Homocysteine May Contribute to Pathogenesis of RNA Damage in Brains with Alzheimer's Disease

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Key Words

Alzheimer's disease • Homocysteine, free • Homocysteine, total • 8-Hydroxyguanosine • Cerebrospinal fluid

Abstract

Background: The pathogenesis of Alzheimer's disease (AD) is related to homocysteine (HC), but the details are unknown.

Objective: We aimed to measure the cerebrospinal fluid (CSF) concentrations of 8-hydroxyguanosine (8-OHG), considering RNA oxidative damage marker, free HC and total HC in the CSF of patients with AD and in normal control subjects.

Method and Patients: Subjects were 18 untreated patients with AD (M/F = 7/11) and 15 age-matched controls (M/F = 9/6), with a mean age $\pm$ SD of 67.4 ± 5.0 years for patients and of 65.7 ± 9.2 years for controls. The concentrations of free HC, total HC and 8-OHG in the CSF of AD patients were measured by high-performance liquid chromatography using an electrochemical detector. The control subjects were neurologically normal patients who underwent lumbar spinal anesthesia for minor surgery. **Results:** Total HC and 8-OHG concentrations were significantly increased, and there was a significant positive correlation between total HC and 8-OHG concentrations. However, the concentration of 8-OHG in the CSF showed no correlation with 8-OHG in serum

and was not significantly altered in AD patients. **Conclusion:** These results suggest that total HC and 8-OHG are positively correlated and may be related to AD pathogenesis due to RNA-associated oxidative damage linked to total HC.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that represents the most common form of dementia. The pathogenesis of AD has been linked to homocysteine (HC), but the details are unknown [1].

HC is an amino acid that contains an SH group and is generated from the metabolism of methionine in the sulfur-containing amino acid as well as cysteine. Oxidative stress due to excessive oxidation, resulting from the production of free radicals upon oxidation of HC, may lead to cell damage [2, 3]. Most HC in plasma is of the oxidized type, and 70–80% of the HC is bound to albumin. The remaining 20–30% exist as total HC in the reduced HC form (sulfhydryl form) and in the oxidized form, and as a mixed disulfide in HC-cysteine and other such combinations. Free HC, i.e. the reduced type, comprises only about 1% of total HC [4] (fig. 1).

Fig. 1. Synthesis and metabolic pathway associated with HC. Partially revised version of Parnetti et al. [5].

Using mouse models of AD, Kruman et al. [6] recently reported that HC promotes neurodegeneration in the hippocampus. Previous studies have identified a point mutation from C to T in the 677th nucleoside (C667T) in the methylenetetrahydrofolate reductase (MTHFR) gene. Enzyme activity has also been shown to change due to MTHFR gene polymorphisms, and the plasma total HC concentration is high in polymorphic homo (T/T) individuals [7]. Plasma total HC concentrations increase with age and, thus, the relationship between the pathogenesis of AD and HC is under investigation [8]. Studies have also suggested that plasma total HC is significantly higher in AD patients than in normal controls, and a significant correlation has been found between plasma total HC concentration and dementia scale [9]. We recently reported that the increased total HC concentration in the cerebrospinal fluid (CSF) of AD patients was of brain origin [10].

On the other hand, several studies have established an association of oxidative stress with AD. Oxidative damage results from an impaired oxidative balance in which the reactive oxygen production exceeds cellular antioxidant defenses, leading to damage to proteins, lipids and nucleic acids [11]. The free radical theory of aging suggests a major role for oxidative stress in age-related cellular dysfunction. The central nervous system is particularly vulnerable to oxidative damage because of its higher

energy requirements, higher oxygen consumption rate and less active antioxidant defense system as compared to other organs [12]. Increases in the production of nitric oxide and 3-nitrotyrosine, an oxidative stress marker of peroxynitrite, have been reported in various neurodegenerative disorders including AD [13, 14] and in normal aging [14]. Several studies, mainly using cultured cell lines, have also shown that apoptosis is caused when β -protein is added to neuronal cultured cells. The mechanism for this is believed to be the reduction of β -protein to its elements by metal ions or hydrogen peroxide under oxidative conditions. This causes the production of reactive oxygen species and reactive nitrogen species [15, 16].

8-Hydroxyguanosine (8-OHG) is a good marker of hydroxyl radical damage to RNA. This molecule has been proposed as a useful systematic marker of oxidative stress [17]. Since RNA is turned over rapidly, the level of 8-OHG reflects the steady-state oxidative balance at the time of determination rather than the previous oxidative damage [18, 19]. Recent histochemical studies showed a marked accumulation of 8-OHG cytoplasmic RNA within the cerebral neurons of patients with AD [20].

We measured the CSF concentrations of free HC, total HC and 8-OHG in each specimen [10, 21]. Based on the *in vivo* findings, we reported that an increase in total HC and metabolites (such as homocysteic acid) [5] might be related to the pathogenesis of AD [10]. Moreover, we dem-

onstrated the importance of RNA oxidation in the early phase of AD development [21].

In the present study, we measured free HC, total HC and 8-OHG using the same sample as in the above-mentioned analysis in order to determine their concentrations in the CSF of patients with AD. We thus investigated whether HC contributes to RNA disorders in the pathogenesis of AD.

Materials and Methods

Patients

The subjects were 18 untreated patients with AD (M/F = 7/11) and 15 age-matched controls (M/F = 9/6), with a mean age $\pm$ SD of 67.4 ± 5.0 and of 65.7 ± 9.2 years, respectively. The control subjects were neurologically normal patients who underwent lumbar spinal anesthesia for minor surgery. Diagnostic criteria for AD were defined according to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th revision (DSM-IV) [22], the National Institute of Neurological and Communicative Disorder and Stroke (NINCDS), the AD and Related Disorders Association (ADRDA) [23] and Hachinski's ischemic score [24]. Cognitive function was assessed based on the Mini-Mental State Examination (MMSE) score [25]. In the patients with AD, the duration of illness $\pm$ SD was 3.1 ± 2.2 years and the mean MMSE score $\pm$ SD 16.5 ± 3.5 . All the patients were admitted to a hospital and were maintained on a standard diet. All the patients or their families provided informed consent, and the study protocol was approved by the Committee for Ethics in Biomedical Research at Iwate Medical University (Morioka, Japan).

CSF Analysis

CSF was obtained by lumbar puncture with the patients in a lateral decubitus position between 9.00 and 10.00 a.m., after overnight bed rest and before breakfast. For serum preparation, venous blood samples were centrifuged at 1,000 g for 5 min at 4°C. First, a 3-ml CSF sample was used for general examination, and a further 1-ml CSF sample and serum samples taken from the patients were rapidly frozen and stored at -80°C prior to being assayed. Cell counts and protein concentrations in CSF were within their normal ranges in both AD patients and controls ($1.6 \pm 1.1 \text{ mm}^3$ and $35.2 \pm 5.2 \text{ mg/dl}$; $1.4 \pm 1.2 \text{ mm}^3$ and $29.4 \pm 8.2 \text{ mg/dl}$, respectively).

Free and Total HC

A tube filled with 100 μl of distilled water was used to determine the free HC concentration, and a tube filled with 75 μl of distilled water and 25 μl of Tris(2-carboxy-ethyl)phosphine to determine the total HC concentration. To each of these tubes, 300 μl of CSF was added. Then, 300 μl was added after mixing for 60 s with a vortex mixer and shaking by hand for 10 min, followed by the addition of 500 μl of 0.3 N HClO_4 . After shaking for 1 min, 100 μl was centrifuged at 25°C and 10,000 g for 10 min. Finally, 100 μl of supernatant was taken and 20 μl of it applied to a high-performance liquid chromatography column (MCM C_{18} reversed-phase column, $80 \times 4.6 \text{ mm}$; MC Medical, Tokyo, Japan) with an electrochemical detector (Coulchem II Model 5300; ESA, Inc.,

Bedford, Mass., USA) system to determine the concentration of HC. The electrode potentials were maintained at 750 mV for detector II. The mobile phase consisted of 0.15 M phosphate (pH 2.7) and phosphoric acid-methanol (92/8 v/v); buffered saline was used. The flow rate was 1.0 ml/min, and the column temperature kept at 26°C . The detection limits for free HC and total HC were both 0.1 nM. The standard of HC was obtained from Sigma Chemical Co. (St. Louis, Mo., USA).

8-Hydroxyguanosine

The free 8-OHG concentration was determined according to the method of Shigenaga et al. [26] with some modifications.

Briefly, 1 ml of CSF or serum was absorbed in a solid-phase extraction cartridge (Bond Elut C_{18} 3 ml/200 mg; VARIAN, Harbor City, Calif., USA) and eluted with 1.5 ml of methanol. The eluate was concentrated with a centrifugal evaporator and dissolved in 100 μl of distilled water. After filtration with a 0.45- μm membrane filter, 40 μl of the solution were analyzed using high-performance liquid chromatography (MCM C_{18} reversed-phase column, $250 \times 4.6 \text{ mm}$; MC Medical) with an electrochemical detector (Coulchem II Model 5200; ESA, Inc.). The mobile phase consisted of 10 mM NaH_2PO_4 and 6% (for CSF) or 4% (for serum) methanol. The electrode potentials were maintained at 0.3 V for the guard cell, 0.15 V for detector I and 0.25 V for detector II. The flow rate was 1.0 ml/min and the column temperature was kept at 20°C . The limit of detection for 8-OHG was 20 pM. The 8-OHG standard was obtained from Cayman Chemical Co. (Ann Arbor, Mich., USA).

Statistical Analysis

In brief, the statistical analysis was performed using the non-parametric Mann-Whitney U test or the Spearman rank correlation coefficient (r_s) with Stat View 5.0 software (SAS Institute, Inc., Cary, N.C., USA). $p < 0.05$ was considered to be statistically significant.

Results

Free and Total HC Concentrations in CSF

The concentration of free HC did not differ significantly between controls ($10.9 \pm 3.4 \text{ nM}$) and AD patients ($10.6 \pm 6.0 \text{ nM}$). The concentration of total HC in AD patients ($110.6 \pm 31.6 \text{ nM}$) was significantly increased as compared to $84.9 \pm 24.5 \text{ nM}$ in controls ($p < 0.05$) (table 1). There was no significant correlation between the duration of illness and the concentration of total HC in AD. In patients with AD, there was no significant correlation between MMSE score and the concentration of total HC [10].

8-OHG Concentration in CSF

The concentration of 8-OHG in the CSF of the control subjects ranged from 40 to 140 pM. There was no significant correlation of the 8-OHG concentration with age

Fig. 2. Correlations between total HC and 8-OHG in the CSF from patients with AD.

Table 1. Concentrations of free HC and total HC in the CSF of patients with AD

	Free HC, nM	Total HC, nM
Controls	10.9 ± 3.4	84.9 ± 24.5
AD patients	10.6 ± 6.0	110.6 ± 31.6* (+31%)

Values are means ± SD. * p < 0.001.

Table 2. Concentrations of 8-OHG in serum and CSF of patients with AD

	8-OHG in serum, nM	8-OHG in CSF, pM
Controls	1.42 ± 0.59	97 ± 32
AD patients	1.53 ± 0.60	500 ± 213* (>500%)

Values are means ± SD. * p < 0.001.

($r_s = -0.25$; $p = 0.35$). The concentration of 8-OHG was significantly and remarkably increased in the CSF of the patients with AD as compared to the controls (500 ± 213 vs. 97 ± 32 pM; $p < 0.001$) (table 2). The difference between the groups was clear: each of the 18 AD patients had a concentration of 8-OHG that was >250 pM. The concentration of 8-OHG in the CSF of AD patients showed a significant negative correlation with the dura-

tion of illness ($r_s = -0.48$; $p < 0.05$; data not shown) and a significant positive correlation with the MMSE score ($r_s = 0.67$; $p < 0.01$; data not shown).

However, the concentration of 8-OHG in the CSF did not correlate with that in the serum of controls ($r_s = -0.10$; $p = 0.71$) or AD patients ($r_s = -0.12$; $p = 0.61$). In addition, the concentration of 8-OHG in the serum of AD patients (1.53 ± 0.60 nM) was not significantly altered as compared to the controls (1.42 ± 0.59 nM) [21].

Correlation between Total HC and 8-OHG Concentrations in CSF

We observed a significant positive correlation between total HC and 8-OHG concentrations in the CSF ($r_s = 0.58$; $p < 0.02$) (fig. 2). This relationship was not observed in controls (data not shown).

Discussion

The involvement of HC in AD has not been evaluated in autopsied brains, while in vivo studies have been performed on plasma total HC. Selley et al. [27] recently examined the CSF of 8 patients with AD and found that total HC in the CSF was high in AD patients, and that a positive correlation existed between the total HC concentration and the concentration of 4-hydroxy-2-nonenal, which is a biochemical marker of the neurotoxic products of lipid peroxidation. The results of that study suggest that HC may induce neuronal damage via oxidative stress [27]. HC has also been reported to enhance the susceptibility of the hippocampus to neuronal damage and may promote the peroxidation of amyloid β -protein (the major protein component in senile plaques) or enhance neurotoxicity [28].

Recently, we showed that total HC and HC metabolites such as homocysteic acid might be related to the pathogenesis of AD. Based on these results, it appears that an increase in total HC in the brain characterizes AD and that total HC is a contributor to degeneration; however, this could also reflect an epiphenomenon [10].

We recently demonstrated a significant fivefold increase in 8-OHG concentration in the CSF of AD patients as compared to controls. But the concentration of 8-OHG in the CSF showed no correlation with that in the serum in both the controls and AD patients. In addition, the concentration of 8-OHG in the serum was not significantly altered in the AD patients compared to that in the controls, suggesting that the 8-OHG concentrations in CSF do not reflect those in serum and may probably re-

flect those in brain tissue. Also, we observed a negative correlation between the concentration of 8-OHG and the duration of illness as well as a positive correlation between the concentration of 8-OHG and the MMSE score [21]. Although we cannot specify the origin of 8-OHG in the CSF, its concentration in the CSF may, for the most part, reflect that in the brain rather than in the blood because 8-OHG is hydrophilic. The concentrations of 8-OHG in serum showed no correlation with those in CSF in both the controls and AD patients. Further, the concentrations of 8-OHG in serum did not significantly differ between controls and AD patients.

The present data suggest the possibility that RNA oxidation is abnormally accelerated in the cerebral tissue of AD patients and that an increased oxidation of RNA occurs in the early stages of AD. These findings are consistent with those of previous reports on semiquantitative immunohistochemical studies using anti-8-OHG monoclonal antibody in autopsied brains [29]. These previous studies reported that 8-OHG immunoreactivity was significantly increased in the hippocampus and in neurons within the frontal, temporal and occipital neocortex of AD brains compared with controls [29]. During aging, nerve cells in the cerebral cortex of patients with Down's syndrome demonstrate neuropathological changes similar to those found in AD [20, 30].

Whether oxidative stress plays a role in the early phase of AD pathogenesis or is secondary to neuropathological changes in AD remains to be determined. RNA is more vulnerable to oxidative stress than DNA both in vitro and in vivo [15, 16] probably because, unlike nuclear DNA,

RNA is single-stranded and not covered with protective histones. The relative paucity of oxidative damage to DNA may be explained by a DNA repair mechanism, while the only known compensation for an increased oxidation of RNA is its higher turnover rate [17]. The fidelity of RNA, a molecule that can be viewed as the disposable soma of genetic information, has been much less studied than that of DNA, which is the heritable germ line. However, there may be unidentified RNA repair mechanisms that are involved in important RNA modifications such as editing and splicing [31]. Therefore, RNA oxidation is abnormally accelerated in the cerebral tissue of AD patients, and an increased oxidation of RNA occurs in the early stage of AD.

In summary, the untreated AD patients showed that total HC and 8-OHG concentrations were elevated in the CSF. In addition, total HC and 8-OHG were significantly increased in the AD patients. Our results demonstrate a significant positive correlation between concentrations of total HC and 8-OHG in CSF. These findings suggest that RNA disorders due to associated HC may result from oxidative damage mediated by HC in the etiopathogenesis of AD.

Acknowledgments

We wish to thank Miss Yoko Iwa for her technical and secretarial assistance. This study was supported in part by the Ministry of Education, Science and Culture, and the Ministry of Health, Labor and Welfare in Japan.

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