expression can also be impacted by antemortem inflammatory conditions that may require anti-
inflammatory drug administration. There were a few patients in the study who had evidence of antemortem non-steroid anti-inflammatory drug/glucocorticoid use, and their cytokine profiles did not statistically differ \((P > .05)\) from those of noninflammatory cohorts (data not shown). Cause of death was considered for all the patients to rule out the possibility that infection or other inflammatory events might impact the cytokine levels measured in the study. Of the 64 patients included in the study, 51 died from acute "cardiac failure" (including cardiorespiratory failure/arrest, ventricular fibrillation, cardiopulmonary arrest, myocardial infarct, and congestive heart failure), 2 from cancer, 2 from heart disease (specified), 1 from pneumonia, and 8 from unknown cause. Therefore, long-term inflammatory events are not a confounding factor in the study.

**BASAL CYTOKINE LEVELS IN COGNITIVELY NORMAL PATIENTS**

Total RNA was isolated from the entorhinal cortex, superior temporal gyrus, and occipital cortex and subjected to RPA analysis. Results of a representative RPA analysis are presented in Figure 1. For TNF-β, TNF-α, IL-1α, IL-1Ra, and IFN-γ, mRNA expression was below the level of detection for this assay system \((Table 2 and Figure 1)\). Levels of IL-6, TGF-β1, and IL-1β mRNA were determined for the 3 brain regions and expressed as a ratio to mRNA from the housekeeping gene GAPDH \((Table 2, Figure 1, and Figure 2)\).

Expression of IL-6 mRNA was greater than 10-fold higher in the entorhinal cortex and superior temporal gyrus as compared with the occipital cortex \((Figure 2, A)\). In contrast, expression of TGF-β1 mRNA was relatively lower than that of IL-6 mRNA and was consistent across all 3 brain regions \((Figure 2, B)\). Expression of IL-1β mRNA was of low abundance and showed no regional differences \((Figure 2, C)\).
Increased cytokine mRNA expression in entorhinal cortex and superior temporal gyrus as a function of CDR score

IL-6 mRNA expression

Interleukin 6 mRNA expression was measured in the entorhinal cortex, superior temporal gyrus, and occipital cortex from cases at different stages of AD dementia as assessed by the CDR score (Figure 3, A–C). For the entorhinal cortex, there was an overall difference in IL-6 mRNA expression across all CDR groups examined (ANOVA: \( P = .001, F_{5,60} = 8.3 \)). In addition, cases characterized by severe/terminal dementia (CDR, 5) had significantly elevated IL-6 mRNA levels (\( P < .01; \) Figure 3, A) as compared with cognitively normal patients (CDR, 0).

In the superior temporal gyrus, as in the entorhinal cortex, there was also an overall difference in IL-6 mRNA expression among the different CDR groups (ANOVA: \( P = .03, F_{5,63} = 2.7 \)). Cases characterized by severe/terminal dementia (CDR, 5) had significantly elevated IL-6 mRNA levels (\( P < .01; \) Figure 3, B) as compared with cognitively normal cases (CDR, 0).

No detectable change in IL-6 mRNA expression was found in the occipital cortex (Figure 3, C).

TGF-β1 mRNA expression

A pattern similar to that of IL-6 mRNA expression was found for TGF-β1 mRNA expression in the 3 brain regions examined (Figure 3, D–F). Although TGF-β1 mRNA levels in the entorhinal cortex of cases characterized by severe/terminal dementia (CDR, 5) increased greater than 1-fold relative to cognitively normal cases (CDR, 0), the change was not statistically significant by ANOVA (\( F_{5,60} = 1.948; P = .01 \)) (Figure 3, D).

Similar to the results for IL-6 mRNA expression, in the superior temporal gyrus, there was an overall difference in TGF-β1 mRNA expression among the different CDR groups (ANOVA: \( P = .009, F_{5,63} = 3.4 \)), and cases characterized by severe/terminal dementia (CDR, 5) had significantly elevated TGF-β1 mRNA levels (\( P < .01; \) Figure 3, E) as compared with cognitively normal cases (CDR, 0).

There was no change in TGF-β1 mRNA expression in the occipital gyrus of patients who were very severely demented (CDR, 5; Figure 3, F).

In both the entorhinal cortex and superior temporal gyrus, TGF-β1 and IL-6 mRNA expression in cases characterized by questionable (CDR, 0.5), mild (CDR, 1), moderate (CDR, 2), or severe (CDR, 4) dementia did not differ from cognitively normal (CDR, 0) cases (Figure 3, E and F).

### Table 2. Basal (CDR, 0) Cytokine mRNA Levels Normalized to GAPDH mRNA*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Entorhinal Cortex (BM 36/38) (n = 13)</th>
<th>Superior Temporal Gyrus (BM 22) (n = 14)</th>
<th>Occipital Cortex (BM 17) (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.041 ± 0.005</td>
<td>0.050 ± 0.006</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>0.023 ± 0.003</td>
<td>0.015 ± 0.002</td>
<td>0.018 ± 0.004</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.005 ± 0.001</td>
<td>0.004 ± 0.001</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>TNF-β</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-1α</td>
<td>ND</td>
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<tr>
<td>IL-1Ra</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Values are ratios and are expressed as mean ±SEM. CDR indicates Clinical Dementia Rating; mRNA, messenger RNA; GAPDH, glyceraldehyde-3-phosphodehydrogenase; BM, Brodmann area; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; ND, not detectable under standard assay conditions; and IFN, interferon.
IL-1β mRNA Expression

Interleukin 1β mRNA expression was very low in all brain areas examined and did not change as a function of CDR score (data not shown).

Correlation of IL-6 and TGF-β1 mRNA Levels

In the entorhinal cortex, IL-6 and TGF-β1 mRNA levels showed a high degree of correlation (Pearson R, 0.64; P<.001). This correlation was more pronounced in the superior temporal gyrus (Pearson R, 0.84; P<.001).

Cytokine Expression as a Function of AD Neuropathology (CERAD)

IL-6 mRNA Expression

In the entorhinal cortex, there was an overall difference in IL-6 mRNA expression across groups stratified by CERAD neurofibrillary tangle rating (ANOVA: P=.04, F[2,60]=3.4). In addition, patients with severe neurofibrillary tangles (CERAD, 5) had significantly elevated IL-6 mRNA expression (P<.05; Figure 4, A) as compared with patients with absent or sparse neurofibrillary tangles.
Figure 4. Changes in IL-6 and TGF-β1 messenger RNA (mRNA) expression in the Alzheimer disease brain as a function of the severity of neurofibrillary tangle and neuritic plaque pathological features rated according to a 4-point scale based on the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) regional sampling scheme. Data are expressed as a ratio of expression of the mRNA of interest normalized to GAPDH mRNA. Values are mean ± SEM. See Figure 1 legend for expansions of additional abbreviations.
There was no change in IL-6 mRNA expression with increasing neuritic plaques (Figure 4, B).

This same pattern of changes was found in the superior temporal gyrus, with an overall difference in IL-6 mRNA expression across groups stratified by CERAD neurofibrillary tangle rating (ANOVA: P = .002, F(2,63) = 6.7). As observed in the entorhinal cortex, cases with severe neurofibrillary tangles (CERAD, 5) had significantly elevated IL-6 mRNA expression (P < .01; Figure 4, C) compared with cases with absent or sparse neurofibrillary tangles (CERAD, 0-1). No detectable change in IL-6 mRNA expression with increasing neuritic plaque levels was found in the superior temporal gyrus (Figure 4, D).

### TGF-β1 mRNA Expression

Transforming growth factor β1 mRNA expression in the entorhinal cortex did not change with increasing neurofibrillary tangles or neuritic plaques (Figure 4, E and F, respectively).

In the superior temporal gyrus, there was an overall inverse relationship (ANOVA: P < .001, F(2,63) = 12.2) between TGF-β1 mRNA expression and neurofibrillary tangles. Cases with moderate to severe neurofibrillary tangle pathological features (CERAD, 3 or 5) had significantly lower TGF-β1 mRNA expression (P < .01; Figure 4, G) as compared with patients with absent or sparse neurofibrillary tangles (CERAD, 0-1). There was no relationship between TGF-β1 mRNA expression and neuritic plaques (Figure 4, H).

Because there are relatively few neuropathological changes caused by AD in the occipital cortex, the corresponding neurofibrillary tangle and neuritic plaque data are unavailable.

Expression of TNF-β, TNF-α, IL-1α, IL-1Ra, and IFN-γ mRNAs were all below the level of detection for this assay system (Table 2 and Figure 1) and did not change with dementia or neuropathological features (data not shown).

### Comment

Inflammatory cytokine gene expression was measured in 3 different brain regions from cases characterized by different stages of AD clinical dementia (CDR) and different levels of neuritic plaques and neurofibrillary tangles. For TNF-β, TNF-α, IL-1α, IL-1Ra, and IFN-γ, mRNA expression was below the level of detection for our assay system in the cognitively normal brain and did not change with dementia or increasing plaques and tangles. In cases characterized by normal cognitive status, we found that IL-6 mRNA expression was greater than 10-fold higher in the entorhinal cortex and superior temporal gyrus relative to the occipital cortex. The level of IL-6 and TGF-β1 mRNA was increased only in the entorhinal cortex and superior temporal gyrus of cases characterized by severe/terminal clinical dementia (CDR, 5) but not questionable, mild, or moderate dementia. Furthermore, IL-6 and TGF-β1 mRNA levels were highly correlated with each other. In contrast, while the IL-6 mRNA level correlated with increased levels of neurofibrillary tangles in the entorhinal cortex and superior temporal gyrus, the TGF-β1 mRNA level was inversely associated with neurofibrillary tangles in the superior temporal gyrus.

This study suggests that cytokine expression in the brain may play an important role as a conditional factor for neurodegenerative events in the later stages of clinical disease. Our data are consistent with a localized inflammatory response to late AD neurodegeneration but do not support a role for cytokine-mediated destructive inflammation in the early progression of AD.

Interleukin 6 mRNA expression was measured from the same group of patients after stratification by either CDR or CERAD neuropathological ratings. Elevation of IL-6 mRNA was observed in cases defined by severe/terminal dementia or high levels of neurofibrillary tangles in the entorhinal cortex or superior temporal gyrus. The fact that IL-6 mRNA expression was increased in severe AD cases, defined by either dementia or neuropathological features, further supports the use of the CDR scale to assess disease progression and confirms previous studies correlating neuropathological features with CDR.

Like IL-6, TGF-β1 mRNA levels were increased in the superior temporal gyrus and entorhinal cortex of severely demented cases. In contrast to IL-6, however, TGF-β1 mRNA expression decreased in the superior temporal gyrus as a function of neurofibrillary tangle levels. This suggests that the 2 cytokines may be linked to different neuropathological mechanisms and that there may be qualitatively different mechanisms active in the 2 brain regions. Previous studies have shown that TGF-β1 can be produced by both neurons and glia. Ongoing immunocytochemical and in situ hybridization studies in our laboratory will further clarify the cell-type expression of TGF-β1 and other cytokines during the clinical progression of AD dementia.

Previous studies have suggested that microglial IL-1 expression is up-regulated in the AD brain and support the hypothesis that IL-1 may be involved in the maturation of neuritic plaques. In the present study, we found no elevation of IL-1β mRNA expression during the clinical progression of AD. This apparent conflict may be related to methodological issues. Our study is a broad screening of cases at various levels of cognitive dysfunction using the RPA technique to measure cytokine mRNA expression. The earlier studies used primarily immunocytochemical techniques, which cannot easily be scaled up to the level used in our study and may not be reliable indicators of cytokine expression (particularly in view of non-specific adsorption of many molecules to plaques). Furthermore, careful clinical staging of cases was not available in the earlier studies. Moreover, recent data show that TGF-β1 knockout mice have elevated IL-1β mRNA levels, suggesting that the 2 cytokines may have a reciprocal relationship. The lack of IL-1β mRNA in our study may be related to the increases we found in TGF-β1 mRNA.

The present data suggest that inflammatory cytokine genes are not up-regulated in early AD. Moreover, the lack of a correlation between IL-6 mRNA expression and neuritic plaque density challenges the hypothesis that amyloid deposition initiates the cerebral acute-phase response in AD. Surprisingly, this study suggests that IL-6 mRNA
expression may be related to neurofibrillary tangle levels by a mechanism that is not presently understood. It has been suggested that drugs that suppress cytokine expression, such as glucocorticoids, may be beneficial in the treatment of AD. However, the absence of inflammatory cytokine up-regulation in early to moderate stages of AD dementia found in this study does not support a central role for such mediators in disease progression. Indeed, this hypothesis is consistent with the failure of prednisone therapy in the treatment of mild to moderate AD. It may be more appropriate to direct anti-inflammatory treatment strategies at target molecules that seem to be involved in the early stages of the disease, such as those found for neuronal cyclooxygenase 2 (Ho et al, personal communication).

Much effort has been directed toward clarifying the role of inflammatory cytokines in neurodegenerative disorders such as AD. Careful characterization of the temporal and regional regulation of cytokine expression is necessary to further elucidate cytokine mechanisms and the potential of anticytokine therapy. In addition, because mRNA levels do not necessarily reflect changes in bioactivity, future studies will attempt to explore these relationships at the protein level. Nonetheless, the present studies reveal an interesting regional pattern of cytokine gene expression in the AD brain that may be relevant to neurodegenerative disease but do not support the theory that cytokines are intimately involved in plaque maturation in early AD or other processes central to the progression of early AD.

Accepted for publication March 13, 2000.

This work was supported by grants AG13799, AG14329, and AG16743 (Dr Paisnetti), AG05138 (Alzheimer Disease Research Center [ADRC] of Mount Sinai [Dr Davis], and AG02219 (program project) [Dr Mohs]), National Institute on Aging, National Institutes of Health, Bethesda, Md.

The authors thank Daniel Perl, MD, Mount Sinai Alzheimer’s Disease Research Center, New York, NY, for neuropathological support.

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