

Double-Labeling Immunohistochemical Studies on Canine Senile Plaques and Cerebral Amyloid Angiopathy

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ABSTRACT. The relationship of senile plaques to neuronal cells, neurites, glial cells, or capillaries was examined using double labeling-immunostaining methods on the Bouin's solution-fixed serial brain sections from dogs. Compact deposits of beta protein (amyloid plaques) in the cerebral cortex always contained microvessels labeled by anti-collagen type IV antibody and some of them might be formed as the result of fusion of several perivascular beta amyloid deposits. In the periphery of those plaques swollen neurites recognized with anti-neurofilament antibody were sometimes present, but the relation between such plaques and neuronal cells or glial cells were unclear. Diffuse deposition of beta protein (diffuse plaques) was frequently developed beside neuronal cells, while most plaques did not contain glial cells. Some of those plaques were closely contact with microvessels, but some had no relation. Intact or irregularly arranged neurites were present in diffuse plaques. Such irregularity of the neurites were obvious in the plaques in the hippocampus as compared with those in the cerebral cortex. These results indicate the possibility that canine amyloid plaques would be formed as the result of amyloid degeneration of cortical capillaries, and diffuse parenchymal deposition of beta protein would originate from neuronal or neuritic processes.—**KEY WORDS:** amyloid angiopathy, beta protein, canine, double-immunostaining, senile plaque.

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Senile plaques and amyloid angiopathy appear frequently in the brain of aged dogs [3, 18, 19, 25]. Several investigators [5, 6, 20, 24] have revealed that these brain lesions were formed by the aggregation of the beta protein like those in the patients with Alzheimer's disease or aged people. Canine senile plaques could be differentiated histopathologically into several subtypes. Diffuse plaques suggested as initial form of senile plaques in human cases [25] were visualized by periodic acid-methenamine (PAM) or modified Bielschowsky silver and beta protein immunostaining with formic acid pretreatment [5, 18-20, 24]. Another type of senile plaques (amyloid plaques) mimicking human primitive or classical plaques were defined by the presence of amyloid deposits and degenerative neurites [23].

Although there were many reports describing histological or immunohistochemical features of canine senile plaques, their histopathogenesis was not well elucidated. Recently, Shimada *et al.* [17] examined the brains of aged dogs with serial paraffin sections histopathologically or immunohistochemically and indicated that canine diffuse plaques originate from neuronal or glial cells and amyloid plaques (called as matured or perivascular plaques) might be related to cerebral amyloid angiopathy.

In human cases double or triple-labeling immunostain techniques have been employed to know the histogenesis of senile plaques [2, 4, 7-9], while such examinations have not been performed in canine cases. In the present study, we investigated the relationship between canine senile plaques and neuronal cells, neurites, glial cells or capillaries using double-labeling immunostaining on serial brain sections of dogs and discuss the histogenesis of canine senile plaques.

MATERIALS AND METHODS

Dog(s): Three autopsies cases of dogs were examined. The first dog (case No. 1), 8 year-old, female, suffered from mammary gland adenocarcinoma with systemic metastases. The second (case No. 2), 15 year-old, female, showed dirofilariasis. The third (case No. 3), 16 year-old, male, was involved in liver cirrhosis and chronic nephritis. Bouin's solution-fixed brain sections which obtained from these dogs, were employed in this study, because we previously showed the paraffin sections fixed with the fixative were useful to detect diffuse plaques immunohistochemically [21].

Histopathology: The brain sections of 8 μ m thick were stained with hematoxyline and eosin, alkaline Congo-red, and PAM for the detection of amyloid and senile plaques.

Antisera: As primary antisera, immunized rabbit serum against a synthetic 1-28 amino acid peptide of beta protein, kindly provided from Dr. Nobuyuki Nukina, Department of Neurology, School of Medicine, the University of Tokyo, monoclonal antibody (MAb) against rat type IV collagen (1:50, Dako, Carpinteria, CA, U.S.A.), MAb against high and low molecular weight of neurofilament (NF) protein (1:50, Dako, Carpinteria, CA, U.S.A.) and MAb against human glial fibrillary acidic protein (GFAP; 1:50, Dako, Carpinteria, CA, U.S.A.) were used. For secondary antibodies of avidin and biotin peroxidase complex (ABC) methods, biotinylated anti-rabbit IgG goat serum and biotinylated anti-mouse IgG goat serum were employed.

Immunostaining: Single-labeling immunohistochemical studies were attempted by ABC methods to know the immunoreactivity of canine brain tissues with the primary

antibodies examined in the present study.

Double-labeling immunostaining: Double-labeling immunostaining was performed using an universal Dako double-stain kit, system 40 (Dako, Carpinteria, CA, U.S.A.). The serial brain sections pretreated with 0.1% trypsin were immersed in 3% hydrogen peroxide for 5 min to inhibit endogenous peroxidase activity. The sections were also incubated with normal swine serum for the blocking of non-specific reactions. Then, the sections were reacted with anti-beta protein rabbit serum, and MABs for collagen type IV, NF, or GFAP at 4°C, overnight. As negative control, normal rabbit and mouse sera were employed, though significant immunoreactivities were not detected. After the incubation with the mixture of goat antiserum to mouse immunoglobulins and swine antiserum to rabbit immunoglobulins, the sections were reacted with provided alkaline phosphatase anti-alkaline phosphatase complex. Then, the sections were also incubated with horseradish peroxidase anti-horseradish peroxidase complex. The alkaline phosphatase activity was developed with fast red chromogen and peroxidase activity was visualized with diaminobenzidine. As counterstain Mayer's hematoxylin was employed.

RESULTS

Histopathology: In the brain of case No. 1, diffuse plaques were distributed in the cerebral cortex and hippocampus, but amyloid deposition was not detected both in vessels and brain parenchyma. On the other hands, diffuse plaques, amyloid plaques containing swollen neurite-like structures, and amyloid deposition in arteriolar or capillary walls were observed in the brain of case No. 3. The brain of case No. 2 exhibited only amyloid angiopathy.

Single-labeling immunostaining: The immunoreactivity of amyloid deposits in the vessels and senile plaques of the canine brains with anti-beta protein antisera were reported previously [20]. Amyloid plaques appeared as compact deposits of beta protein in the brain parenchyma. Diffuse deposits of the protein representing diffuse plaques were also detected without formic acid pretreatment. The distribution of beta protein in the brain of the dogs examined was summarized in Table 1. Anti-collagen type IV MAB labeled intensely the basement membrane of blood vessels in the brain. NF-immunostaining recognized the neurites and some axons and GFAP-immunostaining labeled glial fibers and the cytoplasm of glial cells which showed the morphological characteristics of astrocytes.

Double immunostaining

1) Cerebral amyloid angiopathy: Serial sections of cerebral cortex double-stained for beta protein and collagen type IV obviously revealed the topographical relationship between vessels and vascular or perivascular beta amyloid. In the arterioles, beta protein deposits were localized within the media tunica (Fig. 1), but beta protein deposited around basement membrane of capillaries

Table 1. Distribution of beta protein deposits in dogs examined

Case No.	Age (years)	Region	Beta protein deposits		
			vascular	diffuse	compact
1	8	Cerebral cortex	- ^{a)}	+ ^{b)}	-
		Hippocampus	-	+	-
2	15	Cerebral cortex	+	-	-
		Hippocampus	+	-	-
3	16	Cerebral cortex	+	+	+
		Hippocampus	+	-	-

a) -: Not detected; b) +: Detected.



Fig. 1. Beta protein deposition (arrows) observed in the tunica media of meningeal arterioles. The basement membrane of the vessels stained with fast red (arrow head). Double-immunostaining with beta protein and collagen type IV. $\times 125$. Case No. 2.

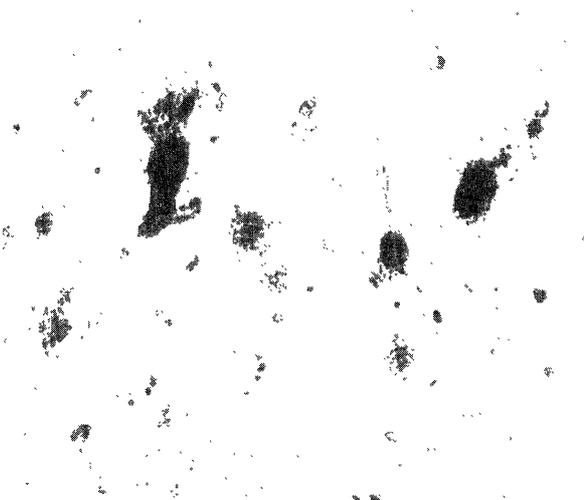


Fig. 2. Beta protein deposition in or around the wall of cerebral capillaries. Double-immunostaining of beta protein and collagen type IV. $\times 125$. Case No. 2.

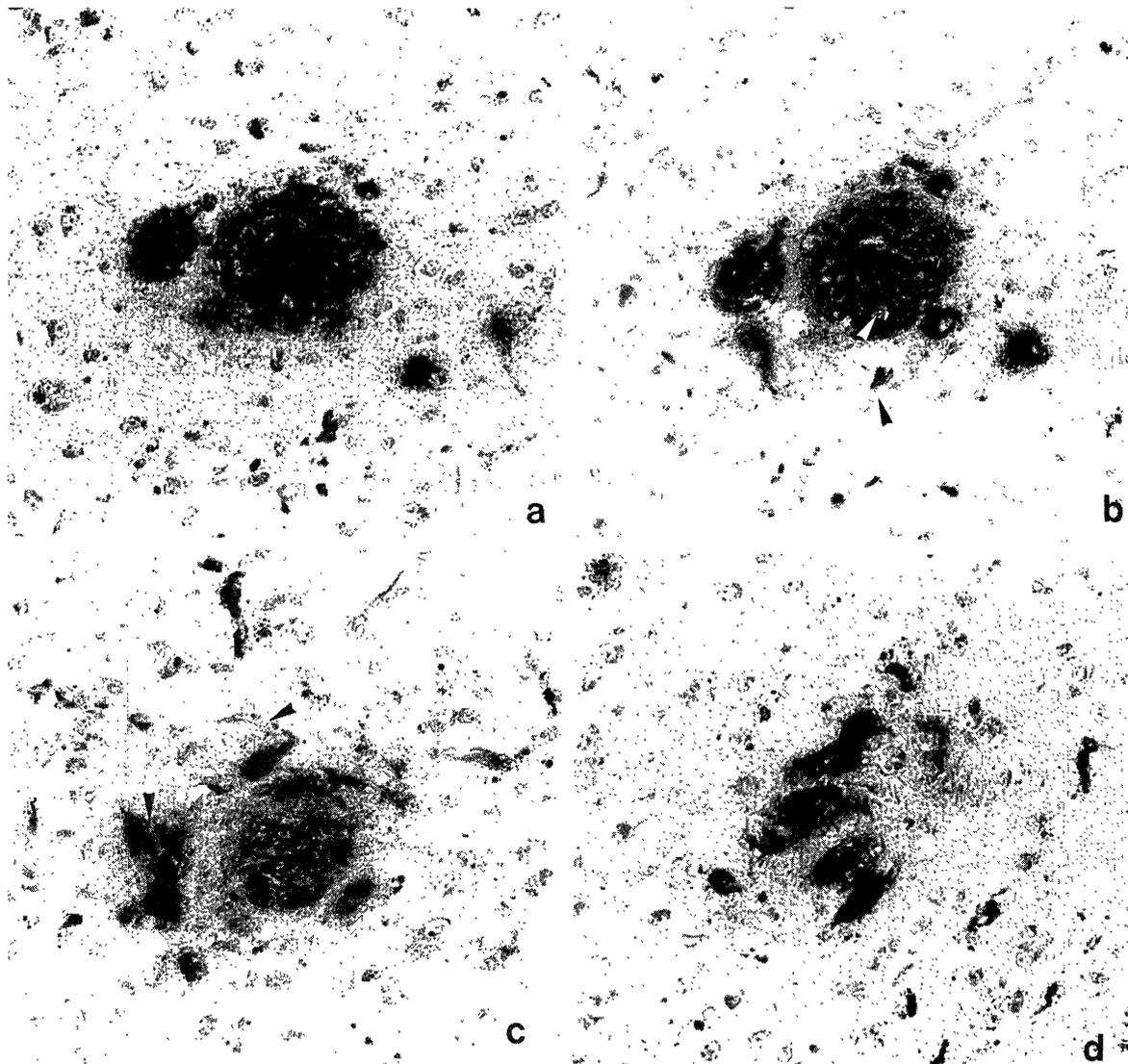


Fig. 3. Serial sections of amyloid plaques in the cerebral cortex (a to d). The plaque contained several capillaries (arrow heads) and is appeared to be made by the fusion of beta protein deposited around 4 or 5 numbers of capillaries (d). Double-immunostaining of beta protein and collagen type IV. $\times 125$. Case No. 3.

distributed in the surrounding cortical parenchyma (Fig. 2).

2) Amyloid plaques: The double-immunostaining also revealed the connection between compact beta protein deposition (amyloid plaques) and capillaries. The amyloid plaques frequently contained capillaries. Perivascular beta protein deposits appeared to combine each other resulting in formation of plaque-like lesions which were indistinguishable from amyloid plaque (Figs. 3a, 3b, 3c and 3d). Around these plaques, a small number of GFAP positive cells thought to be astrocytes and some neuronal cell were occasionally present, but the relation between amyloid plaques and these brain cells was not clear. At the periphery of the amyloid plaques, NF-positive swollen neurites were sometimes present.

3) Diffuse plaques: Some of diffuse beta protein deposits (diffuse plaques) in the cerebral cortex and

hippocampus showed close contact with capillaries (Fig. 4a), but some of them appeared to have no connection with capillaries (Fig. 4b). By contrast, many diffuse plaques in the cerebral cortex were formed beside one or two neuronal cells (Fig. 4b), though in the hippocampus, such close-relationship between diffuse plaques and neuronal cells was not obvious. However, almost all diffuse plaques both in the cerebral cortex and hippocampus contained intact or slightly swollen neurites recognized by anti-NF MAb. Moreover, in the hippocampus irregularly arranged neurites were observed in or around diffuse plaques (Fig. 5). Close relationship between GFAP-positive cells and diffuse plaques was not detected, although one or only a few GFAP-positive cells were occasionally present inside of some diffuse plaques.

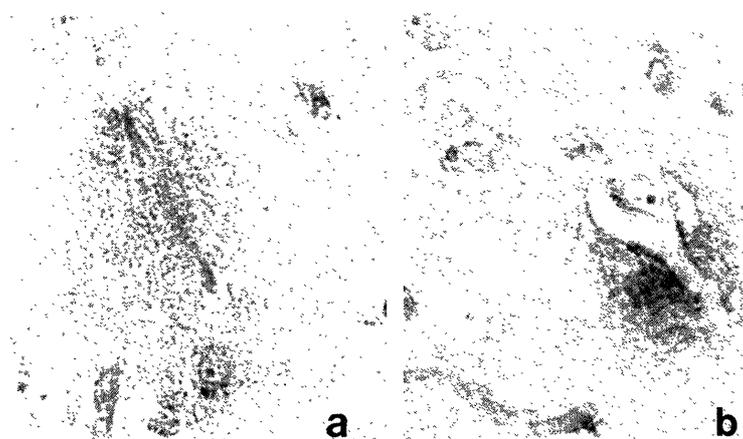


Fig. 4. Diffuse beta protein deposition around a capillary (a) and that contacts only to a neuron (b). Double-immunostaining of beta protein and collages type IV. $\times 320$. Case No. 3.

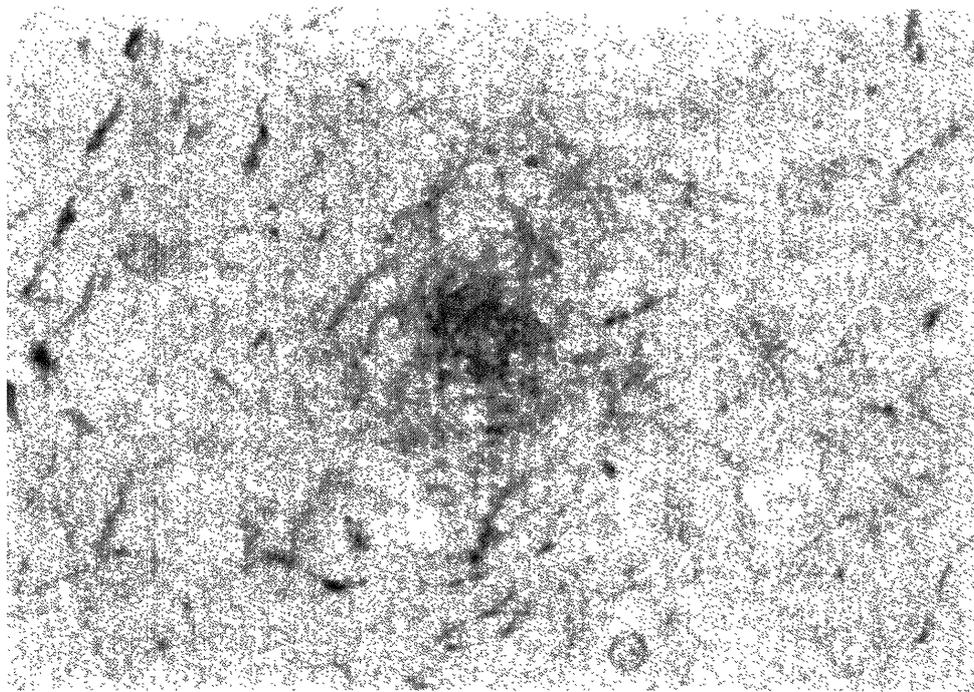


Fig. 5. Diffuse plaque in the stratum moleculare of hippocampus. Irregularly arranged neurites are present in or around the plaque. Double-immunostaining of beta protein and NF. $\times 320$. Case No. 1.

DISCUSSION

In the present study, we showed the close relationship between canine amyloid plaques and capillaries. Amyloid plaques contained several capillaries. Moreover, perivascular amyloid deposits often made plaque-like lesions which might be caused by their fusion. These findings suggest that canine amyloid plaques are developed as a result of amyloid degeneration of cortical capillaries. Recently, Shimada *et al.* [17] investigated the topographical relationship between canine senile plaques and cerebral vessels or brain cells and also emphasized the

importance of amyloid angiopathy on the formation of canine amyloid plaques.

In human cases, amyloid plaques named as primitive (neuritic) and classical (matured) plaques, which are commonly seen in the brain of patients with Alzheimer's disease, were thought to be formed by the development of diffuse plaques [25]. However, the brain of patients with amyloid angiopathy also exhibited amyloid plaque-like lesions called as "Drusige Entartung" [16]. These lesions were considered to appear as a result of amyloid degeneration of capillaries and the invasion of amyloid deposited around the vessels to the cortical parenchyma.

Although histological or immunohistochemical similarity of canine amyloid plaques to human primitive or classical plaques has been mentioned [18, 19, 23, 24], the histopathogenesis of canine amyloid plaque could be in conformity with that of plaque-like lesions originating from cerebral amyloid angiopathy. There may be possibility that human primitive or classical plaques are also related to amyloid angiopathy or originate from cerebral vessels [10, 11], but recent several reports showed negative correlation between these plaques and cerebral vessels [8, 15].

Many investigators' interests are focused on the pathogenesis of diffuse plaques because those have been supposed as the initial lesion of cerebral parenchymal beta protein-deposition [25]. Pappolla *et al.* [12] examined the distribution patterns of beta protein in serial sections from brains of patients with Alzheimer's disease and non-demented aged people using image analysis microscopy. They showed 4 patterns of beta protein deposition and indicated that neurons were related to the formation of a subset of diffuse plaque (called as "early primitive plaques"). Moreover, recent reports concerning beta protein deposition in the brain of transgenic mice [14, 22] support the interpretation that production of the protein commences in neurons in our study, canine diffuse plaques also seemed to be related most closely to neuronal cells. This finding may indicate an important role of the neuronal cells during diffuse plaque formation. Occasionally, capillaries and glial cells were contained within canine diffuse plaques, but relationships of these elements to the plaques were not clear.

Moreover, in the hippocampus irregularly arranged neurites were seen in or around diffuse plaques. The abnormalities of neurites were also described in the brain of aged primates and the relation of those to senile plaque formation had been discussed [1, 13]. Price *et al.* [13] described that abnormal neurites or fiber abnormalities appeared at earlier ages and were more wide-spread than beta protein deposits in monkeys and supposed that these fiber abnormalities might be, in some way, related to some of the intraparenchymal deposits of beta amyloid. Although similar interpretation for the presence of neurites in canine diffuse plaques may be suggested, further studies will be necessary to discuss the meaning of the neuritic lesions in dogs.

Present study shows the close relation of canine amyloid plaques to cerebral amyloid angiopathy and suggests the importance of neuronal cells or neurites for the development of diffuse plaques. Although the origin of beta protein in cerebral vessels must be elucidated to understand the mechanism of cerebral beta amyloid deposition in details, the observation in this report may provide useful informations to investigate the pathogenesis of senile plaques.

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