Association Between Complement Regulatory Protein Factor H and A M 34 Antigen, Detected in Senile Plaques

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Background. We have previously shown that monoclonal antibody AM34, which is reactive with senile plaques, may recognize the C terminus of complement factor H. In this study, we investigated the expression of factor H in tissue from a human brain and the relation between AM34 antigen and factor H.

Method. Total ribonucleic acid (RNA) was extracted from a normal human brain. A reverse transcriptase-polymerase chain reaction method was employed for detecting messenger RNAs coding for factor H and related proteins. Protein extracts from a normal human brain were also analyzed to detect factor H and related proteins by means of Western blotting. The cerebrospinal fluid from an Alzheimer’s disease patient was immunoprecipitated with AM34 and anti-factor-H antibodies, and then it was subjected to gel electrophoresis followed by immunoblotting with AM34 and anti-factor-H antibodies.

Results. 26 clones of complementary DNA fragment were obtained by reverse transcriptase-polymerase chain reaction. Among them, seven clones were identical to factor H, and the others were related proteins and unreported sequences. A Western blot analysis of protein extracts from the normal brain tissue exhibited a 150-kd band, indicating the presence of factor H. AM34 was immunoreactive with the 150-kd molecule contained in the immunoprecipitates with anti-factor H antibodies, and vice versa. These results suggest that AM34 antigen could be identical to complement factor H.

Conclusions. The results of our experiments indicate that factor H is possibly detected in the human brain, and that the AM34 antibody could recognize factor H. Because AM34 is capable of staining senile plaques positively, factor H is suggested to be associated with senile plaques in the human brain.

ALZHEIMER’S disease (AD) is a progressive neurodegenerative disorder that is characterized by deposition of β-amyloid protein (Aβ) in the form of senile plaques (1). Aβ is a 39- to 43-amino acid peptide (2) proteolytically produced from amyloid precursor protein (APP) (3). C-terminally elongated Aβ, including Aβ1-42 or Aβ1-43, is inclined to form insoluble amyloid fibrils more rapidly than Aβ1-40 (4). Mutations of APP, presenilin 1 and presenilin 2, which are located on chromosomes 14 and 1, respectively, are found to be mutated in familial Alzheimer’s disease, enhancing the extracellular concentration of C-terminally elongated Aβ in vivo (5). These observations have led to the conclusion that the abnormal metabolism of APP produces elongated Aβ and results in an accumulation of aggregated Aβ to generate the pathological changes of AD; however, the precise mechanism whereby deposits of aggregated Aβ cause injury to neuronal cells has not been fully elucidated.

The implication of various inflammatory mediators has been proposed in the progression of AD. Complement factors C1q, C3, and C4 have been detected in senile plaques (6), and an increased amount of C1qB and C4 messenger ribonucleic acid (mRNA) in the AD brain has been demonstrated (7). Moreover, an interaction of Aβ with C1q/C3 leading to the activation of the complement classical/alternative pathway (8–10) and to the enhancement of Aβ aggregation (11) has been shown. Therefore, it is conceivable that Aβ may activate the complement pathway, leading to the neuropathology of AD.

We previously established monoclonal antibody AM34 by using the kidney of secondary amyloidosis as an antigen, and we reported that AM34 was capable of reacting with not only the amyloid deposit of secondary amyloidosis but also senile plaques in the brain of AD (12). In the report, we screened the human liver complementary deoxyribonucleic acid (cDNA) library (human adult liver cDNA library; Clontec, Palo Alto, CA, #HL 1001b, Lot 2102) with AM34 by the use of an immunoscreening method, and we demonstrated that obtained positive clones contain an identical structure to the C terminus of complement factor H, a regulator of complement alternative pathway. Factor H, a 150-kd serum glycoprotein, binds with C3b to dissociate C3bBb (C3/5 convertase; decay acceleration activity) (13), and C3b-bound factor H accelerates the inactivation of C3b by factor I (factor I–cofactor activity) (14). The primary structure of factor H shows that this polypeptide is composed of 20 homologous regions consisting of 60 amino acid residues, termed short consensus repeats (SCRs) (15). There are some other proteins that are structurally and immunologically related to factor H, named factor-H-related (FHR) proteins, including FHR-1 to FHR-4 (16,17). They are composed of four or five SCRs and are lacking in the domain corresponding to the SCR1-4 of factor H, which is required for cofactor activity (18). Because their two C-terminal SCRs are highly homologous to the SCR19-20 of factor H, it is possible that AM34 is capable of detecting the related proteins but not factor H in the AD brain.
To clarify whether factor H or factor-H-related proteins are present in senile plaque, we investigated the expression of mRNA encoding for factor H and related proteins by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, using antisera against factor H, we detected factor H protein in the human brain immunologically. We also demonstrated, by the use of an immunoprecipitation method, that AM34 antigen is identical to factor H in the cerebrospinal fluid of an AD patient.

Methods

Human Brain Samples

Human brain tissues were obtained at autopsy from AD patients and non-AD elderly persons at the Sapporo Medical University hospital. Samples from 10 cases of AD and two apparently normal brain tissues were utilized for immunohistochemical analysis, and those from one case of non-AD were utilized for western blotting. The cerebrospinal fluid from a patient with AD was utilized for immunoprecipitation and western blotting.

Immunohistochemistry

The deparaffinized sections were reacted with AM34 overnight at 4°C after blocking endogenous peroxidase by treatment with 0.3% H2O2 and nonspecific binding with 10% normal rabbit serum. After washing, the sections were incubated with biotinylated rabbit IgG against mouse IgG (Dako, Glostrup, Denmark) for 30 minutes and then with an avidin biotin peroxidase. They were developed with 0.03% 3,3-diaminobenzidine tetrahydrochloride in 50 mM of Tris-Cl (pH 7.6) containing 0.006% H2O2. Counterstaining was performed with hematoxylin.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from a human brain tissue by the use of the acid guanidinium thiocyanate phenol chloroform method. Randomly primed cDNA was prepared from 1 μg of total RNA by Moloney-murine leukemia virus reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT) and was amplifed by PCR (19). The sense primer was 5′-AAATgTggg(C/g,C/g)CCCTCCACC-3′, a 21-mer nucleotide corresponding to the region encoding amino acid residues KCGPPP in the SCR19 of factor H, and the antisense primer was 5′-CATTTTggTggg(T/g)C(T/C)GACCA, a 20-mer nucleotide corresponding to the region encoding amino acid residues WSEPPK in the SCR19 of factor H. Both regions are highly conserved among the factor H and related proteins. PCR was performed for 30 cycles (denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute). The PCR product was purified and subcloned into p-Bluescript (Stratagene, La Jolla, CA).

Characterization of Isolated cDNA Fragments

We obtained 26 clones of cDNA fragments as mentioned above. They were sequenced in a double-stranded form by dideoxy chain termination, using α-35S-dATP and Sequenase II (USB, Cleveland, OH).

Immunoprecipitation

A 1:1 suspension of Protein G-sepharose was prepared in a solubilizing buffer (Radioimmunoprotein assay, or RIPA, buffer: 10 mM Tris-HCl, pH 7.4/1% Nonidet P-40/0.1% sodium deoxycholate/0.1% sodium dodecyl sulfate, or SDS/0.15 M of NaCl/1 mM of ethylenediamine tetra-acetic acid/10 μg/ml of aprotinin/0.3 M of Phenyl-methyl-sulphonyl fluoride). 50 μl of the 1:1 suspension of Protein G-sepharose was added to 200 μg of the cerebrospinal fluids from the AD patient and incubated for 24 hours at 4°C on a rocking platform. After centrifugation at 2000 × g, 10 μg of AM34 or sheep anti-human factor H antibody was added to the supernatant. The mixture was shaken and incubated for 1 hour at 4°C; then 50 μl of the 1:1 suspension of Protein G-sepharose was added to the samples. Incubation was continued for 1 hour at 4°C on the rocking platform. The beads were harvested by centrifugation at 2000 × g for 1 minute and washed five times with RIPA buffer at 4°C.

Western Blotting

20 μg of protein from brain tissue or immunoprecipitated protein obtained as described above were suspended in a 2 × sample buffer (20% glycerol/10% 2-mercaptoethanol/6% SDS/130 mM of Tris-HCl, pH 6.8) and boiled for 5 minutes. After centrifugation for 1 minute at 2000 × g, 35 μl of the supernatant were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in a miniature slab gel apparatus (SDS-PAGE minigel, Ikabi glass, Tokyo, Japan), with appropriate molecular-weight standards. The electrotransfer of proteins from the gel to nitrocellulose (pore size 0.45 μm; Schleicher & Schull, Dassel, Germany) was performed as described previously. The nitrocellulose blots were incubated with AM34 (20 μg/ml) or polyclonal anti-factor-H antibody (sheep anti-human factor H; The Binding Site, Birmingham, England; 1:1000 dilution) overnight at 4°C and then washed at room temperature. The blots were then incubated with peroxidase-conjugated rabbit IgG against mouse IgG for 30 minutes, and then with an avidin biotin peroxidase. Bound antibodies were visualized by using the enhanced chemiluminescence reagents for Western blot analysis (Amersham, Arlington Heights, IL).

Results

AM34 Immunoreactivity in the AD Brain

Brain specimens from 10 cases of autopsied AD patients were investigated by immunohistochemical analysis. Figure 1 shows immunoreactivity for AM34 in the AD brain specimens. Specific staining was observed in senile plaques (Figure 1A), which is confirmed by bodian staining of the same section (data not shown), and amyloid angiopathy was also stained positively (Figure 1B); other regions revealed no positive staining. All samples from AD specimens exhibited positive staining with AM34. Two apparently normal brain tissues were also investigated, but immunoreactivity with AM34 was not detected. Thus it was
suggested that the distribution of AM34 antigen is localized around the deposited Aβ.

Analysis of mRNA for Factor-H and FHR Proteins in Human Normal Brain Tissue

We investigated the expression of mRNA encoding for factor-H and factor-H-related proteins by RT-PCR. Factor-H-related proteins are designated FHR-1 (H-36 cDNA), FHR-2 (DDES59 cDNA), FHR-3 (DOWN16 cDNA) (16) and FHR-4 (Sac6 cDNA) (17). These proteins are composed of five or four SCRs, and all contain the SCR corresponding to the SCR-19 and SCR-20 of factor H. Oligonucleotide primers were designed to amplify the 167bp fragment contained in the SCR-19 of factor H and the corresponding region of factor-H-related proteins. The PCR product was purified and analyzed by agarose-gel electrophoresis (Figure 2). The obtained fragment was about 167 base pair in size. Thus we obtained 26 clones of cDNA fragment, which had been subcloned into the p-Bluescript vector, and then these clones were analyzed by sequencing. The results are represented in Table 1. Among the obtained cDNA clones, seven clones were identical to the cDNA sequence of factor H, whereas other clones were FHR-2 (DDES59) and FHR-3 (DOWN16). In addition, two unreported sequences, 1 and 2, which resemble FHR-3 or FHR-4 in the amino acid sequence, were identified (Figure 3). Thus mRNA encoding for factor-H and FHR proteins were detected in human brain tissue, suggesting that factor-H is transcribed locally in the brain, which is compatible with the previous report detecting factor-H mRNA in cultured human astrocytes (20). However, the possibility cannot be ruled out that these messages are derived from blood. In order to draw a conclusion, detection of cells producing factor-H mRNA will be required.

Detection of Factor-H Protein in the Non-AD Brain

Protein extracts from a non-AD brain were subjected to SDS-PAGE followed by immunoblotting with antisera raised against the factor-H protein. A positive band migrating at a molecular mass of 150 kd was identified (Figure 4), revealing that factor-H protein is present in the non-AD brain. The bands indicating other factor-H-related proteins did not appear in this experiment.

Analysis of the Relationship between AM34 Antigen and Factor H

We immunoprecipitated the cerebrospinal fluid with AM34 and anti-factor-H antibody. Then the precipitated material was analyzed by SDS-PAGE, followed by immunoblotting with AM34 or anti-factor-H antibodies. Immunoprecipitates with anti-factor H were immunoreactive with AM34, representing the positive band of 150 kd in molecular size (Figure 5, lane 1). In addition, immunoprecipitates with AM34 were capable of reacting with anti-factor-H antibody, representing the 150-kd protein, the same as above (Figure 5, lane 4). As a control, precipitated materials with TB-1 (anti-Aβ monoclonal antibody) were analyzed as described above, but no positive signals were identified (data not shown).
not shown). These results suggest that AM34 and anti-factor-H antibody were capable of reacting with the same 150-kd protein in the cerebrospinal fluid, indicating that AM34 antigen may be identical to factor H.

**DISCUSSION**

In this study, we detected mRNA encoding for factor-H and factor H-related proteins in a human brain, and we identified factor-H protein in human brain extracts by Western blotting. Furthermore, we demonstrated here that AM34 antigen might be identical to factor H. Because senile plaques were capable of being stained with AM34, it is indicated that factor H may be associated with senile plaques in the AD brain.

Factor H has also been detected in cultured cells derived from human brain tissues (20). However, there are few reports describing involvement of an alternative pathway in the AD brain. Recently, it has been shown that factor H could modulate the activity of the classical complement pathway. In the fluid phase, factor H binds to C4b and serves as a cofactor for factor I-mediated inactivation of C4b (21). Factor H is also an inactivator of C3b derived from activation of the classical pathway (22). Therefore, factor H may restrict the activation of the classical pathway and protect surrounding neurons from injury by the aggregated Aβ, even if an alternative pathway is not involved in the pathogenesis of AD. Although factor H is a downregulator of complement activation, some authors have demonstrated that factor H may serve as an immunopotentiator. Ohtsuka and colleagues (23) showed that monocyte chemotactic activity was generated from factor H after incubation with thrombin. They proposed that factor H might convert to a monocyte chemotactic factor and play a role in the delayed-type hypersensitivity reaction in the skin. Nabil and colleagues (24) demonstrated that factor H contained in the pleural effusion of patients with malignant mesothelioma showed monocyte chemotactic activity. These studies regard factor H as a potential upregulator of the inflammatory response. It is conceivable that the chemotactic factor H could recruit and activate microglia and contribute to the progression of pathological changes in AD. Additional experiments will be required before we can draw a conclusion about the role of factor H in the pathogenesis of AD.

We prepared AM34 by immunizing mice with tissue extracts from a kidney with secondary amyloidosis (12). Secondary amyloidosis is characterized by extracellular accumulation of amyloid A protein, which is derived from serum precursor, serum amyloid A protein (SAA). Interestingly, the local production of SAA in the AD brain, but not in the brain affected by other neurological diseases, has been reported recently (25). As we previously reported, AM34 is reactive with tissues from secondary amyloidosis and AD, whereas any lesions with primary amyloidosis, myeloma-associated amyloidosis, familial amyloid polyneuropathy, localized amyloid, and skin amyloid were not stained positively, indicating that the expression of AM34 antigen may be specific for AA and Aβ. Thus, detection of SAA in the AD brain may become a clue to a connection between AM34 antigen (factor H) and AD.
The neurodegenerative process in AD may be caused by inflammatory responses mediated by the complement and glial activation, which originates in the deposition of aggregated Aβ. We demonstrated here the presence of factor H in the AD brain; however, the role of factor H is presently unknown. Recent reports have concluded that anti-inflammatory drugs are effective in retarding the progression of AD (26). Elucidation of the precise inflammatory process will contribute to the development of more effective anti-inflammatory therapies against AD.

Acknowledgment

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References