

Comparative Study of Cerebellar Degeneration in Canine Neuroaxonal Dystrophy, Cerebellar Cortical Abiotrophy, and Neuronal Ceroid-Lipofuscinosis

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ABSTRACT. The cerebellar lesions of three dogs with canine neuroaxonal dystrophy (NAD), one dog with cerebellar cortical abiotrophy (CCA), and 4 dogs with neuronal ceroid-lipofuscinosis (NCL) were examined to understand their pathogenesises. Purkinje cell loss was most severe in the vermis of a dog with CCA, and granule cell loss was most prominent in the cerebellar hemisphere of dogs with NCL. Immunohistochemically, CD3- and HLA-DR-positive cells were most frequent in the dogs with NCL, and moderate in dogs with NAD, but not in a dog with CCA. The number of cleaved caspase 3-positive cells was prominent in a dog with CCA, but no significant in the dogs with NAD. The results indicate different pathway of neuronal loss of these canine neuronal disorders.

KEY WORDS: canine, cerebellar cortical abiotrophy, cerebellar degeneration, ceroid-lipofuscinosis, neuroaxonal dystrophy.

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Canine neuroaxonal dystrophy (NAD) and canine cerebellar cortical abiotrophy (CCA) are extremely rare neurodegenerative diseases that are suspected to be hereditary disorders. The Rottweiler, Kerry blue terrier, Jack Russell terrier, and Papillion breeds are predisposed to canine NAD [4, 7, 9, 23, 26] and CCA [5, 23]. Neuronal ceroid-lipofuscinosis (NCL) is also a hereditary neurodegenerative disorder and is relatively common compared to NAD and CCA in dogs [14, 31]. These neuronal degenerative diseases share some common clinical features. In general, most dogs affected with NAD, CCA, or NCL exhibit early onset of progressive neurological signs and take a lethal course. In computed tomography (CT) and magnetic resonance imaging (MRI) examinations, cerebellar atrophy is a significant finding to suggest the possible diagnoses of these diseases [8–10, 32].

Histopathological lesions of the cerebellum of canine NAD, CCA, and NCL are characterized by moderate to severe neuronal loss. Canine NAD is characterized by severe axonal degeneration with numerous spheroid formation throughout the central nervous system (CNS), whereas, there is no such feature in CCA [28]. NCLs are characterized severe lysosomal storage of autofluorescent lipopigments in neurons and macrophages, various degrees of neuronal loss, and astrocytosis. Current molecular biological studies revealed the genetic features of this unique hereditary disease in both the medical and veterinary fields [33]. In dogs, mutations in CLN5 and CLN8 have been confirmed in Border collies [21] and English setters [15], respectively. In spite of this molecular biological evidence of neurodegenerative disorder, the detailed mechanism that

causes the neuronal loss remains unclear. Recently, cellular injury caused by a combination of oxidative stress and autophagy leading to lysosome dysfunction-induced neuronal death has been proposed in the pathogenesis in NCL [1].

To examine cell death processes, several histological procedures to detect an apoptosis are available. Together with the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method [12, 18], immunohistochemical staining using antibodies against single stranded DNA (ssDNA), phosphorylated P53 and cleaved caspase 3 are common for the detection of apoptotic cells. In addition, oxidative stress can be evaluated by the use of antibody for 8-hydroxydeoxyguanosine (8-OHdG). Although these techniques have been very useful in experimental studies using rodent models and cell cultures, their application to clinical cases, especially in necropsied case, is still questionable. The aim of this study is to clarify the differences in cerebellar lesions between canine NAD, CCA, and NCL.

Brain tissues from eight dogs including three dogs with NAD, one dog with CCA, and four dogs with NCL were examined. Cerebellar tissues from a 4-month-old necropsied Papillion dog without any neurological signs were used as a normal control. A summary of the clinical information of these cases is shown in Table 1. The clinicopathological details of the dogs with NAD and CCA have been described previously [24]. In addition, the clinicopathological features of NCL in two chihuahuas dogs have been also reported [22]. Under the microscope, the number of survived Purkinje and granule cell in vermis and hemisphere was calculated the number of averages in 10 fields under high power magnification (x400), respectively. Selected sections were also stained with Sudan Black B, Luxol fast blue (LFB), and periodic acid Schiff (PAS). Immunohistochemistry were performed by the envision polymer method with rabbit polyclonal antibodies against glial fibrillary acidic

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Table 1. Clinical features of cases investigated in this study

Dog No.	Breeds	Gender*	Age**	Diagnoses
1	Papillon x Chihuahua	M	8 m	NAD
2	Papillon	F	6 m	NAD
3	Papillon	F	9 m	NAD
4	Papillon	M	2y9 m	CCA
5	Border collie	M	2y	NCL
6	Border collie	MC	2y1m	NCL
7	Chihuahua	MC	1y11m	NCL
8	Chihuahua	MC	2y	NCL
Control	Papillon	M	1y6m	Peritonitis by perforated duodenal ulcer

*: M; male, MC; Castrated male, F; Female. **: m; months, y; years.

protein (GFAP; Prediluted, Dako-Japan), cleaved form caspase 3 (1:10, Chemicon International Inc., Temecula, CA., U.S.A.), phosphorylated caspase 9 (1:50, Abcam, Tokyo, Japan), and phosphorylated p53 (1:25, Abcam, Tokyo, Japan) and mouse monoclonal antibodies against HLA-DR (1:25, Dako-Japan), CD3 (1:25, Dako-Japan), ss-DNA (Dako-Japan), and 8-OHdG (Nikken Zeil Co., Furu-roi, Japan). TUNEL assay were performed with the in situ cell death detection kit (Roche Molecular Biochemicals, Indianapolis, Ind.). Visualization was then performed with 3,3'-diaminobenzidine tetra hydrochloride (Sigma), and light green was used for counterstaining.

Histopathologically, in dogs with NAD (dogs 1 to 3) demonstrated degenerative changes in the residual Purkinje cells such as pyknosis and vacuolation. The morphological changes of the granule cells were minimal except the dog 3 showing karyopyknosis. Diffuse astrocytosis was prominent throughout the cerebellar cortex. In the cerebellar white matter in all dogs with NAD, a number of swollen axons were observed. A dog with CCA (dog 4) demonstrated most severe loss of Purkinje cells, and surviving Purkinje cells exhibited degenerative changes including pyknosis. The remaining granule cells were also pyknotic. No torpedo or spheroid shaped axons were observed. Dogs with NCL (dogs 5 to 8) were commonly characterized neuronal degeneration with intracytoplasmic accumulation of ceroid-lipofuscin-like materials in the surviving Purkinje cells and microglia/macrophages. These materials demonstrated autofluorescence and were positive for PAS, Sudan black B, and LFB. Purkinje cell loss was severe next to CCA. The granule cell loss was most prominent among three diseases. A few degenerative axons were scattered in the granule cell layer and white matter. Diffuse astrocytosis was prominent in the Purkinje cell layer and white matter. The Purkinje and granule cell loss was more prominent in the cerebellar vermis than in the cerebellar hemisphere in dogs with CCA or NAD, whereas the lesion was more dominant in the cerebellar hemisphere in the dogs with NCL (Figs. 1 and 2).

Immunohistochemically, the number of GFAP-positive astrocytes was significantly increased in all affected dogs. The sharpest increase was found in the cerebellar hemisphere of the dogs with NCL. Moderate number of GFAP-

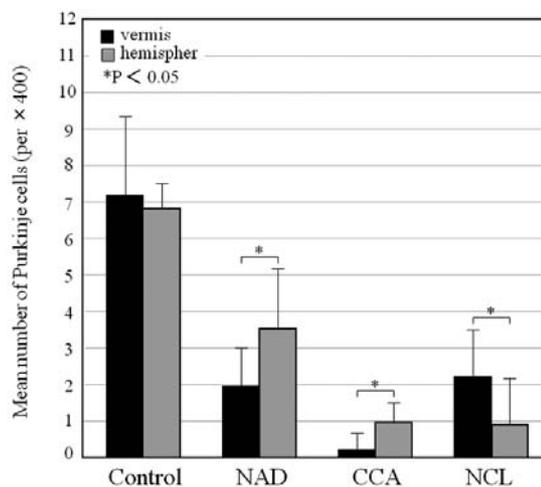


Fig. 1. The mean number of Purkinje cells in the cerebellar vermis and cerebellar hemisphere of dogs with CCA, NAD, or NCL, and the control dog. All data were plotted as means \pm the standard error, and statistical significance was assessed by the Student's *t*-test: * $P < 0.05$.

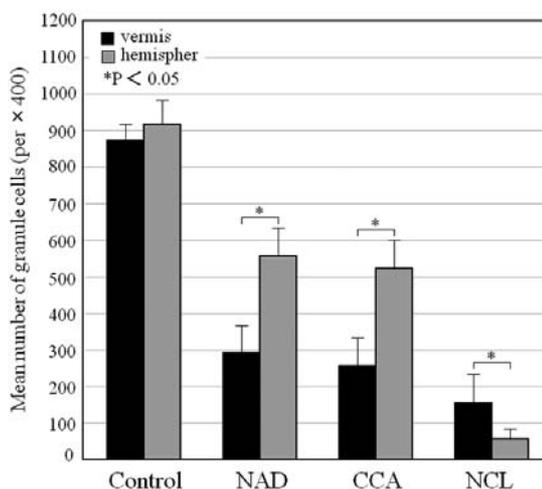


Fig. 2. The mean number of granule cells in the cerebellar vermis and cerebellar hemisphere of dogs with CCA, NAD, or NCL, and control dog. All data were plotted as means \pm the standard error, and statistical significance was assessed by the Student's *t*-test: * $P < 0.05$.

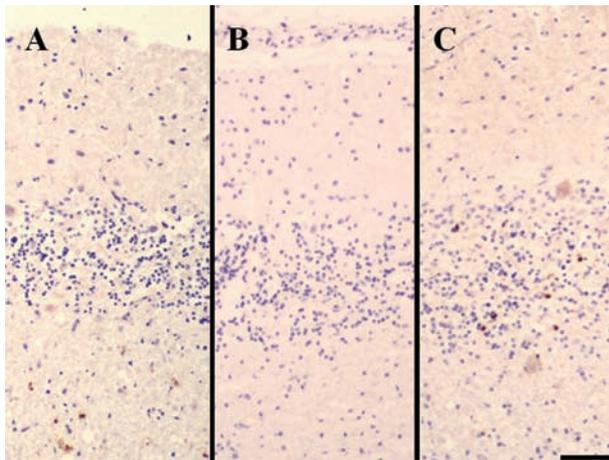


Fig. 3. Cerebellar cortex (vermis). Immunohistochemistry for CD3. CD 3-positive cells were observed in NAD (A, dog 1) and NCL (C, dog 8), but not in CCA (B). Envision polymer method. Counterstained with Mayer's hematoxylin. Bar=100 μ m.

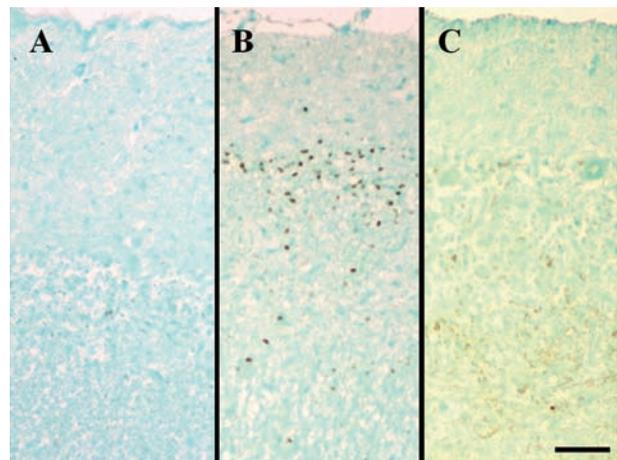


Fig. 4. Cerebellar cortex (vermis). Immunohistochemistry for cleaved caspase-3. Many caspase 3-positive cells were observed in a dog with CCA (B), and a few positive cells were also found in NCL (C, dog 8). No positive cells were detected in NAD (A, dog 1). The caspase 3-positive cells were distributed in the Purkinje and granule cell layers. Envision polymer method. Counterstained with light green. Bar=100 μ m.

positive astrocytes was observed in the vermis of the dogs with NCL and the dog of CCA. Among all three diseases, the increase in GFAP-positive cells was relatively mild in the dogs with NAD. The results for CD3-positive cell (T-cell) infiltration were analogous to those for HLA-DR-positive cells (macrophages or microglia) in the cerebellar cortex in dogs with NAD, CCA, or NCL (Fig. 3). The infiltration of CD3- and HLA-DR-positive cells was most severe in the dogs with NCL, especially in the cerebellar hemisphere. The increase in the number of CD3- and HLA-DR-positive cells in the dogs with NAD was milder than that in the dogs with NCL. On the other hand, in a dog with CCA, there were no CD3-positive cells and a few HLA-DR-positive cells as shown in the control dog. The number of cleaved caspase 3-positive cells was significantly increased in a dog with CCA among all dogs examined (Fig. 4). In this dog, cleaved caspase 3-positive cells were detected predominantly in the vermis, which corresponded to the degree of Purkinje and granule cell loss. These cells were mainly distributed in the Purkinje cell and granule cell layers. In dogs with NCL, a small number of caspase 3-positive cells were also detected. In dogs with NAD, no significant increase in the number of caspase 3-positive cells was recognized. Concerning the immunostainings for cleaved caspase 9, phosphorylated P53, and 8-OHdG, no specific staining patterns were obtained in the affected cases. TUNEL assay and ssDNA-immunostaining also demonstrated intense non-specific nuclear staining was detected in all dogs including the control dog.

The present study revealed the different degree and distribution of cerebellar degeneration among canine CCA, NAD, and NCL. Purkinje cell loss was most evident in a dog with CCA, especially in the vermis, and granule cell loss was most prominent in the dogs with NCL, especially in the cerebellar hemisphere. The neuronal loss of the dogs

with NAD was intermediate between CCA and NCL. A different phylogenetic characteristics of the vermis and cerebellar hemisphere might be involved is consider as one of the reason of the differences of severity of the lesions [27]. The cerebellar vermis is also called as the archicerebellum, which is an older region in evolutionary terms than cerebellar hemisphere, which is sometimes called as neocerebellum. One possibility is that differences in certain metabolic mechanisms may cause the neuronal omission.

This study also indicate different pathway of neuronal loss in each diseases as evidenced by the specific features of cerebellar degeneration in canine NAD, CCA, and NCL. In the dog with CCA, many caspase 3-positive cells without any apparent inflammatory reactions were observed in both the granule and Purkinje cell layers. It indicates that the apoptotic pathway was involved in the pathogenesis of canine CCA in the Papillion dog. Together with excitotoxic neuronal injury [5], an apoptosis of precursor cells in the outer granule cell layer [27] have been suggested as a pathogenesis of canine CCA in the previous reports. Complete identification of caspase 3-positive cells remained unclear in this study. In dogs with NAD, moderate inflammatory reactions without caspase 3-positive reaction were observed in both the granule and Purkinje cell layers. It might indicate non-apoptotic pathways, such as necrosis or oncosis, are mediated. In the dogs with NCL, prominent infiltration of the inflammatory cells including CD3-positive cells (T lymphocytes) and HLA-DR-positive cells (macrophages/microglia) were observed. It might indicate that inflammation participated in the neuronal loss of canine NCL.

Human spinocerebellar degeneration (SCD) shows some similar clinicopathological features to canine CCA [6, 31]. However, in histology, neither inclusions nor aggregated

polyglutamine which is a feature of human SCD has not reported in canine CCA previously [34]. Causative gene of PANK2 in human NAD [11, 13, 17, 19] associates the synthesis of CoA that participates in fatty acid synthesis and energy metabolism. Thus, the altered composition of CoA may lead to degeneration of the plasma membrane and the membranes of cell organelles [17, 19]. Another possible target of the diseases can be PLA2G6 which encodes phospholipase A₂ (iPLA₂), an important enzyme for maintaining phospholipid membranes [3, 16]. Therefore, iPLA₂ deficiency may alter the phospholipid composition of membranes or lead to a failure to repair oxidative damage to membrane phospholipids [2, 20, 29, 30]. In human NCL, several hypotheses including apoptosis and oxidative stress related neuronal cell death have been proposed, although these opinions have not been fully accepted [12, 18, 25, 33]. Very recently, an autophagosome-associated cell death pathway has been suggested [28, 35, 36]. The utility of several immunohistochemical markers including ssDNA, cleaved caspase 9, phosphorylated P53 and TUNEL assay to detect apoptotic pathway was questionable in this study. In addition, reliable data could not be obtained by immunostaining for 8-OHdG in both affected and control dogs. These immunohistochemical markers and special stains might be inadequate for the evaluation for the cell-death processes in necropsied cases. Thus, further studies other than morphological analysis will be required to know the detailed processes of neuronal cell death in these canine neurodegenerative diseases.

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