Immunohistochemical Analysis of Constituents of Senile Plaques and Cerebro-Vascular Amyloid in Aged Dogs

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ABSTRACT. Immunohistochemical analysis of constituents of senile plaques and cerebro-vascular amyloid in the brain of aged dogs was performed using antisera against beta protein, cystatin C, ubiquitin, tau, and neurofilament (NF). All types of senile plaques and cerebro-vascular amyloid in aged dogs were labeled by anti-beta protein serum. Cystatin C immunoreactivity was detected in neuronal cell bodies, primitive or classical plaques, and amyloid deposited around cerebral capillaries, but not in diffuse plaques and amyloid deposited in the media tunica of cerebro-meningeal arterioles. Ubiquitin-positive granules distributed widely in both gray and white matter of aged dogs, while they were very small in number in young dogs. Swollen neurites-like materials in primitive plaques or classical plaques were immunoreactive for anti-ubiquitin serum. Tau immunostaining labeled commonly axons and several neuronal or glial cells after hydrate autoclave pretreatment. Tau-positive components were observed rarely in the corona of classical plaques. Most of swollen neurites-like structures of primitive or classical plaques were not reactive for anti-NF serum, and only a few plaques contained small numbers of NF-positive elements. Key words: amyloid angiopathy, beta protein, cystatin C, senile plaque, ubiquitin.


Senile plaques, cerebral amyloid angiopathy, and neurofibrillar tangles have been known to appear in the brain of aged people and patients with Alzheimer's disease [4, 27]. Senile plaques and cerebro-vascular amyloid consisted of amyloid beta-protein [10] and neurofibrillary tangles are composed of paired helical filaments (PHF) [34]. Recently, a modified form of microtubule-associated protein, tau protein [12], and a small peptide correlated to the degradation of alternated or denatured protein, ubiquitin [5, 11, 15], have been acknowledged to be the major components of PHF. Moreover, recent immunohistochemical studies revealed the existence of tau [16, 22, 36], ubiquitin [7, 18] as well as neurofilament (NF) [23, 25] in degenerative neurites of senile plaques.

In addition, several protease inhibitors such as alpha 1-antichymotrypsin, serin protease inhibitor [28], and cystatin C (gamma-trace), cystein protease inhibitor [3, 13], were shown to coexist in senile plaques and cerebro-vascular amyloid together with beta protein [1, 14, 19]. Shoji et al. [24] described that all types of senile plaques including diffuse plaques and some cerebro-vascular amyloid were labeled by alpha 1-antichymotrypsin immunostain-

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MATERIALS AND METHODS

Dogs: Ten dogs, 3 to 20 years old, 4 male and 6 female, were examined. The age and sex of the dogs examined in this study were summarized in Table 1. Brain samples from 9 dogs were fixed in 10% neutral buffered formalin (NBF), and those from 1 dog in Bouin's solution.

Histopathology: Paraffin sections of 6 to 8 μm, stained with alkaline Congo-red were observed under polarized light to confirm the birefringence of amyloid and those stained with periodic acid-methenamine-silver (PAM) were used for the detection of senile plaques. The occurrence of senile plaques and cerebral amyloid angiopathy in the dogs examined was shown in Table 1. Senile plaques were classified into 3 subtypes as described previously [30].

Antiserum: As the primary antibody, rabbit sera against synthetic peptide of amyloid beta-protein (kindly supplied from Dr. N. Nukina, Department of Neurology, School of Medicine, The University of Tokyo), ubiquitin (Dakopatts, Carpinteria, CA, U.S.A.), cystatin C (Dakopatts, Carpinteria, CA., U.S.A.), and tau (Sigma, St. Louis, MO., U.S.A.) and a mouse monoclonal antibody against high and low molecular weight of NF (Dakopatts, Carpinteria, CA., U.S.A.) were used. Biotinylated goat sera against rabbit IgG (Kirkegaard and Perry laboratories, Gaithersburg, MD., U.S.A.) or mouse IgG (Vector Laboratories, Burlingame, CA., U.S.A.) were used as the secondary antiserum. The immunoreactivity of canine cerebro-vascular amyloid and senile plaque with anti-beta protein serum was described in the previous report [31].

Pretreatment for immunostaining: For the detection of beta protein, deparaffinized sections were dipped in 90% formic acid for 10 min. To enhance the immunoreactivity of tau protein, sections soaking in distilled water were autoclaved at 121°C, for 5 min [21]. The pretreatment with 0.25% proteinase K (Boehringer Mannheim, Germany) at 37°C for 5 min, was also attempted for each immunostaining, except for beta protein.

Immunostaining: Brain sections with or without the pretreatments were incubated with 0.3% hydrogen peroxide in methanol for 10 min and normal goat serum at 37°C for 30 min to exclude non-specific reaction. Then, the sections were incubated with primary antisera at 4°C over night, with secondary antisera at 37°C for 30 min, and with avidin-biotin peroxidase complex (ABC, Vector laboratories, Burlingame, CA., U.S.A.) at 37°C for 30 min. The visualization was done in 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO., U.S.A.) solution. For counter stain, hematoxylin and alkaline Congo-red stain were employed.

RESULTS

The immunoreactivity of cerebro-vascular amyloid, senile plaques and brain tissues of dogs with antisera used in this study was summarized in Table 2.

Beta protein immunostain: Beta protein immunostain labeled diffuse and compact deposits of the protein in neuropil representing senile plaques in the brain of aged dogs (Figs. 1a and 1b). Although diffuse plaques in paraffin sections of the brain tissue fixed with NBF were detectable only after formic acid pretreatment, those plaques in the sections fixed with Bouin's solution were detected.

<p>| Table 1. Distribution of cerebral amyloid angiopathy and senile plaques in the dogs examined |
| --- | --- | --- | --- | --- |
| Case No. | Age (Years) | Sex | Senile plaques | Vascular amyloid |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>diffuse</th>
<th>primitive</th>
<th>classical</th>
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<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>F</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>4</td>
<td>13</td>
<td>F</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>M</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>F</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
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<td>9</td>
<td>18</td>
<td>M</td>
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<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

F: female; M: male. -: detected (+) and not detected (-).
Table 2. Immunoreactivity of senile plaques, vascular amyloid and brain tissues of dogs with antibodies examined

<table>
<thead>
<tr>
<th>Antibodies for</th>
<th>Senile plaque</th>
<th>Vascular amyloid</th>
<th>Brain tissues</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DP</td>
<td>PP</td>
<td>CP</td>
</tr>
<tr>
<td>Beta-protein</td>
<td>+*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tau</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
</tr>
<tr>
<td>NF</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
</tr>
</tbody>
</table>

DP: Diffuse plaque; PP: Primitive plaque; CP: Classical plaque; Cap: Capillaries; Art: Arterioles; NC: Neuronal cells; GC: Glial cells; ACT: Antichymotrypsin. 1: A few pericytes of the capillaries were positive. 2: Many granules in neuropil were positive in aged dogs. *: detected (+) and not detected (-).

Fig. 1. Diffuse plaques in the hippocampus (a), and a primitive plaque (b) in the cerebral cortex. Beta protein immunostain. A: × 70, B: × 130.

Fig. 3. A neuronal cell showing intense immunoreactivity for cystatin C. Immunostain. × 130.

Fig. 2. Vascular amyloid in the meningeal arterioles (a) and cerebral capillaries (b). Beta protein immunostain. A: × 70, B: × 130.

Fig. 4. Amyloid around the wall of capillary labelled by cystatin C immunostain. × 130.

without the pretreatment (Fig. 1a). Amyloid deposited in cerebro-meningeal arterioles and around the wall of cerebral capillaries of aged dogs also reacted intensely with anti-beta protein serum (Fig. 2).

Cystatin C immunostain: Anti-cystatin C serum labeled neuronal cells of the cerebral cortex (Fig. 3) and pericytes of cerebral arterioles or capillaries of all dogs examined. In the brain of aged dogs with senile plaques and/or amyloid angiopathy, amyloid deposited around capillaries (Fig. 4) and primitive
Fig. 5. Immunoreactivity of primitive plaques and perivascular amyloid for antibodies against beta protein (a) and cystatin C with proteinase treatment (b). Immunostain. × 70.

Fig. 6. Ubiquitin-positive granules in the cerebral cortex (a) and white matter (b) of aged dogs. Immunostain. × 130.

Fig. 7. Swollen neurites-like structures in a classical plaque reacting with anti-ubiquitin serum. Immunostain with Congo-red. × 130.

Fig. 8. Immunoreactivity of axons and neuronal cells in the hippocampus for anti-tau serum after hydrated autoclave pretreatment. Immunostain. × 130.

Fig. 9. Very small numbers of tau-positive materials (arrows) detected in the corona of a classical plaque. Immunostain with Congo-red. × 130.
or classical plaques (Fig. 5b) were immunoreactive with anti-cystatin C serum. The immunoreactivity was faint as compared with that with anti-beta protein serum (Figs. 5a and 5b), while by the pretreatment with proteinase K, the immunoreactivity for cystatin C was enhanced. Amyloid deposited in the tunica media of cerebro-meningeal arterioles and diffuse plaques did not react with anti-cystatin C serum, but showed intense immunoreactivity with anti-beta protein serum. In 2 aged dogs, beta protein-positive capillaries did not react with anti-cystatin C serum.

Ubiquitin immunostain: Ubiquitin-positive granules distributed widely in the cerebral cortex (Fig. 6a) and more numerous in the white matter (Fig. 6b) of all aged dogs, while they were very small in number in the brain of young dogs. Swollen neurites-like structures in primitive plaques or classical plaques showed intense immunoreactivity with anti-ubiquitin serum (Fig. 7). In diffuse plaques or cerebro-vascular amyloid, the accumulation of ubiquitin-positive granules were not observed.

Tau immunostain: Small numbers of neuronal cells and axons were weakly stained with anti-tau serum, while, after hydride autoclave pretreatment, these immunoreactivity were enhanced (Fig. 8). Glial cells in the white matter were immunoreactive for tau. Tau-positive materials were rarely detected in the coronas of classical plaque (Fig. 9), but diffuse plaques had no tau positive materials. In the cytoplasm of neuronal cells in the cerebral cortex or hippocampus, neurofibrillary tangle-like immunoreactivity of tau was never detected.

NF immunostain: Axons and dendrites in the white or gray matter were highly immunoreactive for the monoclonal antibody against NF. Only few NF-positive materials were detected in primitive plaques or classical plaques, but the swollen neurites-like materials in these plaques were negative for NF (Fig. 10).

DISCUSSION

Several investigators have reported that in cerebro-meningeal arterioles cystatin C coexisted with beta protein in patients with Alzheimer's disease or cerebral hemorrhage with amyloid angiopathy [14, 32], and considered that cystatin C might play an important role in the pathogenesis of cerebral hemorrhage in these patients. In the present study, cystatin C immunoreactivity was detected in two types of senile plaques, primitive and classical plaques, in addition to the capillaries with amyloid deposition. However, unlike human cases [14, 32], amyloid consisting of beta protein deposited in meningeal arterioles was not labeled by cystatin C immunostaining in the dogs. In addition, Vinters et al. [32] demonstrated that gamma trace peptide was rarely seen in cortical capillaries with amyloid deposition in human cases. The cause of these differences between human cases and the present results is unclear. The biological function of cystatin C has been known as a lysosomal cystein protease inhibitor [3]. Maruyama et al. [14] supposed that the accumulation of beta protein might alter the homeostasis of proteases and their inhibitors, increasing the accumulation of these proteins in the cerebro-vascular walls. Variant cystatin C is known to form amyloid fibrils itself in human hereditary cerebral hemorrhage with amyloid angiopathy of Icelandic type [9]. However, similar pathogenesis is not applicable in aged dogs. The localization of cystatin C was thought as secondary events in capillaries with beta amyloid deposition, because the immunoreactivity of these capillaries for cystatin C was faint and cystatin C deposition could not be detected in 2 dogs.

Tau protein shown as the important antigenic elements of PHF forming neurofibrillary tangles [12] and NF distributed in dystrophic neurites of senile plaques [16, 22, 23, 25, 36]. In aged dogs, neurofibrillary tangles have been never observed, though degenerative neurites appeared in primitive plaque or classical plaques [33]. Wisniewski et al. [35] investigated the brain sections of aged dogs using
anti-tau protein antibodies and Selkoe et al. [20] examined the immunoreactivity of those for PHF or NF. However, significant immunoreactive elements had not been observed in both reports. It has been considered from these studies that senile plaques of aged dogs may contain no PHF elements because there was no immunoreactivity for tau, NF, and PHF.

In the present study, axons and neuronal or glial cells reacted intensely with anti-tau serum after hydrate autoclave pretreatment [21], though only few tau or NF-positive elements were seen in primitive or classical plaques. These findings support the hypothesis that senile plaques of aged dogs are lacking for PHF elements. Even in human, tau and NF elements have been shown to be rare or absent in senile plaques of non-demented aged people and the abnormality of these cytoskeletal proteins is thought as specific features in Alzheimer's disease [2, 21]. Namely, the structural characteristics of senile plaques of aged dogs are considered to be in conformity with those of non-demented aged people.

Ubiquitin is also major element of PHF together with tau protein and detected in senile plaques in aged people or patients with Alzheimer's disease [7, 18]. In this study, ubiquitin immunostain labeled swollen neurites-like structures of primitive plaques and the coronas of classical plaques. Suenaga et al. [26] also observed similar senile plaques containing ubiquitin-positive and tau-negative neuritic elements in the cerebellum of patients with Alzheimer's disease, while PHF had not been detected in cerebellar senile plaques [37]. As the biological function of ubiquitin is the degradation of alternated or denatured proteins [11], ubiquitin might recognize degenerated neurites in senile plaques. Moreover, ubiquitin-positive granules widely distributed in both gray and white matters of aged dogs as compared with those in young dogs just like the observation in man [17]. These findings show that abnormal ubiquitinated proteins may increase and accumulate with the advance of age in the brain of dogs, while the pathogenic significance is still unknown.

In conclusion, we clarified the immunohistochemical features of the constituents other than beta protein of cerebro-vascular amyloid and senile plaques in aged dogs in the present study. The findings in this study may provide useful information to elucidate the pathogenesis of cerebral hemorrhage correlated with amyloid angiopathy in aged dogs and histogenesis of senile plaques, although further studies are necessary to make clear the significance of the constituents other than beta protein of cerebro-vascular amyloid and senile plaques.

REFERENCES


