Intrathecal Chemokine Synthesis in Mild Cognitive Impairment and Alzheimer Disease

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Background: Immuno-reactivity for several chemokines and for their related receptors has been demonstrated in resident cells of the central nervous system, and the up-regulation of some of them is associated with pathological changes found in Alzheimer disease (AD).

Objective: To determine interferon-γ-inducible protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), and interleukin 8 (IL-8) levels in cerebrospinal fluid (CSF) from subjects with amnestic mild cognitive impairment (MCI) and patients with AD as compared with age-matched controls.

Patients: Thirty-eight subjects with amnestic MCI, 36 patients with AD, and 41 age-matched subjects with non-inflammatory affections of the nervous system.

Design: Evaluation of CSF chemokine production at time of diagnosis of MCI and AD; correlation with clinical and personal data. Longitudinal evaluation of subjects with MCI until conversion to AD.

Results: Cerebrospinal fluid IP-10 concentration was significantly increased in patients with MCI and mild AD but not in patients with severe AD (Mini-Mental State Examination score <15), whereas MCP-1 and IL-8 levels were increased in patients with MCI and all patients with AD. A significant positive correlation between Mini-Mental State Examination score and CSF IP-10 or MCP-1 concentration was observed in patients with AD. No correlation between IP-10 levels and age was found, whereas MCP-1 and IL-8 levels correlated positively with age. Out of 38 subjects with MCI, 19 developed AD within a 1- to 3-year follow-up.

Conclusions: The presence of inflammatory molecules is likely to be a very early event in AD pathogenesis, even preceding the clinical onset of the disease, as demonstrated by subjects with MCI who developed AD over time. Interferon-γ-inducible protein 10 is specifically increased in MCI and seems to decrease with the progression of AD, whereas MCP-1 and IL-8 are up-regulated also in late stages of the disease, suggesting a role in phases in which neurodegeneration is prevalent.

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Alzheimer Disease (AD) is the most common form of dementia in the elderly. β amyloid (1-42) deposition into the brain is considered a crucial step during its development because it begins a cascade of events leading to irreversible neuronal damage. It is widely accepted that a chronic inflammatory reaction plays an important role in the pathogenesis of AD, and a variety of inflammatory factors, including cytokines and chemokines, have been detected in and around plaques and tangles. Chemokines are low molecular weight chemotactic cytokines that have been shown to play an important role in early inflammatory events. So far, microglial cells were shown to display an increased migratory response to β chemokines, including monocyte chemotactic protein 1 (MCP-1), suggesting that these molecules may play an important role in the trafficking of mononuclear phagocytes within the brain. Immunoreactivity for chemokines and their related receptors has been demonstrated in resident cells of the central nervous system, and the up-regulation of some of them is associated with pathological changes found in AD. In particular, MCP-1 was detected immunohistochemically in mature senile plaques and reactive microglia (but not in immature senile plaques) in brain tissues from patients with AD. Both interferon-γ-inducible protein 10 (IP-10) and its CXC chemokine receptor 3 (CXCR3) have been detected in normal and AD brains. Interferon-γ-inducible protein 10 immunoreactivity was markedly increased in reactive astrocytes in AD brains as well as the level of its expression. Astrocytes positive for IP-10 were found to be...
associated with senile plaques and showed an apparently coordinated up-regulation of macrophage inflammatory protein 1B. Interleukin 8 (IL-8) enhances the survival of hippocampal neurons in vitro and increases the proliferation of glial cells. Strong immunoreactivity for CXCR2, the IL-8–related receptor, has been demonstrated in both AD and control brains; in particular, CXCR2 expression in AD is close to neuritic plaques, surrounding deposits of β amyloid.

Recently IP-10, MCP-1, and IL-8 levels were evaluated in cerebrospinal fluid (CSF) from a small group of patients with AD and compared with age-matched controls. Although CSF MCP-1 and IL-8 levels were higher in all patients with AD, CSF IP-10 levels were increased only in a subgroup of patients and tended to correlate positively with the Mini-Mental State Examination (MMSE) score, suggesting a role of IP-10 in early stages of the disease during which inflammation is likely to be more pronounced. In this regard, emerging evidence suggests that intrathecal inflammation precedes the clinical development of AD because cytokine dysregulation has been observed in patients with mild cognitive impairment (MCI), which can be defined as an isolated deficit in memory often associated with decline to AD. In particular, increased production of the proinflammatory cytokine tumor necrosis factor α together with decreased production of the anti-inflammatory cytokine transforming growth factor β in CSF from subjects with MCI has been shown. Other studies demonstrated that elevated plasma levels of α1-antichymotrypsin and interleukin 6 increase the risk of developing dementia. Besides cytokines, an alteration of some biochemical factors involved in oxidative stress has been found in patients with AD, including an increase in plasma total homocysteine levels and a decrease in total antioxidant capacity. Similar to cytokines, both total homocysteine and total antioxidant capacity modifications seem to be early events in the pathogenesis of AD because a trend toward an alteration of these parameters was observed in MCI as well.

To further study the role of chemokines in AD pathogenesis, we evaluated IP-10, MCP-1, and IL-8 levels in CSF samples from individuals with AD and amnestic MCI, which is considered the very early stage of AD, and compared them with those of age-matched subjects with no neurological diseases (controls). Intrathecal chemokine expression was subsequently determined by quantitative transcriptional analysis.

### METHODS

### SUBJECTS

Thirty-eight individuals with MCI and 36 with probable AD were consecutively recruited at the Alzheimer Center of the VU University Medical Center (Amsterdam, the Netherlands) and at the Alzheimer unit of Ospedale Maggiore Policlinico (Milan, Italy). All patients underwent a standard battery of examinations, including medical history, physical and neurological examination; screening laboratory tests; neuropsychological evaluation (to assess memory, language, and constructional praxis); brain magnetic resonance imaging or computed tomography; and, if indicated, positron emission tomography. The presence of significant vascular brain damage was excluded (Hachinski Ischemic Score <4). Dementia severity was assessed by the Clinical Dementia Rating and the MMSE. Disease duration was defined as the time in years between the first symptoms (by history) and the lumbar puncture.

Twenty-two of 36 AD patients had an early onset of the disease (age at onset ≤65 years, 9 men and 13 women; mean ± SEM age, 59.4 ± 0.9 years; mean ± SEM disease duration: 3.9 ± 0.4 years; mean ± SEM MMSE score, 15.5 ± 1.5), whereas remainders had a late onset (age at onset >65 years, 4 men and 10 women; mean ± SEM age, 72.4 ± 1.3 years; mean ± SEM disease duration, 3.0 ± 0.6 years; mean ± SEM MMSE score, 18.7 ± 1.5).

Diagnosis of MCI was made in accordance with criteria from Petersen et al. According to recently proposed clinical criteria, all patients with MCI (14 men and 24 women; mean ± SEM age, 68 ± 1.7 years) presented only memory impairment (amnestic MCI) without deficits in other cognitive domains. Their MMSE scores ranged from 26 to 29, and the Clinical Dementia Rating score was 0.5. Patients with AD were diagnosed by exclusion according to NINCDS-ADRDA criteria (National Institute of Neurological and Communicative Diseases and Stroke and Alzheimer’s Disease and Related Disorders Association). Sixteen patients with AD were in a severe stage of the disease, according to Clinical Dementia Rating (≥3) and MMSE scores (mean ± SEM MMSE score, 10.9 ± 1.5), whereas remainders were less severely impaired, having a Clinical Dementia Rating score of 1 or 2 and a MMSE score greater than or equal to 15 (mean ± SEM MMSE score, 21.1 ± 0.8).

To get the best diagnostic uniformity for an accurate phenotypic characterization of patients with AD and MCI, these guidelines were carefully discussed among participants from the 2 centers involved in this collaborative study before starting sample collection. Then, in each center, cases were discussed by a team of clinicians, neuropsychologists, and neuroimaging and laboratory experts. After the recruitment period, an accurate follow-up of patients was done to further confirm clinical diagnoses. None of patients received nonsteroidal anti-inflammatory medication or acetylsalicylic acid inhibitor therapy for at least 1 month before collecting samples, except for 5 patients with AD, who were treated with donepezil hydrochloride (n = 3), galantamine hydrobromide (n = 1), or rivastigmine tartrate (n = 1) at time of sampling.

The control group consisted of 41 subjects matched for ethnic background and age (15 men and 26 women; mean ± SEM age, 64.0 ± 4.6 years) without memory complaints (MMSE score, 28-30) but with other noninflammatory neurological affections: acute headache (12), vertigo (10), nonimmune peripheral neuropathies (9), cerebellar edema (6), compressive radiculopathies (3), and neurofibromatosis type 1 (1). The age of controls did not significantly differ from that of patients with MCI and AD (P > .05). Cerebrospinal fluid composition was in the normal range for all control subjects analyzed (cell number, <5 cells/µL; protein concentration, 150-450 mg/L; glucose, 2.7-4.2 mmol/L). Moreover, chemokine levels in CSF from patients with noninflammatory neurological disorders were previously demonstrated to be similar to healthy controls. None of these individuals developed dementia after 9 to 12 months of follow-up. Informed consent to participate in this study was given by all subjects or their caregivers.

### CSF/SERUM SAMPLE COLLECTION AND ROUTINE ANALYSIS

Samples of CSF were obtained in polypropylene tubes by lumbar puncture at the L4/L5 or L3/L4 interspace and centrifuged at 4°C. Both CSF and serum samples were stored at less than or equal to −30°C until analysis. In a limited number of cases (3 patients with AD and 3 controls), cells were isolated from...
Table. Mean ± SEM Values of IP-10, MCP-1, and IL-8 in Cerebrospinal Fluid From Patients With MCI and AD and Control Subjects

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Controls (n = 41)</th>
<th>MCI (n = 38)</th>
<th>AD (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-10, pg/mL</td>
<td>68.78 ± 4.66</td>
<td>127.90 ± 10.05*</td>
<td>103.38 ± 12.84</td>
</tr>
<tr>
<td>MCP-1, pg/mL</td>
<td>860.79 ± 27.00</td>
<td>1147.20 ± 43.77*</td>
<td>1032.76 ± 50.25*</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>26.15 ± 2.07</td>
<td>76.00 ± 4.27*</td>
<td>33.73 ± 2.07*</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer disease; IL-8, interleukin 8; IP-10, interferon-γ–inducible protein 10; MCI, mild cognitive impairment; MCP-1, monocyte chemotactic protein 1.

*P<.001.

To avoid possible chemokine variations due to multiple freezing/thawing cycles, analyses were carried out after the first thawing cycle and no more than after 1 year of storage, as previously reported. Chemokine levels were measured with human-specific enzyme-linked immunosorbent assay kits (Chemicon, Temecula, Calif [IP-10]; Amersham Biosciences, Piscataway, NJ [MCP-1 and IL-8]), based on the quantitative sandwich enzyme immunoassay technique. The sensitivity of these assays was 16.2, 10.0, and 5.0 pg/mL, respectively. The laboratory technician who did chemokine evaluation was blinded to diagnosis of each patient.

QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION ANALYSIS

Total RNA from CSF cells from 3 patients with mild AD and 3 nondemented subjects was extracted with the single-step acid phenol method using Trizol (Invitrogen, Carlsbad, Calif), and stored at −80°C for subsequent transcriptional analysis. Cerebrospinal fluid cell counts, glucose levels, and protein levels were determined. Albumin was measured by rate nephelometry. To evaluate the integrity of the brain blood barrier and intrathecal IgG production, we calculated the albumin quotient, (CSF albumin/serum albumin)/(CSF IgG/serum IgG). S

CHEMOKINE DETERMINATION

CSF, resuspended in 500 µL Trizol (Invitrogen, Carlsbad, Calif), and stored at −80°C for subsequent transcriptional analysis. Cerebrospinal fluid cell counts, glucose levels, and protein levels were determined. Albumin was measured by rate nephelometry. To evaluate the integrity of the brain blood barrier and intrathecal IgG production, we calculated the albumin quotient, (CSF albumin/serum albumin)/(CSF IgG/serum IgG).

STATISTICAL ANALYSIS

Chemokine levels are expressed as mean ± SEM. A nonparametric Kruskal-Wallis test with post hoc comparisons among groups was used for comparisons. The Spearman test was used for correlations.

RESULTS

The Table lists the mean ± SEM values for IP-10, MCP-1, and IL-8 in CSF from both patients and controls. A trend toward increased levels of IP-10 was observed in patients with AD compared with controls (103.38 ± 12.84 vs 68.76 ± 4.66 pg/mL, P > .05; Figure 1A). However, stratifying patients on the basis of the degree of the cognitive impairment in severe or mild AD according to the MMSE scores at time of sampling (severe, MMSE < 1523), a significant increase of IP-10 was observed in patients with mild AD as compared with severe AD (145.98 ± 17.39 vs 50.12 ± 6.85 pg/mL, P < .01; Figure 1B). Notably, similar to levels in patients with mildly impaired AD, increased IP-10 levels were also found in patients with amnestic MCI vs controls (127.90 ± 10.05 vs 68.76 ± 4.66 pg/mL, P < .01; Figure 1A). Regarding MCP-1, significantly higher levels were found in all patients with AD as compared with healthy subjects (1032.76 ± 50.25 vs 860.79 ± 27.00 pg/mL, P < .01; Figure 2A). The highest MCP-1 peaks were observed in mild compared with severe AD (1129.04 ± 61.06 vs 912.40 ± 74.93 pg/mL, P = .03, Figure 2B). Similar to levels of IP-10, levels of MCP-1 in CSF from subjects with MCI were markedly increased, even to a higher extent than in patients with AD (1147.20 ± 43.77 pg/mL, P < .001, vs con-
A similar pattern was observed with IL-8 levels because they were increased in patients with AD as compared with controls (33.73±2.07 vs 26.15±2.07 pg/mL, \( P < .001 \); Figure 3A) but mostly in subjects with MCI (76.00±4.27 pg/mL, \( P = .001 \), vs controls; Figure 3A). No significant differences in IL-8 CSF concentration were found between patients with mild AD and patients with severe AD (36.12±4.21 vs 29.74±2.73 pg/mL, \( P = .05 \); Figure 3B), although, similar to IP-10 and MCP-1 levels, a trend was shown toward increased levels in patients with mild AD as compared with patients with severe AD.

Serum IP-10 and MCP-1 levels were lower than CSF concentrations. No significant differences were observed among groups, although a trend toward higher MCP-1 levels was found in patients with MCI. Conversely, IL-8 was undetectable in all samples analyzed (data not shown).

An increased amount of MCP-1 mRNA in cells isolated from CSF of a limited group of subjects (3 subjects with mild AD and 3 controls) was demonstrated by real-time polymerase chain reaction (1.32-fold increase over controls, \( P < .05 \); data not shown). Concerning IP-10 and IL-8, expression rates were not detectable with the technique used.

**CORRELATIONS**

In patients with AD, a significant positive correlation between MMSE scores and IP-10 as well as MCP-1 CSF concentration was observed (\( p = 0.37, P = .03 \), and \( p = 0.35, P = .04 \), respectively, Figure 4A). No correlation between IP-10 CSF concentration and age was found (\( p = 0.20, P > .05 \)), whereas mostly MCP-1 but also IL-8 levels correlated positively with age (\( p = 0.47, P = .005 \), and \( p = 0.39, P = .03 \), respectively, Figure 5). Consequently, comparing early age at onset with late age at onset, significant differences in CSF chemokine levels were found with regard to MCP-1 levels (939.15±57.85 vs 1179.86±79.13 pg/mL, \( P = .02 \); data not shown).

Conversely, no correlation was found between chemokines and duration of the disease in AD patients. Chemokine levels were not found to be influenced by sex as well (data not shown).
MCI FOLLOW-UP

After a 1- to 3-year follow-up, 19 subjects with MCI developed AD. 1 died, and others were stable. At time of sampling, mean ± SEM chemokine levels in the subgroup of subjects who developed AD over time were similar to the ones observed in the entire MCI group (IP-10, 126.66 ± 13.7 pg/mL; MCP-1, 1102.40 ± 64.30 pg/mL; IL-8, 75.38 ± 3.03 pg/mL).

According to these results, IP-10 is increased in CSF from patients with MCI and mildly impaired AD, but not in patients with AD with a severe cognitive decline. The significantly positive correlation between MMSE scores and IP-10 levels in patients with AD further strengthen these findings. Conversely, despite a small effect of aging on MCP-1 and IL-8 concentration, their levels are significantly increased in all individuals with cognitive impairment as compared with controls, whose age didn’t significantly differ from that of patients with MCI and AD. The highest chemokine peaks were detected in subjects with amnestic MCI, which might represent the very early stage of AD, 12,13 as well as in mild AD, whereas their levels progressively decrease during late stages of the disease. In all demented patients studied, the blood brain barrier was shown to be intact by the calculation of the albumin quotient and the IgG index, suggesting a possible involvement in a common step leading to neuronal death. Similarly to IP-10, MCP-1 has a strong chemotactic effect, but in contrast to IP-10, its expression alone does not cause inflammatory activation of cells but leads to an enhanced inflammatory response on treatment with other stimuli. Indeed, a possible protective role of MCP-1 has been suggested because it is involved in the regeneration of neural tissue via the induction of differentiation and neurotrophic factors such as basic fibroblast growth factor by astrocytes and microglia. Similarly, IL-8 increase could be related to compensative and reparative mechanisms as well through IL-8 capability to promote neuronal survival.

In conclusion, these findings confirm that chemokine expression represents a crucial step in the pathogenesis and transcriptional activation of the gene. Therefore, it is conceivable that the increased IP-10 levels observed in patients with AD could be linked to amyloid deposition. Notably, IP-10 highest peaks have been detected in patients with AD with a mild cognitive decline, as well as in individuals with amnestic MCI, which is considered a transitional state between the cognition of normal aging and mild dementia, as also demonstrated by the high rate of conversion to AD among our subjects with MCI. These observations suggest that IP-10 increase can be restricted to an early stage of AD, when proinflammatory events are thought to play a more important role in disease development. The same trend, even though less marked than IP-10 up-regulation, has been observed also considering MCP-1 and IL-8 levels, strengthening the hypothesis that intrathecal inflammation precedes the development of AD and represents an initiating factor of the disease rather than a late consequence, as also previously suggested.

This high degree of inflammation during the early stages of AD could represent an attempt of glial cells to remove β amyloid deposits. As the immunological response fails to restore the normal brain environment and neurons start dying, cytokine and chemokine production is down-regulated, as we observe in late stages of the disease. Nevertheless, although IP-10 looks likely to be involved in AD pathogenesis only, MCP-1 is likely to be up-regulated in CSF from patients with different neurodegenerative demening disorders, such as amyotrophic lateral sclerosis, suggesting a possible involvement in a common step leading to neuronal death. Similarly to IP-10, MCP-1 has a strong chemotactic effect, but in contrast to IP-10, its expression alone does not cause inflammatory activation of cells but leads to an enhanced inflammatory response on treatment with other stimuli. Indeed, a possible protective role of MCP-1 has been suggested because it is involved in the regeneration of neural tissue via the induction of differentiation and neurotrophic factors such as basic fibroblast growth factor by astrocytes and microglia. Similarly, IL-8 increase could be related to compensative and reparative mechanisms as well through IL-8 capability to promote neuronal survival.

In conclusion, these findings confirm that chemokine expression represents a crucial step in the pathogenesis and

**Figure 5.** Correlation between age and cerebrospinal fluid (CSF) chemokine levels in patients with Alzheimer disease. A, Monocyte chemotactic protein 1 (MCP-1), p = 0.47, P = .005. B, Interleukin 8 (IL-8), p = 0.39, P = .33.
progression of AD. Chemokine up-regulation is likely to be a very early event in AD pathogenesis, preceding the clinical onset of the disease, as demonstrated in our subjects with MCI, characterized by a high degree of conversion to AD. Evaluation of chemokine levels, combined with other known CSF markers, including β amyloid (1-42), tau, and phospho-tau levels, as well as other possible neuropsychological and neuroimaging tools, may be useful as early markers in MCI to predict conversion to AD. These results will be further confirmed by a longer follow-up of stable subjects with MCI and obviously by the availability of the neuropathological confirmation of diagnoses.

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