An Immunohistochemical and Ultrastructural Study on Age-Related Astrocytic Gliosis in the Central Nervous System of Dogs

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ABSTRACT. The pattern of astrocytic gliosis (AG) was examined in 2-month-old to 18-year-old dogs using glial fibrillary acidic protein (GFAP) immunohistochemistry and electron microscopy. Coronal sections from various levels of the central nervous system (CNS) were stained with hematoxylin & eosin, Luxol Fast Blue, Nissl, and Bodian in addition to GFAP. A consistent pattern of age-related AG was observed in the dogs. The white matter, cortico-medullary junction, and subcortical nuclei in the cerebrum, central nuclei in the cerebellum, various nuclei in the brain stem, and grey matter of the spinal cord showed even and intense GFAP staining. AG was also prominent in the cerebral and cerebellar cortices and thalamus. Moderate AG was observed in the hippocampus and white matter of the cerebellum and spinal cord. Electron microscopy demonstrated increased number of profiles of degenerative neural components in the vicinity of hypertrophic astrocytes in the cerebral cortex of the aged dogs. Moderate to severe AG was consistently shown in the CNS of the aged dogs. In contrast, young normal dogs showed minimum amounts of GFAP-positive astrocytes in the CNS. These findings suggest that the observed AG in the CNS of the dogs is a morphological expression of aging.—**KEY WORDS:** aging, astrocytic gliosis, dog, electron microscopy, glial fibrillary acidic protein.

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Astrocytic gliosis (AG) in the brain has been reported in aged normal humans and in patients with Alzheimer's disease (AD) and senile dementia of the Alzheimer type [2, 7, 13, 21, 28, 32]. The phenomenon is believed to be a reaction to the loss of neurons and dendrites [2, 29]. AG as an aged change was also reported in the brains of animals including rats, macaque monkeys and mice [1, 6, 11, 17, 20, 25]. No consistent results were shown in these studies of animals and this discrepancy was attributed to differing methods used, such as the number of animals, staining and counting techniques [13]. In addition, few studies on age-related ultrastructural changes in glial cells have been performed in both humans and animals [6]. In this study, we employed electron microscopy and immunohistochemistry for glial fibrillary acidic protein (GFAP), which is the primary constituent of glial intermediate filaments and is considered to be exclusively localized in astrocytes [3]. Thus, as Mandybur *et al.* [22] stated, the term 'astrocytic gliosis' in this study refers to 'increase in GFAP-positive astrocytes (GFAP-PA)' or 'increased GFAP immunoreactivity' because not all astrocytic glia is positively stained by the GFAP immunohistochemistry. The aim of the present study is to report the pattern of age-related AG in the central

nervous system (CNS) of dogs.

MATERIALS AND METHODS

Twenty-five dogs, 16 females and 9 males, were used in the present study. Seventeen were old dogs, ranging in age from 11 to 18 years (mean 14.5 years), and eight were young controls, 2 to 12 months of age. No signs of neurological disorders were observed in the dogs. Clinical and pathological data of some dogs have been reported previously [30]. The dogs were killed by euthanasia or died of a variety of disorders including heart worm disease, renal failure, and tumor of visceral or genital organs. Five aged dogs and four young controls were anaesthetized with pentobarbital and perfused through the heart with 2 liters of 4% paraformaldehyde in phosphate buffer (pH 7.2). During the perfusion, the descending aorta was clamped with a hemostat to save fixative. The dogs were decapitated and the exposed brain was re-immersed in perfusate for overnight at 4°C.

Complete autopsies were performed on these dogs. For light microscopy, the CNS, including the cerebrum, cerebellum, brain stem and spinal cord was fixed in 10% neutral buffered formalin at autopsy. A 5-mm-thick coronal slice cut at various

levels of the CNS was dissected. Both left and right cerebral hemispheres of three aged dogs and left cerebral hemispheres of the others were used in this study. The tissue block was then routinely processed and embedded in paraffin. Serial sections ($6\ \mu\text{m}$) were stained with hematoxylin and eosin (HE), Luxol Fast Blue, Nissl and Bodian. Serial sections were also processed for immunohistochemistry using the peroxidase-antiperoxidase (PAP) method with diaminobenzidine (DAB) as chromogen. The primary antibody was anti-human GFAP (rabbit polyclonal, Medac GmbH, West Germany). The antibody was used at a dilution of 1:200. The sections were counterstained with hematoxylin. Method specificity was evaluated by substituting normal rabbit serum with the primary antibody.

For electron microscopy small tissue blocks ($1\ \text{mm}^3$) were taken from the cerebral cortex of the perfused dogs. These tissue blocks were post-fixed in phosphate-buffered 1% OsO_4 for 1 hr, dehydrated in ascending concentrations of ethanol and embedded in Epon. Semithin sections ($1\ \mu\text{m}$) were stained with toluidine blue and were examined light microscopically. Thin sections were stained with lead citrate and uranyl acetate, and were examined with a JEM-100CXII.

At autopsy, tissue samples were also taken from a variety of organs for routine light microscopic studies and fixed in 10% neutral buffered formalin. Paraffin sections ($4\ \mu\text{m}$) were made and stained with HE.

RESULTS

Gross and microscopic examination of the CNS of the aged dogs revealed neither changes indicating ischemia nor signs of neurological disorders other than typical aged changes, such as thickening of the meninges, mild dilatation of the ventricles, occasional dystrophic axons, lipofuscin accumulation in neurons, and nerve cell loss (Fig. 1). No obvious atrophy was observed in the brains examined.

An increase in GFAP-PA in the aged dogs, as compared to young controls, was the most constant histological feature observed in the GFAP immunohistochemistry. No correlation was noted between the intensity of AG and the clinical findings, such as breed, sex and diseases which affected the dogs. AG consisted of hypertrophic astrocytes characterized either by intense GFAP immunostaining in light microscopy or by thick bundles of intermediate

Fig. 1. Cerebral cortices from a 17-year-old dog (a) and an 8-month-old dog (b). Note the apparent neuronal loss in a. Nissl stain. $\times 75$.

filaments in electron microscopy (Figs. 2 and 3). These GFAP-PA were observed both interstitially and perivascularly. In all 17 aged dogs, moderate to severe AG was observed with a consistent distribution pattern in the CNS. The change was symmetric in the dogs whose CNS including left and right cerebral hemispheres was examined.

A marked increase in GFAP-PA was seen in the subcortical and deep white matters in the cerebrum (Fig. 4a), especially at the cortico-medullary junction (Fig. 4b), corpus callosum, internal capsule, subcortical nuclei in the cerebrum, central nuclei in the cerebellum, various nuclei in the brain stem, and gray matter of the spinal cord (Fig. 4c). A prominent increase was also seen in the cerebral and cerebellar cortices (Figs. 2 and 4d) and thalamus (Fig. 4e).

A moderate increase was observed in the hippocampus (Fig. 4f), white matter of the cerebellum and spinal cord, reticular formation, optic tract and subependymal areas (Fig. 4g) and periaqueductal zone throughout the CNS.

Young control dogs showed minimum amounts of GFAP-PA in the CNS; the molecular layer of the cerebrum and white matter throughout the CNS consistently showed a slight increase in GFAP-PA (Fig. 4h).

Electron microscopy revealed a variety of degenerative profiles of neural components, such as dark

Fig. 2. Cerebral cortex from a 17-year-old dog, showing a considerable increase in glial fibrillary acidic protein (GFAP) positive astrocytes. GFAP stain. $\times 120$. Inset: Hypertrophic astrocytes in the cortex. GFAP stain. $\times 240$.

Fig. 3. Transmission electron micrograph of the cerebral cortex from a 14-year-old dog. Hypertrophic astrocytes contain lipofuscin pigments (L) and bundles of intermediate filaments (F). $\times 15,000$.

axon terminals, swollen synaptic vesicles with dense contents, and heterogeneous dense bodies in dendrites in the cerebral cortex of the aged dogs (Fig. 5). These changes were often located in the vicinity

of hypertrophic astrocytic processes (Fig. 6). Few such profiles were observed in the young controls examined.

Fig. 5. Transmission electron micrographs of the cerebral cortices from the aged dogs, showing a variety of degenerative profiles of neural components: swollen synaptic vesicles with dense contents (a; $\times 21,000$); a dilated endoplasmic reticulum (E) (b; $\times 15,000$); heterogeneous dense bodies (D) (c; $\times 22,000$); dark axon terminals (d; $\times 24,000$). Note the increased number of glycogen granules in the astrocytic processes (A).

DISCUSSION

In contrast to slight staining of GFAP in the young dogs, prominent GFAP-immunoreactivity was shown in the CNS of the aged dogs, indicating that age-related AG also occurs in dogs. There are several reports which demonstrated astrocytic hypertrophy in the aging CNS [1, 4, 11, 20]. It was not certain whether there was an increase in the volume of GFAP per astrocyte or just an increase in

astrocyte size [4]. In the present study, we frequently encountered hypertrophic astrocytes with either intense GFAP immunostaining in light microscopy or thick bundles of intermediate filaments in electron microscopy. These findings may suggest that the observed age-related hypertrophy of GFAP-PA is related to an increase in the volume of GFAP per astrocyte. This morphological finding appears to correspond to the results from the biochemical studies of the brains of the experimental animals, in which an age-related increase in the concentration

Fig. 4. GFAP immunohistochemistry of the central nervous system of the dogs. Note the moderate to severe astrocytic gliosis in the aged dogs: subcortical white matter of the cerebrum (a; $\times 150$); cortico-medullary junction of the cerebrum (b; $\times 110$); spinal cord (c; $\times 150$); cerebellum (d; $\times 150$); thalamus (e; $\times 150$); hippocampus (f; $\times 150$); subependymal area of the cerebrum (g; $\times 150$). Only occasional GFAP-positive fibers (arrows) are demonstrated in the cerebral cortex of a 6-month-old dog (h; $\times 110$). GFAP stain.

Fig. 6. Transmission electron micrograph of the cerebral cortex from an 18-year-old dog. Note the process of a hypertrophic astrocyte surrounding the degenerative neural components. $\times 30,000$.

of either GFAP or GFAP mRNA was demonstrated [12, 24].

The brains of the aged dogs showed a normal gross-appearance except mild dilatation of the ventricles and thickening of the meninges. There appeared to be no difference in the thickness of the cerebral cortex between the aged dogs and the young controls. Similarly, no statistical difference as to the cortical thickness was noted between the AD group and the controls in the morphometric studies of human brains [21, 28, 31]. Therefore, the increase of GFAP-PA could not be related to the shrinkage or condensation of the tissue [21, 28].

Studies of the brains of aged rats demonstrated synaptic deterioration and functional changes in synaptic transmission mechanisms [16, 33]. Age-related alterations in neural components including dendrites and synapses have been considered as the stimulus for AG in aging [13, 18]. In this ultrastructural study, we frequently encountered a variety of degenerating profiles of axon terminals and dendrites; some of which were located in the vicinity of reactive astrocytic processes. These findings are in accord with those of aged humans [29]. Recent studies suggested that astrocytes have a phagocytic function, which may contribute to removal of degenerative debris in the CNS [1, 23]. However,

we failed to observe any dense bodies suggestive of ingestion of degenerative neural components even in the cytoplasm of reactive astrocytes occurring among the profiles of degenerating axon terminals. Recent studies demonstrated evidence of new roles for astrocytes, including neurotransmitter uptake [5, 15], synthesis and secretion of trophic factors [9, 10, 14, 27], targets for hormones and immune system cytokines [8, 19, 34], and regulation of cerebral blood flow [26]. Thus, astrocytes may play important roles in the regulation of the microenvironment in the CNS. Therefore, the possibility exists that astrocytic hypertrophy represents a primary effect of aging rather than a mere secondary response to degenerative events in the CNS [24].

As to the patterns of age-related AG, increased numbers of GFAP-PA have been reported in the cerebral cortex and hippocampus in AD and normal aging brains of humans [2, 7, 13, 21, 28, 32]. The pattern of AG observed in the present study is similar to that demonstrated in the recent study of the AD and aging cerebrums, in which extensive AG was observed in the deep cerebral white matter, cortico-medullary junction and subcortical gray matter in addition to the cerebral cortex [2]. Using GFAP immunohistochemistry, Mandybur *et al.* [22] systematically examined the brains of old nude

mice, in which an increase in the number of GFAP-PA and their hypertrophy were also observed in a pattern similar to that found in the dogs except no evidence of AG in the cerebral and cerebellar cortices in the nude mice. This discrepancy may be attributable to the species difference in neuroanatomical and functional specificity of the CNS between the two species.

In animals, occurrence of age-related specific changes, such as senile plaques and cerebrovascular amyloidosis is restricted to some species and in small quantity [22]. We have previously reported that both two changes occur in the brains of aged dogs with high incidence and are morphologically and histochemically very similar to those of humans [30]. In addition, the present study demonstrated the occurrence of age-related AG with a consistent pattern similar to that of humans. It thus appears that aged dogs may be suitable as an animal model for the study of the pathomechanisms involved in CNS aging.

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