Immunohistochemical Studies on Canine Cerebral Amyloid Angiopathy and Senile Plaques

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ABSTRACT. Amyloid protein was isolated from the cerebral meninges of 4 aged dogs with cerebral amyloid angiopathy. By immunoblot analysis, antiserum against synthetic oligo-peptide consisting of 1–28 amino acid of amyloid beta protein recognized prominent wide band ranging from 14 to 18 kilodalton (kd). When amyloid samples were solubilized by formic acid, the antiserum recognized lower molecular weight band ranging from 3 to 4 kd. Immunohistochemical studies on cerebral amyloid angiopathy and senile plaques were performed in 17 aged dogs. Anti-amyloid beta protein serum labeled amyloid deposits in cerebral vessel walls and senile plaques. Compact deposits of beta protein were detected in primitive or classical plaques. After using formic acid pretreatment, diffuse deposits of beta protein in the neuropil representing diffuse plaques were detectable. Classical and primitive plaques reacted with antiserum against glial fibrillary acidic protein, while not with antisera against alpha 1-antichymotrypsin, IgG and IgM. Amyloid deposits in the intestines of aged dogs examined, did not react with anti-amyloid beta protein serum.—**KEY WORDS:** aged dog, amyloid angiopathy, senile plaque, β -protein.

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According to biochemical studies on the protein of cerebrovascular and senile plaque amyloid that is common in patients with Alzheimer's disease or aged people [2, 4, 7, 8, 18], the structure of the amyloid protein is twisted beta-pleated sheet fibril (beta protein). The molecular weight of beta protein (4.2 kd) and the amino acid-sequence of the protein were determined by Glenner and Wong [9]. Moreover, the protein of senile plaque core (A4) in Alzheimer's disease and Down syndrome was shown to consist of multimeric aggregates of a peptide about 4 kd [19]. The amino acid composition of molecular mass and NH_2 -terminal sequence of A4 were mostly identical to those described in cerebrovascular amyloid beta protein. Those resulted in that cerebral amyloid angiopathy and senile plaque amyloid in Alzheimer's disease were formed by the aggregation of beta/A4 protein.

In the brain of aged dogs, senile plaques and cerebrovascular amyloid deposition were seen frequently [5, 30–32]. Previously, we showed that these brain lesions appeared with the advance of age and cerebral amyloid angiopathy was closely correlated to cerebral hemorrhage in aged dogs [30, 31]. The immunoreactivity of canine vascular and senile plaque amyloid with anti-beta protein antibodies has been also documented [7, 13, 25, 33]. However, there were few informations concerning biochemical

nature of the amyloid.

In this paper, we examined the immunoreactivity of canine cerebral amyloid angiopathy and senile plaques for antisera against the synthetic peptide of human amyloid beta protein to confirm the findings of previous reports [6, 13, 23, 33]. In addition, the deposition of other various proteins thought to have some relation to senile plaque components [1, 12, 14, 21–23, 26] was also investigated because there were only few reports concerning those in dogs. Furthermore, we isolated and purified the cerebrovascular amyloid from aged dogs and the molecular weight of these amyloid protein was determined by immunoblot analysis.

MATERIALS AND METHODS

Dogs: Autopsy cases of 21 dogs, 17 aged dogs (8 to 15 years old) and 4 young dogs (3 to 5 years old), were examined for histopathological and immunohistochemical studies. For the isolation of cerebrovascular amyloid, the brain of other 5 dogs (7 to 18 years old) were used.

Histopathology: Tissue samples of the brain and intestines were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections of 6–8 μm thick were made and stained with hematoxylin and eosin (HE), alkaline Congo-red [24] and periodic acid methenamine silver (PAM) for the detection of amyloid deposits or senile plaques. Before alkaline Congo-red stain, selected sections

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were treated with potassium permanganate to distinguish AA amyloid from the other types of amyloid protein [34].

Antisera: Immune rabbit serum against a synthetic 1–28 amino acid peptide of beta protein (kindly provided by Dr. Nobuyuki Nukina, Department of Neurology, School of Medicine, the University of Tokyo) was used. Rabbit sera against glial fibrillary acidic protein (GFAP, Dako, Carpinteria, CA, U.S.A.), dog IgG (Bethyl Lab., TX, U.S.A.), dog IgM (The Binding Site Limited, England) and alpha 1-antichymotrypsin (Bio. Genex Laboratories, San Ramon, CA, U.S.A.) were also used as primary antisera. Biotinylated anti-rabbit IgG goat serum was purchased from a commercial laboratory (Kirkegaard & Perry Lab., Gaithersburg, MD, U.S.A.).

Immunostaining: For the detection of beta protein, deparaffinized sections of the brain and intestines were pretreated with 90% formic acid at 37°C for 10 min. [17]. Endogenous peroxidase activity was blocked by the incubation with 0.3% hydrogen peroxidase in methanol for 30 min. Then, the sections with or without formic acid pretreatment were incubated with the primary antisera at 37°C overnight followed by the reaction with biotinylated antiserum and avidin-biotin peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, U.S.A.). Reaction products were formed with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, U.S.A.) and hematoxyline or alkaline Congo-red were used as counter-stains. The same procedure without formic acid treatment was applied to brain sections to detect the other proteins.

Isolation of amyloid protein: Brain samples of 5 dogs, 7 to 18 years old, obtained at autopsy and stored at –80°C, were used for the isolation of amyloid protein. Histological examinations for the frontal cortex were carried out and cerebral amyloid angiopathy was observed in 4 cases (13 to 18 years old) of them. From the meninges of the 5 dogs, amyloid fibril was isolated by the methods of Glenner and Wong [9]. Isolated amyloid fibril was stained with alkaline Congo-red and monitored under polarized light to confirm the characteristic green birefringence of amyloid. For protein extraction, the amyloid fibril was solubilized in 6 M guanidine-HCl at 37°C for 48 hours, centrifuged at 105,000×G, at 4°C for 60 min. (L5-50B ultracentrifuge, SW41Ti rotor, Beckman, CA, U.S.A.), dialyzed in distilled water, and lyophilized. The resulting powder of amyloid protein was stored at

–80°C until use.

Immunoblot analysis: The amyloid protein was incubated in denaturalizing buffer consisting of 1% sodium dodecyl sulfate (SDS), 8M urea and 1% 2-mercaptoethanol in 0.01 M H₃PO₄, pH 6.8, or 90% formic acid, at 37°C, overnight. The samples solubilized in 90% formic acid were centrifuged with vacuum aspiration at 2000 ×G for 24 hours to remove formic acid and the resulting powder was immersed into the denaturalizing buffer. SDS-urea polyacrylamide gel electrophoresis was performed by Lammi system using 20% polyacrylamide gel containing 8M urea. Electrophoretic transfer was done using clear blot membrane-P (Atto, Tokyo, Japan). The immunoblot analysis using anti-beta protein serum was carried out by the same immunostaining procedure described above, except for blocking by 3% bovine serum albumin.

RESULTS

Immunoblot analysis: The result of immunoblot analysis for the amyloid protein isolated from cerebro-meningeal amyloid, was shown in Fig. 1. Anti-beta protein serum recognized a prominent wide band ranging from 14 to 18 kd of molecular weight on the lanes of 4 samples from aged dogs with amyloid angiopathy (Fig. 1, Lanes 2, 4, 6, and 8). In addition, there were two distinct higher molecular weight bands at about 100 kd and 35 kd on the lanes of most samples including the case without cerebro-vascular amyloid deposition (Fig. 1, Lane 1). When the samples were solubilized by formic acid, the antiserum intensely labeled much lower molecular weight band ranging from 3 to 4 kd on the lanes from 3 aged dogs (Fig. 1, Lanes 5, 7, and 9).

Histopathology: Among 17 aged dogs examined, amyloid angiopathy and senile plaques were detected in 10 and 8 dogs, respectively. Both lesions were observed in 6 of them (Table 1). The wall of cerebral or cerebellar meningeal arterioles and capillaries of cerebral cortex exhibited amyloid deposition (Figs. 2 and 3).

Brain sections stained with PAM revealed 3 types of senile plaques, i.e., classical, primitive, and diffuse plaques. Classical plaques had well-defined amyloid core and neuritic halo, primitive plaques consisted of slight amyloid deposits and neuritic elements, and diffuse plaques were characterized by diffuse accumulation of PAM-positive fibrous sub-

Fig. 1. Immunoblot analysis of cerebro-meningeal amyloid protein isolated from meninges of 5 dogs using beta protein antiserum. Lane 1: sample from a dog without amyloid angiopathy, 7 years old. Lanes 2–10: amyloid protein isolated from aged dogs, 13 (lanes 2 and 3), 14 (lanes 4 and 5), 16 (lanes 6 and 7), and 18 (lane 8, 9, and 10) years old. Lanes 3, 5, 7 and 9; samples were solubilized by formic acid. Lane 10; Normal rabbit serum was employed as a primary antiserum. MW; Molecular weight marker.

stances. Primitive plaques were detected in 2 dogs, and in one of those dogs, classical plaques coexisted (Figs. 4 and 5). Diffuse plaques were observed in the cerebral cortex of 8 dogs (Figs. 6a and 6b). The cases with primitive or classical plaques always exhibited cerebral amyloid angiopathy, while diffuse plaques were seen even in the dogs without vascular amyloid deposition (Table 1). On the other hand, in 4 aged dogs, only cerebral amyloid angiopathy was noted without senile plaques (Table 1, group C). In the brain of young dogs examined as controls, neither amyloid angiopathy nor senile plaques was detected.

In the intestines of aged 10 dogs with cerebral amyloid angiopathy and/or senile plaques, the deposition of amyloid resistant to the potassium permanganate oxidation was found in or around vessel walls of the submucosal layer (Figs. 7a and 7b).

Immunohistochemistry: The immunoreactivity of cerebrovascular amyloid or senile plaques with anti-beta protein serum using formic acid pretreatment was summarized in Table 1. Amyloid in the cerebro-vascular walls and senile plaques were

detected more intensely by immunostaining for beta protein as compared with PAM or Congo-red stain. Amyloid deposits in the wall of cerebro-meningeal arterioles and capillaries of the cerebral cortex reacted intensely with anti-beta protein serum (Figs. 8 and 9). Perivascular beta protein -deposition indistinguishable from senile plaque was often observed around the capillaries (Fig. 9). In 3 dogs which did not show amyloid angiopathy in the histological examination using Congo-red stain, slight beta-protein deposition was found in the wall of capillaries (Table 1).

Both classical and primitive plaques reacted with anti-beta protein serum without formic acid treatment (Figs. 10 and 11). Granular or rod-like beta protein deposition in primitive plaques (Fig. 10) and massive accumulation of the protein in the core and corona of classical plaques were observed (Fig. 11). These immunoreactivity was enhanced by formic acid pretreatment. Moreover, after formic acid pretreatment, anti-beta protein serum labeled diffuse deposits of the protein representing diffuse plaques in the neuropil. Such diffuse deposits of the protein were observed in 2 dogs which did not

Table 1. Results of Congo-red and PAM stains for senile plaques and amyloid angiopathy, and their immunoreactivity with anti-beta protein serum

Group*	Age	Congo-red and PAM				Immunostaining		
		SP			CAA	Beta protein-deposition		
		Diffuse	Primitive	Classical		Diffuse	Focal	Vascular
A	3y	—	—	—	—	—	—	—
	3y	—	—	—	—	—	—	—
	3y	—	—	—	—	—	—	—
	5y	—	—	—	—	—	—	+
	12y	—	—	—	—	—	—	+
	13y	—	—	—	—	—	—	—
	13y	—	—	—	—	—	—	+
	14y	—	—	—	—	—	—	—
B	8y	+	—	—	—	+	—	—
	14y	+	—	—	—	+	—	—
C	10y	—	—	—	+	+	—	+
	13y	—	—	—	+	—	—	+
	13y	—	—	—	+	+	—	+
	14y	—	—	—	+	—	—	+
D	13y	+	—	—	+	+	—	+
	13y	+	—	—	+	+	+	+
	13y	+	—	—	+	+	—	+
	14y	+	—	—	+	+	—	+
	15y	+	+	+	+	+	+	+
	16y	+	+	—	+	+	+	+

*Group: A, dogs without CAA and SP; B, dogs with only SP; C, dogs with only CAA; and D, dogs with both CAA and SP by histological examination.
CAA: Cerebral amyloid angiopathy, SP: Senile plaque.

Fig. 2. Amyloid deposition (arrow heads) in the wall of cerebro-meningeal arterioles. Congo-red stain. × 80.

Fig. 3. Amyloid deposition in or around capillary walls of the cerebral cortex (arrows). Congo-red. × 330.

exhibited diffuse plaques in the histological investigation with PAM stain (Table 1). Some of the diffuse plaques were in close contact with capillaries (Fig. 12a) and contained one or several nerve cells (Fig. 12b). Small amount of beta protein deposition (Fig. 13a) or dendriform deposition (Fig. 13b) around some nerve cells were occasionally recog-

nized. Sometimes, the cell membrane of nerve cells reacted feebly with anti-beta protein serum but the cytoplasm never reacted with the antiserum.

Besides beta protein, some classical and primitive plaques showed positive reaction with the antibody against GFAP (Fig. 14), but diffuse plaques and cerebro-vascular amyloid did not. Alpha 1-

Fig. 4. A primitive plaque in the cerebral cortex. PAM. $\times 210$.

Fig. 5. Two classical plaques in the cerebral cortex. PAM. $\times 210$.

Fig. 6. (a): Many diffuse plaques in the cerebral cortex. PAM. $\times 110$, (b): Higher magnification of a diffuse plaque. PAM. $\times 210$.

Fig. 7. (a): Amyloid deposition around the vessels of intestinal submucosa (arrows). Congo-red. $\times 80$. (b): Higher magnification of amyloid deposition around the vessel. Congo-red $\times 160$.

Fig. 8. Beta protein deposition in the wall of cerebro-meningeal arterioles. Immunostain. $\times 110$.

Fig. 9. Many capillaries reacted with anti-beta protein serum. Plaque like deposition of beta protein in or around capillary walls (arrows). Immunostain. $\times 210$.

Fig. 10. The immunoreactivity of a primitive plaque with anti-beta protein serum. Immunostain. $\times 330$.

Fig. 11. The core and corona of a classical plaque reacted intensely with anti-beta protein serum. Immunostain. $\times 330$.

Fig. 12. Diffuse deposition of beta protein contact with capillaries (a) and including a nerve cell (b). Immunostain after formic acid pretreatment. $\times 330$.

Fig. 13. Slight deposition of beta protein around the cell membrane of a nerve cell (a), and dendriform deposition of the protein around several nerve cells (b). Immunostain after formic acid pretreatment. $\times 330$.

Fig. 14. A classical plaque positive for GFAP. The core of the plaque showed intense immunoreactivity. Immunostain. $\times 330$.

antichymotrypsin, IgG, and IgM could not be detected in senile plaques and vascular amyloid. Immunoreactivity of cerebro-vascular amyloid, senile plaques and nerve or glial cells with the antibodies examined was summarized in Table 2.

Amyloid observed in the intestinal submucosa of aged dogs was not reactive for anti-beta protein serum, while the cytoplasm of nerve cells in the nerve plexuses of submucosal and muscle layers of the intestines weakly or negligibly reacted.

DISCUSSION

Several investigators documented immunohistochemical studies using anti-beta protein antibodies

Table 2. Immunoreactivity of vascular amyloid, senile plaques and brain cells with the antibodies used in this study

Lesion cell* component	Primary antibody against				
	Beta protein	GFAP	Alphal-antichymotrypsin	IgG	IgM
CAA	+	—	—	—	—
DP	+	—	—	—	—
PP	+	—	—	—	—
CP	+	+	—	—	—
NC	++	—	—	—	—
AAC	—	+	—	—	—

Lesion/ cell component*: CAA, cerebral amyloid angiopathy; DP, diffuse plaque; PP, primitive plaque; CP, Classical plaque; NC, nerve cell; and AAC, activated astrocytes.

**: only around the cell membrane or dendrites, not in cytoplasm.

on canine cerebral amyloid angiopathy and senile plaques [7, 13, 25, 33]. Wisniewski *et al.* [33] investigated the brain of aged dogs using monoclonal antibodies against two distinct epitopes of beta protein and found large diffuse beta protein deposition resembling diffuse plaque observed in man [16, 35] and immunoreactivity of those antibodies with primitive plaques or vascular amyloid as well. Giaccone *et al.* [6] also showed diffuse or preamyloid deposition in the cerebral cortex of aged dogs. Our immunohistochemical findings in this study are almost in conformity with those in the previous observations [6, 33]. Recently, Ishihara *et al.* [13] examined the brain of aged dogs immunoelectron microscopically and showed the localization of beta-protein in the cerebral amyloid fibrils. These previous reports [6, 13, 33] including our present study suggest that beta protein is the major component of all types of senile plaques and cerebrovascular amyloid in aged dogs.

In our immunohistochemical studies, diffuse or vascular beta protein-deposition was found even in the cases which did not exhibit diffuse plaques or cerebral amyloid angiopathy in the histological examination with PAM or Congo-red stain (Table 1, group A and C). These results might be attributable to the difference in the detecting capability of the staining specificity between immunohistochemistry and a special histostaining such as PAM or Congo-red. Namely, Congo-red stain can detect only amyloid fibrils but not beta protein and beta protein immunostaining using formic acid pretreatment is thought to be more sensitive for the detection of the protein as compared with PAM.

Although constituents other than beta protein of senile plaques or vascular amyloid had been known

in man [1, 12, 14, 22, 26], we could not detect such elements except for GFAP. However, the plaques with deposition of GFAP were limited to only a few plaques and diffuse plaques supposed to be the initial form of senile plaques [16, 35] did not react with anti-GFAP serum. These might indicate that the deposition of GFAP was a secondary event on the development of senile plaques.

Concerning the origin of beta protein or its precursors, it has been described that the protein might come from the brain cells [3, 10, 27]. In aged dogs, small quantity of beta protein often accumulated around the cell membrane of nerve cells, and in addition, several nerve cells were frequently involved in diffuse plaques. These findings may support the hypothesis that nerve cell will be the primary site of beta protein or its precursor production. On the other hand, in the development of senile plaques, the involvement of cerebro-cortical capillaries can not be ruled out, for close contact of both structures observed in the present study. This finding may also suggest that beta protein in the neuropil arises through cerebral capillaries as indicated in the human cases [28]. Moreover, senile plaques and vascular amyloid deposition did not always coexist (Table 1, Group B, and C). Some cases exhibited only diffuse plaques, and some had only vascular amyloid or beta protein-deposition. These findings might show the possibility that the pathogenesis of senile plaques consisting of beta protein is different from that of vascular amyloid deposition.

By immunoblot analysis, anti-beta protein serum labeled two different molecular weight bands ranging from 14 to 18 kd and from 3 to 4 kd. As the lower molecular weight band appeared by solubilization of

the samples with formic acid which is known to denature or solubilize amyloid protein [19], molecular weight of monomer form of cerebro-vascular amyloid protein isolated from aged dogs might be 3 or 4 kd which is similar to that of human beta protein. The higher molecular weight band ranging from 14 to 18 kd, might show multimeric aggregate, tetramer, formed due to incomplete solubilization. Similar results of electrophoresis were shown in the analysis of amyloid core protein from patients with Alzheimer's disease [19] and the aggregation of the protein was supposed to be a pH-dependent phenomenon. To discuss the natures of cerebro-vascular amyloid of aged dogs, however, further biochemical studies including details of immunoblot analysis will be required.

Beta protein deposition has been observed in the skin and intestines of patients with Alzheimer's disease or aged people [15] and mRNA encoding amyloid beta protein or its precursor was also known to be expressed in wide variety of organs [11, 20, 29]. In aged dogs with cerebral amyloid angiopathy, intestinal amyloid deposition was observed [31], but it did not react with anti-beta protein serum in this study. This fact shows that intestinal amyloid in aged dogs might consist of other protein than beta protein. These findings also indicate that although both of intestinal and cerebral amyloid depositions appear with the advance of age, those amyloidogeneses might be differ from each other.

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