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Codeposition of Cystatin C with Amyloid-β Protein in the Brain of Alzheimer Disease Patients

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Abstract. Immunohistochemical analysis of brains of patients with Alzheimer disease (AD) revealed that the cysteine proteinase inhibitor cystatin C colocalizes with amyloid β-protein (Aβ) in parenchymal and vascular amyloid deposits. No evidence of cerebral hemorrhage was observed in any of the brains studied. Immunoelectron microscopy demonstrated dual staining of amyloid fibrils with anti-Aβ and anti-cystatin C antibodies. Cystatin C immunoreactivity was also observed in amyloid deposits in the brain of transgenic mice overexpressing human β amyloid precursor protein. Massive deposition of the variant cystatin C in the cerebral vessels of patients with the Icelandic form of hereditary cerebral hemorrhage with amyloidosis is thought to be responsible for the pathological processes leading to stroke. Anti-cystatin C antibodies strongly labeled pyramidal neurons within cortical layers most prone to amyloid deposition in the brains of AD patients. Immunohistochemistry with antibodies against the carboxyl-terminus of $A\beta_{x-42}$ showed intracellular immunoreactivity in the same neuronal subpopulation. It remains to be established whether the association of cystatin C to Aβ plays a primary role in amyloidogenesis of AD or is a late event in which the protein is bound to the previously formed Aβ amyloid fibrils.

Key Words: Alzheimer disease (AD); Amyloid β-protein (Aβ); β amyloid precursor protein (βAPP); Cerebral amyloid angiopathy (CAA); Hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D); Hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I).

INTRODUCTION

Cerebral amyloidoses comprise a heterogeneous group of disorders of different etiology marked by amyloid deposition in the brain parenchyma and blood vessel walls. Amyloid β-protein (Aβ) is the major constituent of the amyloid fibrils in aged individuals and patients with Alzheimer disease (AD), Down syndrome and hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D) (1–3). HCHWA-D is a rare, autosomal dominant cerebral amyloid angiopathy (CAA) that is characterized by massive amyloid deposition in leptomeningeal and cortical vessels resulting in recurrent intracerebral hemorrhages (4). In most cases, Aβ also deposits in brain parenchyma, as nonfibrillar preamyloid deposits or diffuse plaques (5–7). The disease segregates with a single mutation resulting in E693Q amino acid substitution at position 22 of Aβ (8). A mutation at the same codon resulting in E to K substitution has been recently found in several members of 2 Italian families that show clinical and pathological features similar to HCHWA-D patients (9). A Flemish family with early onset CAA has an A692G substitution (10). Here the mutation segregates with both presenile dementia and cerebral hemorrhage.

A familial type of HCHWA with a clinical picture and neuropathological changes similar to HCHWA-D (11) was described in patients from Iceland (HCHWA-I) (12). In this condition, the amyloid deposits are restricted to the cerebral and spinal arteries and arterioles and are composed of a variant of cystatin C (13, 14) or γ trace (15), a cysteine proteinase inhibitor (16, 17). The amyloid protein isolated from the leptomeninges of HCHWA-I patients was found to start at position 11 of normal cystatin C and carry an L68Q amino acid substitution (13, 14, 18). The same mutation was identified in the cystatin C gene of an elderly Croatian man, who suffered his first stroke at 63 yr and whose family history showed no indication of hereditary CAA (19).

Previous immunohistochemical studies of patients with CAA due to Aβ deposition have demonstrated dual labeling of vascular amyloid with antibodies to Aβ and cystatin C, and it was suggested that colocalization of cystatin C with Aβ may cause hemorrhagic stroke (20–22). Here we show that cystatin C colocalizes with Aβ in amyloid deposits in the brain parenchyma and vessel walls of AD patients that had neither a clinical history nor pathological evidence of cerebral hemorrhages. Furthermore, strong staining with antibodies to both cystatin C and Aβ was observed in pyramidal neurons, mainly within the third and fourth layers of the cerebral cortex. These data suggest an early role for cystatin C in Aβ amyloidogenesis.

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TABLE
Variability in the Levels of Parenchymal and Vascular Aß Amyloid Deposits in the Brains of AD Patients, an HCHWA-D Patient, and Asymptomatic Aged Individuals

			Aß deposits		
AD patients	Age at death	Disease duration	Parenchymal	Vascular	APOE genotype
1	87	12	H	H	3/4
2	79	9	H	H	4/4
3	68	14	H	M	3/3
4	80	8	H	M	3/4
5	79	5	H	M	3/4
6	33	5	H	M	na
7	84	19	H	L	4/4
8	82	16	H	L	3/4
9	83	14	H	L	4/4
10	72	8	H	L	3/4
11	74	6	H	L	3/4
12	80	6	H	L	3/4
13	73	5	H	L	3/4
14	80	5	H	L	3/4
15	92	14	H	L	na
16	85	16	H	—	3/4
17	81	8	M	H	3/4
18	77	10	M	M	3/4
19	80	7	M	M	3/3
20	87	14	M	L	2/4
21	91	14	M	L	2/4
22	76	11	M	L	3/4
23	79	9	M	L	3/4
24	86	8	M	L	3/4
25	75	5	M	L	na
26	91	11	M	—	3/3
27	83	10	M	—	3/3
28	90	22	L	—	4/4
HCHWA-D Patient					
1	47	0.5	H	H	na
Nondemented Aged Individuals					
1	71	—	H	H	na
2	71	—	M	M	na
3	73	—	—	—	na

Aß amyloid load: high (H), medium (M), low (L), undetectable (—). Tissue was not available (na) for isolation of DNA for some of the samples.

MATERIALS AND METHODS

Source of Tissues

Brain tissue samples were obtained from 28 patients with AD, 1 patient with HCHWA-D and 3 nondemented aged individuals. The levels of parenchymal and vascular amyloid deposits varied between individuals within the 2 groups: the AD group and that of nondemented aged individuals (Table). No evidence of hemorrhages was found in any of these brains. The patient with HCHWA-D exhibited impressive congophilic angiopathy. Brain tissue was also obtained from 2 transgenic mice (females, aged 2 yr) overexpressing β amyloid precursor protein (βAPP) cDNA containing the K670N/M671L mutation driven by hamster prion protein promoter (Tg(HuAPP695.SWE)2576) (23).

Histology and Immunohistochemistry

The study was carried out on 8-μm-thick serial sections from formalin-fixed, paraplast-embedded blocks of cerebral hemispheres of patients and nondemented aged individuals. For immunohistochemistry, adjacent sections were deparaffinized and treated with 100% formic acid for 30 min to enhance amyloid staining. The endogenous peroxidase activity was quenched with 0.3% H_2O_2 in dH_2O for 30 min, and nonspecific binding was blocked with 0.05 M Tris-HCl pH 7.6, 0.9% NaCl buffer containing 10% fetal calf serum and 0.2% bovine serum albumin for 1 h. The sections were then incubated overnight at 4°C with either a rabbit antiserum to cystatin C (Axell, 1:800), the monoclonal 4G8 to $Aβ_{17–24}$ (1:200) (Senetek, Maryland Heights, MO) or rabbit antibodies specific to the carboxyl-terminus of $Aβ_{x-42}$ or $Aβ_{x-40}$ (QCB, 1:100). The immunoreactions were revealed by either biotin-conjugated anti-rabbit (1:800) or anti-mouse (1:200) IgG (Sigma, St. Louis, MO), followed by ABC kit (Vector Laboratories, Burlingame, CA), each for 60 min at room temperature. Peroxidase activity was developed with either 3,3′-diaminobenzidine containing or lacking 0.005% cobalt hexachloride (Sigma), or Vector SG substrate (Vector Laboratories). Negative controls included substitution of the primary antibody with either normal rabbit serum, mouse serum (1:800 and 1:200 dilution, respectively), or the anti-cystatin C antiserum (1:800) preabsorbed with cystatin C expressed as glutathione S-transferase fusion protein and immobilized on agarose beads.

Transgenic mice were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer pH 7.4. The brains were incubated in 4% paraformaldehyde overnight and cryoprotected with 30% sucrose in phosphate buffer pH 7.4 for 24 h. The brains were then embedded in M-1 #1310 matrix (Lipshaw Manufacturing, Detroit, MI) and frozen at −80°C for storage. 30 μm coronal sections were stored in 0.1 M phosphate buffer pH 7.4, 0.01% NaN_3 at 4°C. For immunohistochemistry, floating sections were incubated in 0.1 M Tris pH 7.4, 150 mM NaCl, 0.25% Triton X-100 for 15 min at room temperature, and nonspecific binding was blocked with 3% goat serum in the same buffer. Sections were incubated with either rabbit antiserum to cystatin C (Axell, 1:40,000), the monoclonal 4G8 to $Aβ_{17–24}$ (Senetek, 1:200) or rabbit antibodies specific to the carboxyl-terminus of either $Aβ_{x-42}$ (QCB, 1:500) or $Aβ_{x-40}$ (QCB, 1:100), overnight at room temperature. The immunoreactions were revealed by either biotin-conjugated anti-rabbit or anti-mouse IgG (Sigma, 1:1,000) for 30 min followed by the Vectastain kit (Vector Laboratories).

Immunoelectron Microscopy

A paraffin-embedded block of brain tissue from an AD patient was deparaffinized in xylene and rehydrated through graded ethanol solutions. Samples of cerebral cortex were dissected, post-fixed in 1% osmium tetroxide for 1 h and embedded in Epon 812. Silver-gray ultrathin sections were mounted on nickel grids and pretreated with 4% solution of aqueous sodium metaperiodate for 1 h before immunocytochemical labeling. After washing in 0.1 M phosphate buffered saline, pH 7.4, the sections were incubated for 1 h in 1% bovine serum albumin in phosphate buffered saline, pH 7.4, to block nonspecific binding sites. The sections were then transferred onto a drop of

primary antibody (rabbit antiserum to cystatin C, 1:20, and/or monoclonal 4G8 to $A\beta_{17-24}$, 1: 20) and incubated overnight at 4°C in a moist chamber. After spin washing in 10 mM Tris pH 7.5, 150 mM sodium chloride, 0.1% Tween-20, sections were incubated for 2 h with either anti-rabbit IgG, anti-mouse IgG, or protein A (1:50) (Amersham, Piscataway, NJ) conjugated with 1-, 10- or 15-nm gold particles, respectively. The immunogold labeling was silver enhanced (Vector Labs) for 20 s. The sections were stained briefly with uranyl acetate and lead citrate and were examined under a Zeiss-EM10 electron microscope. The specificity of the immunoreactions was confirmed by replacing the primary antibody with either rabbit serum, mouse serum, or with preabsorbed anti-cystatin C antiserum.

Genetic Analysis—Apolipoprotein E (APOE) Genotyping

Genomic DNA was extracted from frozen brain tissue and APOE genotyping was performed essentially as described (24). Polymerase chain reaction (PCR) products (227 bp) were digested for 2 h at 37°C with HhaI (New England Biolabs, Beverly, MA), subjected to electrophoresis on 4% Methaphor (FMC) and photographed under ultraviolet light.

Genetic Analysis—Search for the HCHWA-I Mutation

Genomic DNA was extracted from frozen brain tissue and PCR was performed using the forward 5′ ATC GTA GCT GGG GTG AAC 3′ and reverse 5′ CTT TTC AGA TGT GGC TGG TC 3′ primers. PCR products (113 bp) were digested for 2 h at 37°C with AluI (New England Biolabs), subjected to electrophoresis on 4% Methaphor (FMC) and photographed under ultraviolet light.

RESULTS

Immunolabeling with antibody 4G8 showed Aβ deposits in the neuropil of the cerebral cortex and in leptomeningeal and cortical vessel walls of AD patients and 2 nondemented aged individuals. The amount of Aβ deposits varied extensively between AD patients (Table; Fig. 1B, D). Genetic analysis of the apolipoprotein E alleles (APOE) of 25 of the AD patients revealed a high frequency of APOEϵ4 allele (50%): 4 APOEϵ4 homozygotes, 2 cases with APOEϵ2/ϵ4, 15 APOEϵ3/ϵ4 and only 4 cases with APOEϵ3/ϵ3 (Table). This finding is in agreement with APOEϵ4 being highly associated with late-onset familial and sporadic AD (25, 26). It was suggested that APOEϵ4 is also a risk factor for the development of CAA (27–29). Furthermore, APOEϵ2 allele may have a role in hemorrhage (27) by causing amyloid-laden vessels to undergo vasculopathic changes that lead to rupture (30). However, in the population of AD patients studied here, we did not find an association between a specific APOE genotype and parenchymal or cerebrovascular amyloid load. The number of patients studied and the fact that 21 out of 25 patients had at least 1 APOEϵ4 allele may be the reason for lack of association between APOE allele and amyloid load.

A comparison of serial sections, one immunostained with anti-Aβ antibody 4G8 and the other immunostained with anti-cystatin C antiserum, revealed in all cases that cystatin C immunopositive deposits were less frequent than 4G8 immunopositive ones (Fig. 1A, C). Furthermore, cystatin C immunoreactivity was less intense than Aβ immunoreactivity and varied between patients, as well as between amyloid deposits in the brain of the same patient. Normal rabbit serum and anti-cystatin C antiserum preabsorbed with cystatin C did not stain the amyloid deposits, indicating the specificity of the reaction (data not shown). Genetic analysis of 25 of the AD patients revealed that none of them had the HCHWA-I mutation in the cystatin C gene. Thus, the immunoreactive variability between patients is not due to the presence of this mutation in any of the patients tested.

Immunohistochemistry with end-specific antibodies to the carboxyl-termini of Aβ showed that the vast majority of amyloid deposits in the neuropil was labeled by anti-$A\beta_{x-42}$ antibody, while only a limited proportion was immunoreactive with either anti-$A\beta_{x-40}$ antibody or with both antibodies to $A\beta_{x-40}$ and $A\beta_{x-42}$ (Fig. 2B, C, E, F). The anti-cystatin C antibody decorated some of the $A\beta_{x-42}$ immunoreactive plaques, some of the $A\beta_{x-40}$ immunoreactive plaques and some of the plaques labeled by both antibodies (Fig. 2A, D), without a distinct staining pattern corresponding to one of the anti-Aβ antibodies.

Colocalization of cystatin C and Aβ was also observed in amyloid deposits of 2-yr-old transgenic mice overexpressing human βAPP_{695} cDNA containing the K670N/M671L mutation (Tg(HuAPP695.SWE)2576) (23). These mice have more than 5-fold overexpression of the transgene compared to endogenous βAPP and develop age-related amyloid deposition in the brain. Similar to the staining pattern observed in AD patients, only some of the Aβ deposits were immunoreactive with the anti-cystatin C antibody (Fig. 2G–I).

Immunoelectron microscopy of samples of cerebral cortex from an AD patient showed that the monoclonal antibody 4G8 to Aβ strongly labeled the amyloid fibrils (Fig. 3A). Immunostaining with anti-cystatin C antibody also resulted in decoration of the fibrils (Fig. 3B). Although the intensity of cystatin C immunoreactivity was noticeably weaker than that of Aβ, it was specific since no immunostaining was detected when either the antiserum was preabsorbed with cystatin C (Fig. 3D) or normal rabbit serum was substituted for the primary antibody (not shown). Double immunostaining revealed that some fibrils were exclusively labeled by either the antibody to Aβ or to cystatin C, while other fibrils were decorated by both antibodies (Fig. 3C).

Immunohistochemical analysis of brain sections of AD patients unveiled that the anti-cystatin C antiserum also labeled neurons, astrocytes, and cells within the walls of large leptomeningeal vessels. In particular, strong punctate immunoreactivity within the cytoplasm and cell processes was observed in a subpopulation of pyramidal neurons of the

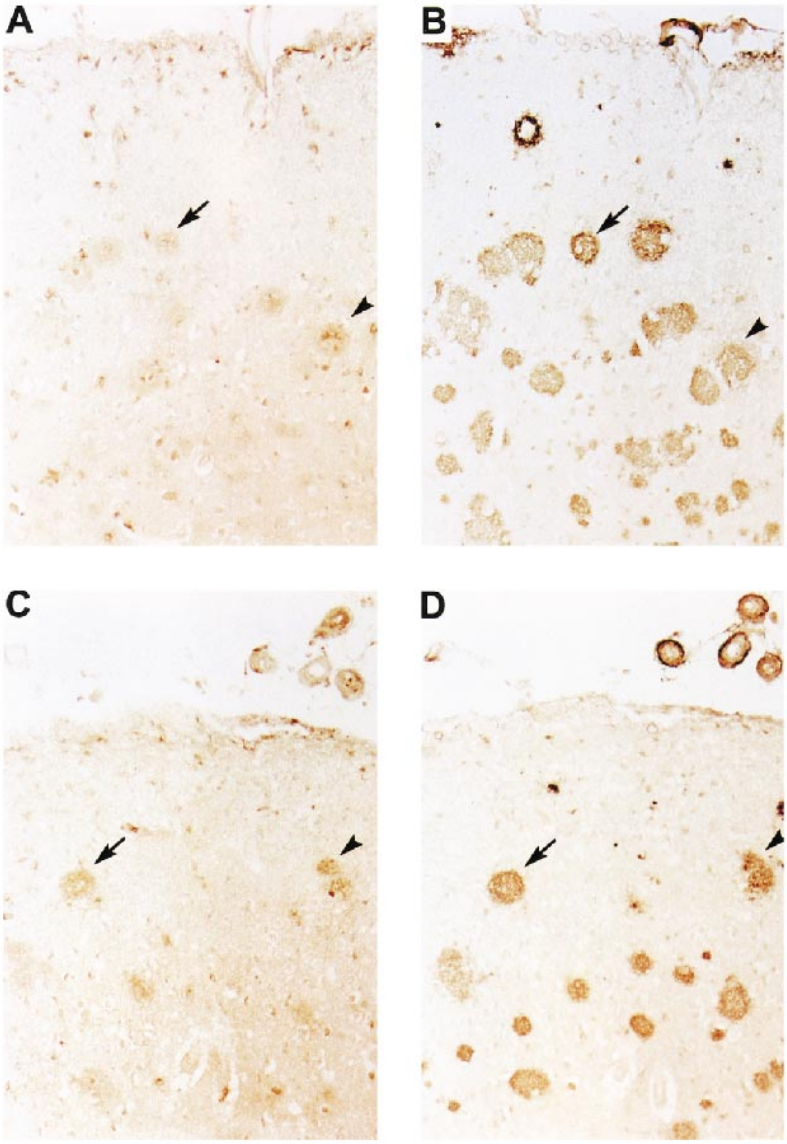

Fig. 1. Immunohistochemical staining of neuropil and cerebrovascular amyloid deposits in the brain of AD patients with the anti-Aβ antibody 4G8 (B, D) and with the anti-cystatin C antibody (A, C). Arrows and arrowheads indicate same plaques stained with both antibodies. Original magnification: ×10.

cerebral cortex. Although these cells were located among amyloid deposits (Fig. 4), their number was not related to the extent of amyloid burden in the individual patient. Exclusively in subjects with high amyloid load, very few neurons were labeled by the anti-$A\beta_{x\text{-}42}$ antibody and showed a punctate staining pattern similar to that of cystatin C (Fig. 4). Conversely, no intracellular immunoreactivity was observed with anti-$A\beta_{x\text{-}40}$. Double immunohistochemistry showed that cystatin C and $A\beta_{x\text{-}42}$ immunostaining colocalized in some cells; however, most cystatin C-positive neurons were not immunoreactive with anti-$A\beta_{x\text{-}42}$ (Fig. 4). To investigate whether cystatin C immunoreactivity occurred in neurons containing hyperphosphorylated tau, the main component of neurofibrillary tangles, brain sections were probed with the antibody AT8. The study showed that neurons immunoreactive with anti-cystatin C antibody were more numerous than those labeled by the anti-tau antibody and exhibited normal morphology (Fig. 5).

Strong immunoreactivity of pyramidal neurons with the anti-cystatin C antibody was also observed in the cerebral cortex of a nondemented aged individual without amyloid deposits (Fig. 6A, D). These pyramidal neurons were located mainly in layers 3 and 4 of the cerebral cortex. The end-specific antibody to the carboxyl-terminus of $A\beta_{x\text{-}42}$ also labeled a subpopulation of neurons in this subject (Fig. 6B, E). The intracortical distribution and staining pattern of the $A\beta_{x\text{-}42}$-positive cells were very similar to those observed in adjacent sections probed with

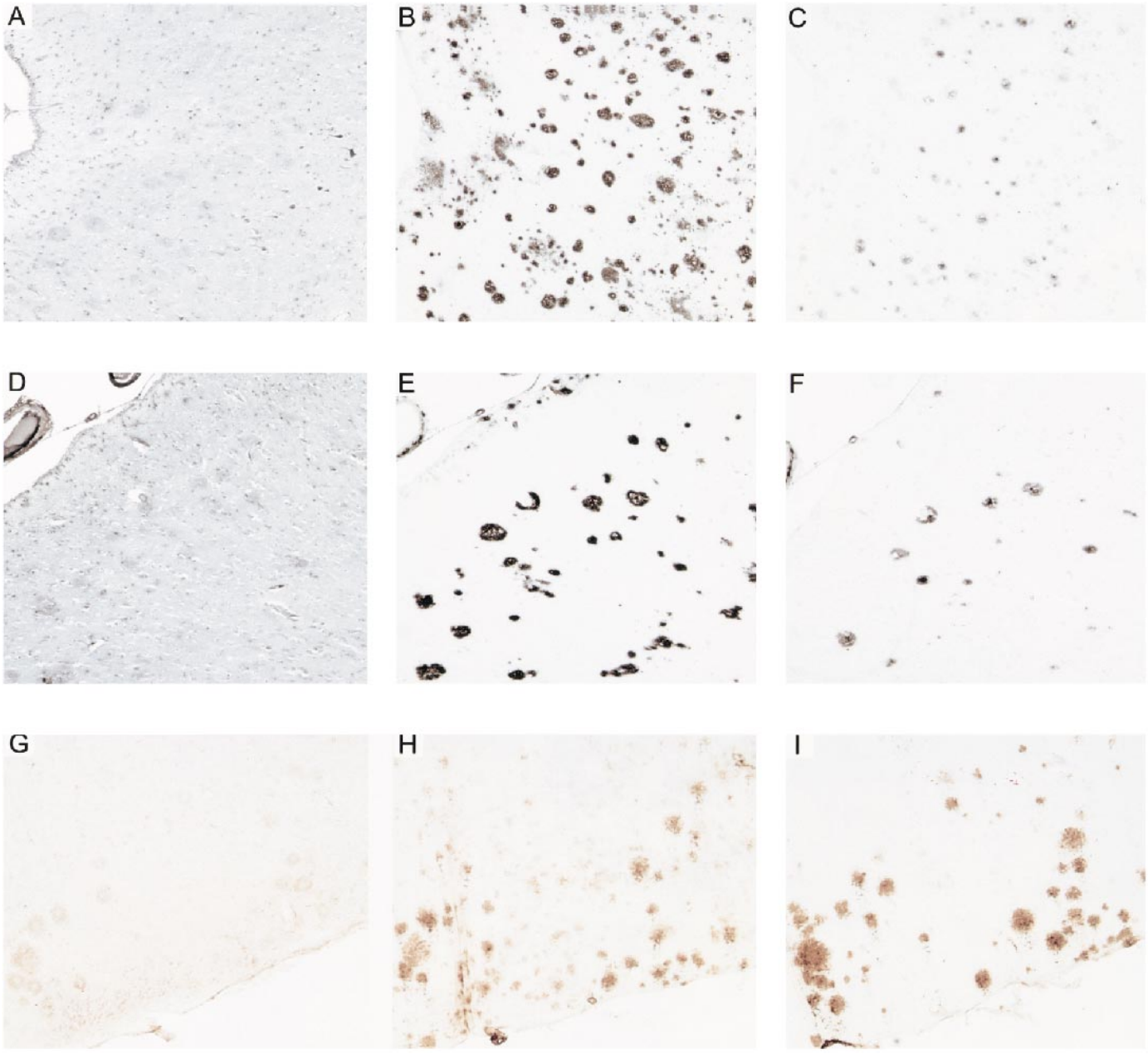

Fig. 2. Immunohistochemical staining of neuropil and cerebrovascular amyloid deposits in the brain of AD patients (A–F) and the brain of a βAPP transgenic mouse (G–I) with anti-cystatin C antibody (A, D, G), with anti-A$\beta_{x\text{-}42}$ antibody (B, E, H) and with anti-A$\beta_{x\text{-}40}$ antibody (C, F, I). Original magnification: ×10.

the anti-cystatin C antibody (Fig. 6A, D). In contrast, no intracellular immunoreactivity was detected with anti-A$\beta_{x\text{-}40}$ antibody (Fig. 6C, F). It is noteworthy that both anti-A$\beta_{x\text{-}40}$ and anti-A$\beta_{x\text{-}42}$ have negligible cross-reactivity with full-length βAPP.

DISCUSSION

Our data indicate that codeposition of cystatin C with Aβ amyloid is a common finding in AD and occurs both in the neuropil and vessel walls. This extends previous observations that cystatin C is often associated with Aβ amyloid in microvessels, but only rarely in senile plaques (20, 21). Furthermore, we found that codeposition of cystatin C with Aβ amyloid also occurs in the brain of nondemented aged individuals. In agreement with earlier studies, cystatin C immunoreactivity was restricted to only some of the Aβ amyloid-laden vessels and was generally weak (20–22). Immunoelectron microscopy demonstrated dual staining of amyloid fibrils with antibodies to cystatin C and to Aβ. Consistent with the light microscopy finding, the labeling with the anti-cystatin C antiserum was less abundant than that with the anti-Aβ

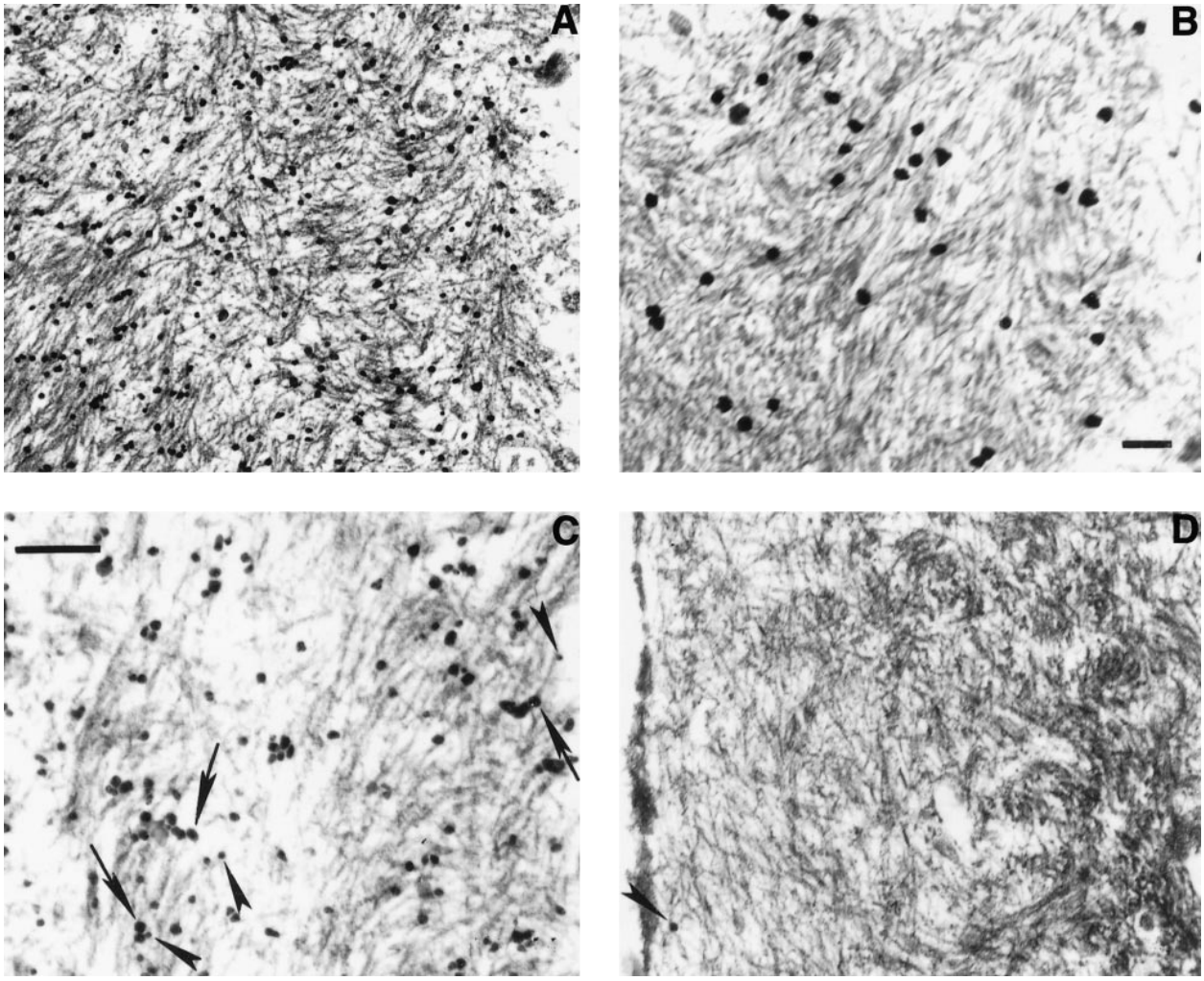

Fig. 3. Immunoelectron microscopy of amyloid fibrils from cerebral cortex of an AD patient with the monoclonal anti-Aβ antibody 4G8 followed by rabbit anti-mouse IgG conjugated with 10 nm gold particles (A, C); with the polyclonal anti-cystatin C antibody and protein A conjugated with 15 nm gold particles (B); with the polyclonal anti-cystatin C antibody (C), and with the polyclonal anti-cystatin C antiserum preabsorbed with cystatin C (D) and goat anti-rabbit IgG conjugated with 1 nm gold particles. Arrows and arrowheads indicate big (anti-Aβ antibody) and small (anti-cystatin C antibody) gold particles respectively (C). Arrowhead in (D) denotes background gold particles. Bars correspond to 200 nm (A, B and D have the same magnification).

antibody. We (EL) have previously shown colocalization of cystatin C with Aβ amyloid deposits in the brain of aged rhesus monkeys and squirrel monkeys (31). We now demonstrate dual staining of amyloid deposits with antibodies to Aβ and to cystatin C also in the brain of transgenic mice overexpressing human βAPP.

A previous study of patients with AD, Down syndrome, intracranial hemorrhage or cerebral infarction, and elderly patients without neurologic disorders, revealed that cerebrovascular amyloid deposits with heavy Aβ immunostaining also reacted with an anti-cystatin C antiserum (22). Patients with heavy amyloid burden suffered a fatal subcortical hemorrhage. Therefore it was advanced that colocalization of cystatin C with Aβ may play a role in the development of cerebral hemorrhages (22). Further studies showed co-occurrence of Aβ and cystatin C in cerebral microvessels of patients with CAA-associated intracerebral hemorrhage (32, 33), AD patients, including probable familial cases (34) and patients with HCHWA-D (35). To determine the significance of CAA as a cause of senile intracranial hemorrhage, Itoh et al (36) analyzed 1,000 brains from normotensive aged subjects (average age 82.9 yr) and found hemorrhages in 101 cases. About 11% of these patients had CAA. All vascular amyloid deposits were Aβ-immunoreactive of which 91% were immunostained by anti-cystatin C antibodies. Based on this observation it was suggested that cystatin C deposition occurs secondarily to Aβ amyloid formation and may play a role in the development of cerebral hemorrhages. Unlike the previous studies, the

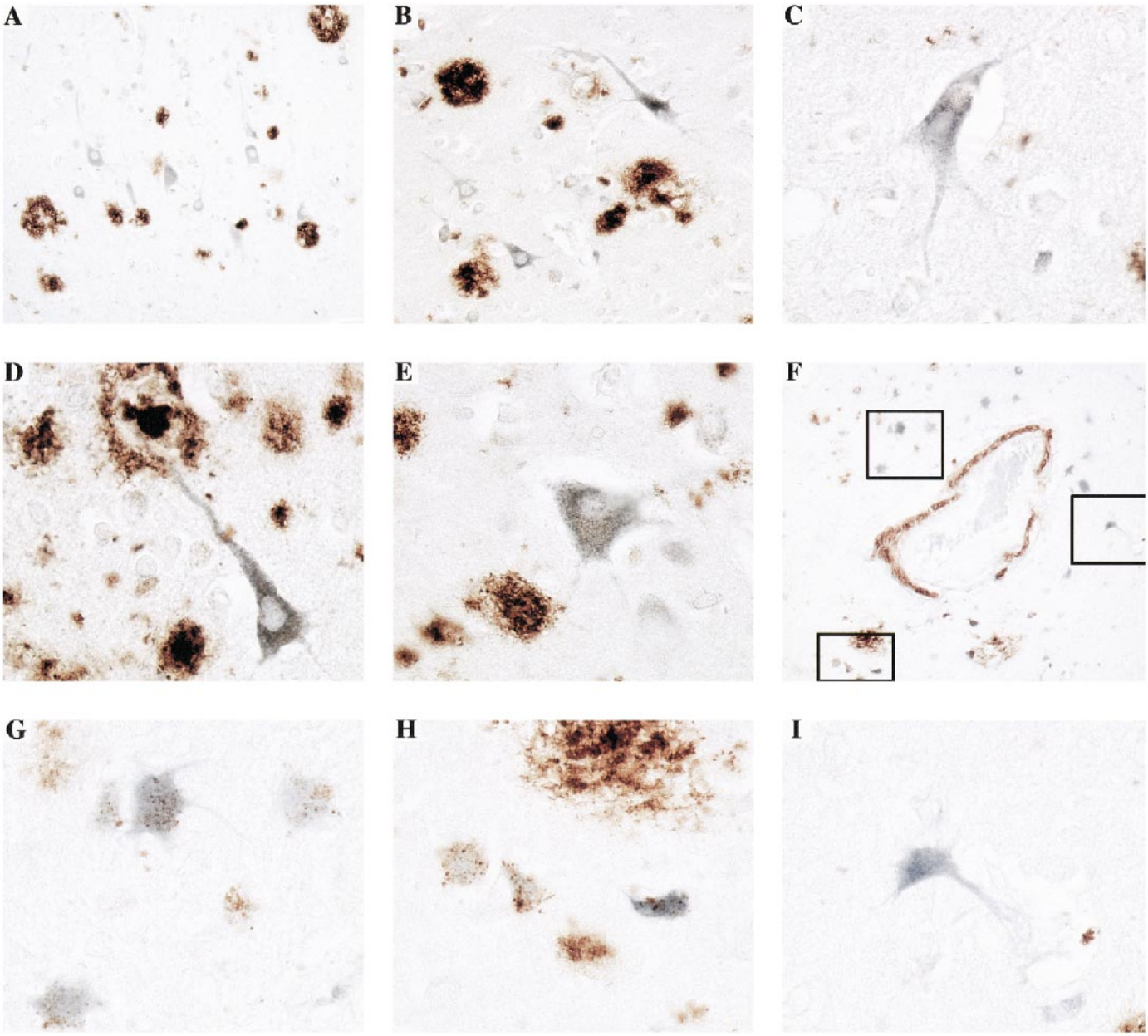

Fig. 4. Dual staining of neurons and amyloid deposits in the brain of AD patients (A–E) and an HCHWA-D patient (F –I) with anti-cystatin C antibody (blue) and with anti-$A\beta_{x-42}$ antibody (brown). High magnification pictures of the regions boxed in F are presented in G–I. Original magnifications: A, F, ×20; B, ×40; C–E and G–H, ×100.

present investigation does not provide evidence of a substantial relationship between cystatin C association to Aβ in vessel walls and occurrence of hemorrhages, because none of the brains analyzed had either signs of old or recent bleedings.

The discrepancy between previous studies and ours might be due to the fact that the effect of cystatin C on cerebral vasculature could depend on the extent of amyloid load. Previous investigations revealed that the difference between mild and severe CAA is in the area of $A\beta_{x-40}$ immunoreactivity per affected vessel rather than in the number of affected cortical vessels (37). This suggests that development of CAA is related to a progressive amyloid deposition in the vessel wall (37). Other studies have shown a statistically significant over-representation of cystatin C-positive vessels, and activated microglia have been found in sections from patients with CAA-related hemorrhage, compared with CAA patients without hemorrhage, AD patients, and controls (38). It is therefore conceivable that the risk of cerebral hemorrhage increases when high level of cystatin C is present in cerebrovascular Aβ amyloid deposits.

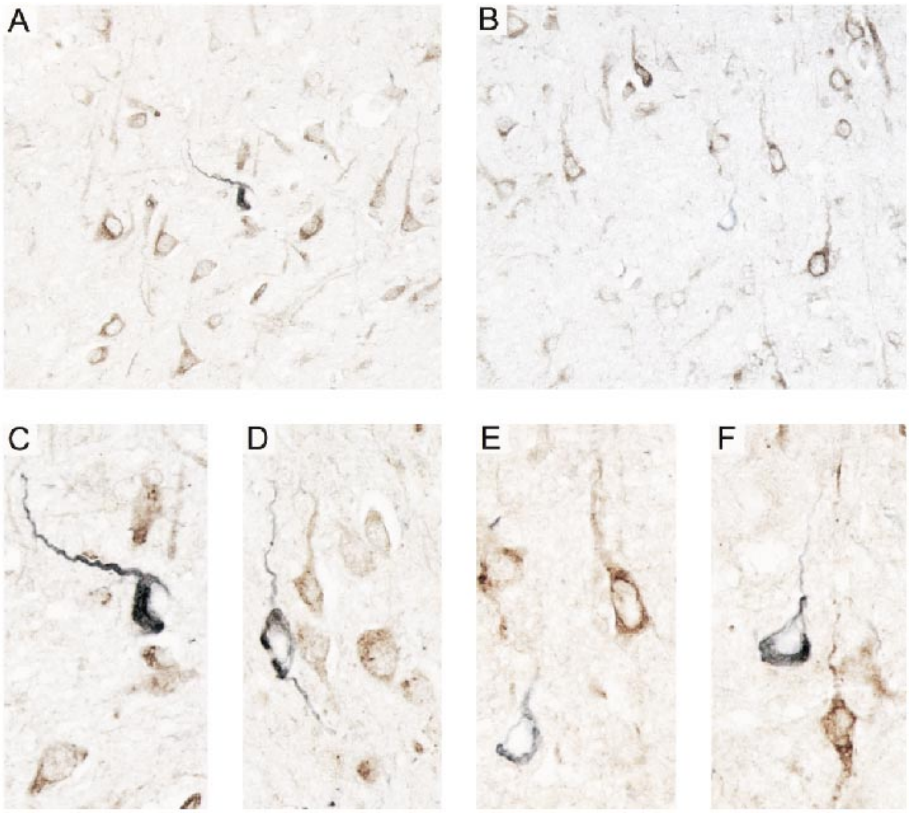

Fig. 5. Dual staining of neurons in the brain of AD patients with anti-cystatin C antibody (brown) and with the antibody AT8 to hyperphosphorylated tau (blue). Original magnifications: A, B, ×40; C–F, ×100.

Immunohistochemistry with anti-cystatin C antibody showed intracellular staining in pyramidal neurons mainly in layers 3 and 4 of the cortex of aged individuals and AD patients. It was previously suggested that cystatin C accumulates in degenerating rat hippocampal neurons (39). However, we found that neurons, which were strongly stained by the anti-cystatin C antibody, had a normal morphology. Furthermore, most of the cystatin C immunoreactive cells did not contain hyperphosphorylated tau, the principal component of neurofibrillary tangles.

Using end-specific antibodies to the carboxyl-terminus of $A\beta_{x\text{-}42}$, we observed intracellular immunoreactivity in a subpopulation of cortical neurons of an aged individual who did not have amyloid deposits. The topographical distribution and staining pattern of these cells were very similar to those observed with the anti-cystatin C antibody. Double immunohistochemistry verified that both proteins colocalized in the cytoplasm and cell processes of the same cells. On the other hand, only a few neurons were immunoreactive with the anti-$A\beta_{x\text{-}42}$ antibody in AD brains, and exclusively in patients with high concentration of amyloid deposits.

It has been recently reported that, in AD, neurons accumulate $A\beta_{x\text{-}42}$ independently from the presence or absence of neurofibrillary tangles (40). The $A\beta_{x\text{-}42}$ immunoreactivity was especially evident within pyramidal cells of the hippocampus and entorhinal cortex, which are prone to develop early AD pathology. Neurons from neurologically normal individuals showed intraneuronal $A\beta_{x\text{-}42}$ staining that appeared to increase with the subject's age. Furthermore, intraneuronal $A\beta_{x\text{-}42}$ immunoreactivity was found to occur with early AD pathology and to become less noticeable with disease progression; consequently it was suggested that $A\beta_{x\text{-}42}$-containing neurons are lost and/or replaced by ghost tangles and/or plaques (40). However, in another study it was demonstrated that $A\beta_{x\text{-}42}$ immunoreactivity was localized to some cells in the brains of AD patients only (41). Our data suggest that $A\beta_{x\text{-}42}$ accumulates in a specific population of pyramidal neurons in the brain, the same cell type in which cystatin C is highly expressed.

In addition to the major amyloid component Aβ, other molecules have been identified in amyloid deposits of AD patients, including α1-antichymotrypsin (42), P-component (43), apolipoprotein E (44), apolipoprotein J (45, 46) and proteoglycans (47). In vitro studies suggest that some of these molecules, such as apolipoprotein E, (44), feature in amyloid fibril formation. Whether the association of cystatin C to Aβ plays a primary role in amyloidogenesis of AD or is a late event in which the protein is bound to the previously formed Aβ amyloid fibrils is unknown.

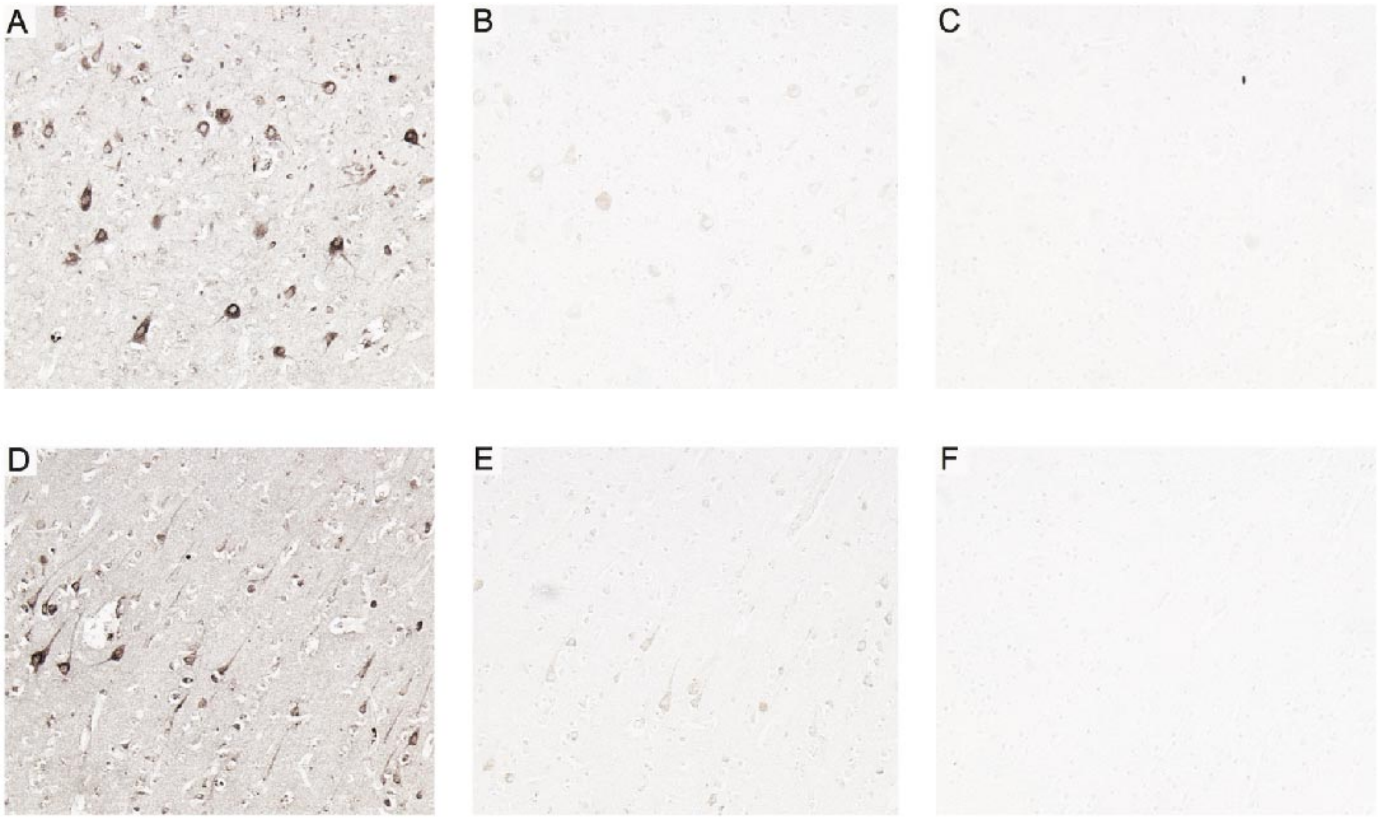

Fig. 6. Immunohistochemical staining of neurons in the brain of a nondemented aged individual with anti-cystatin C antibody (A, D), with anti-A$\beta_{x\text{-}42}$ antibody (B, E), and with anti-A$\beta_{x\text{-}40}$ antibody (D, F). Original magnification: ×20.

Some molecular mechanisms underlying AD may be involved in initiation of the pathologic process, while others, termed progression factors (48), may be associated with amplification of cell stress in an environment created by the pathogenic factors. Progression factors include cytokines (49), complement activation (50–52), and reactive oxygen intermediates (53–56).

In summary, we found that (i) cystatin C codeposits with Aβ amyloid in the neuropil and vessel walls of AD patients and asymptomatic aged individuals, and (ii) a subpopulation of neurons in AD-vulnerable brain regions is strongly immunoreactive with both anti-cystatin C and A$\beta_{x\text{-}42}$ antibodies. This may suggest an early role of cystatin C in local accumulation of the amyloidogenic Aβ peptide. The hypothesis that the association of cystatin C to Aβ plays a primary role in amyloidogenesis of AD is further supported by the observation that cystatin C has the intrinsic ability to form amyloid fibrils. Furthermore, it has been proposed that the interaction between proteases and protease inhibitors may be involved in amyloid formation and/or the pathogenesis of relevant tissue changes (57, 58). It is conceivable that deposition of the protease inhibitor cystatin C might cause an imbalance between proteases and their inhibitors in the vessel walls, contributing to degeneration of cerebral microvessels (59). The demonstration of cystatin C also in parenchymal Aβ amyloid deposits, may suggest a more general involvement of the protease inhibitor in the process leading to cellular degeneration.

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