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# Glial pathology in development of cerebellar dysplasia in the hereditary cerebellar vermis defect (CVD) rat

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**Abstract** The cerebellar vermis defect (CVD) rat is a new neurological mutant characterized by a cerebellar vermis defect and dysplasia in the cerebellum, especially at the cerebellopontine junctions. In this study, the cytokinetics of glia in terms of the development of cerebellar dysplasia in the CVD rat was investigated using glial fibrillary acidic protein (GFAP) and vimentin immunohistochemistry. In the cerebellar hemispheres, dislocation of the Bergmann glia was observed from postnatal day 5 (P5) in lesions with abnormally aggregated external granule cells (EGCs). Rearranging Bergmann glia were often seen around the EGCs penetrating into the white matter. In the cerebellopontine junctional areas, Bergmann glia were induced after penetration of the Purkinje cells, identified with calbindin immunohistochemistry, and EGCs into the pons from P10. Bergmann fibers were frequently arranged perivascularly. In the clusters of Purkinje cells without EGC settlement in the pons, a small number of Bergmann fibers were observed and their alignment was completely disturbed. These findings suggest that morphological changes in the Bergmann glia depend on their contact with Purkinje cells, but that the orientation of their processes may be influenced by EGC settlement. These glial fibers in the CVD rat may play an important role in the aberrant migration of EGCs, resulting in the development of cerebellar dysplasia.

**Key words** Bergmann glia · Cell migration · Cerebellar dysplasia · Immunohistochemistry · Mutant rat

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## Introduction

During the development of the nervous system, neurons migrate before they settle in their final positions [18]. Recently, some studies have shown that cell adhesion molecules, extracellular matrix proteins and proteolytic enzymes or their activators play important roles in the processes [5, 21]. However, the precise mechanisms for the neuronal migration are not fully understood. The development of cerebellum has been investigated in great detail because of its simply laminated structures, the presence of limited neuronal cell types and the abundant knowledge of the morphological transformation during the development [1, 4, 6, 24–26]. In particular, cerebellar granule cells have been focused on to study the morphogenesis in the mammalian brain since the ordered and sequential extending of the parallel fibers is suitable for studying the axonogenesis [4]. Furthermore, it is considered that Bergmann fibers assist the inward migration of immature granule neurons from the external to internal granular layers during the cerebellar development. Thus, the morphological kinetics of granule cells and Bergmann glia in cerebellar development may be the best index to investigate the neuron-glial interaction in the neurogenesis [6, 25].

We have established a new neurological mutant rat with a cerebellar vermis defect (CVD) and fused cerebellar hemispheres. This mutant was named CVD rat [9]. Histopathologically, CVD rats had dysplastic lamination and cell positioning abnormalities of the cerebellar cortex, especially at the cerebellopontine junctions; these findings are similar to those of human cerebellar dysplasia [23]. Although the CVD mutation lacks any cerebral anomaly, the pathological findings and the autosomal recessive inheritance of the mutation in the CVD resemble some characteristics seen in the human Walker's lissencephaly and Neu-Laxova syndrome [2, 14, 15, 19]. Therefore, the CVD mutation may provide a useful animal model for understanding the pathogenesis of the cerebellar vermis defect and cerebellar cortical dysplasia due to the genetic insults.

In the present study, to clarify the role of glia in cerebellar dysplasia, we analyzed the morphological kinetics of the glia in the CVD using immunohistochemistry for vimentin and glial fibrillary acidic protein (GFAP) as markers for the Bergmann glia and astrocyte [3].

## Materials and methods

### Animals

The mutant colony of CVD rats originated from spontaneously ataxic rats found in a stock of the Lewis strain. The colony has been kept as a hybrid strain with both Lewis and Donryu genetic backgrounds [10]. We mated heterozygous females *(+/cvd)* and homozygous males *(cvd/cvd)* and obtained the affected mutants used in this study. The day with the first sperm-positive vaginal smear was counted as embryonic day 1. Age-matched heterozygous littermates *(+/cvd)* which showed neither clinical nor pathological abnormalities were used as controls.

Histopathological and immunohistochemical examinations were conducted on rats every day from postnatal day 0 (P0) to P40. At least four affected mutant rats were examined at each point.

### Histopathology

Neonatal rats were killed under ether anesthesia. Brains from these rats were fixed with methacarn [16] or 10% neutral buffered formalin, and embedded in paraffin. Paraffin-embedded sections were cut at 4 μm and stained with hematoxylin and eosin (H&E) for histopathology.

### Immunohistochemistry for the Bergmann glia and astrocytes

For immunohistochemistry, rats were anesthetized with ether and perfused through the left ventricle with 4% paraformaldehyde in phosphate-buffered saline (PBS). The cerebellum, midbrain, and brain stem were removed and postfixed in the same paraformaldehyde fixative for 3 h and 30-μm-thick sections were cut using a vibratome (Technical Products International, USA). Free-floating sections were incubated with rabbit polyclonal antibody against GFAP (Dako, 1:1000) or monoclonal antibody against vimentin (Dako, 1:1000) at 4 °C overnight. After washing in PBS, the floating specimen were incubated with biotinylated anti-rabbit IgG (Dako) for GFAP or biotinylated anti-mouse IgG (Dako) for vimentin for 1 h at room temperature. The samples were then incubated with streptavidin biotin-peroxidase complex (LSAB kit, Dako, Japan) for 1 h at room temperature. Immunoreactions were developed by 0.03% diaminobenzidine (DAB) in 0.05 M TRIS buffer (pH 7.6) containing 0.005% hydrogen peroxide. Some sections were counterstained lightly with hematoxylin. They were mounted on glass slides, dehydrated, and coversliped.

To clarify the relationship between the Bergmann glia and Purkinje cells, double immunolabeling of GFAP and calbindin [8, 22] was performed using the Doublestain kit (Dako): the peroxidase-antiperoxidase method with DAB as chromogen for detection of GFAP-positive glia, and the alkaline phosphatase-antialkaline phosphatase (APAAP) method with fast red TR as chromogen for detection of calbindin-positive Purkinje cells. Mouse monoclonal anti-calbindin antibody (BioMakor, Israel, 1:200) was used. After visualization with these chromogens, the sections were counterstained lightly with hematoxylin.

Negative control sections were prepared by omitting the primary antibodies or applying normal rabbit or mouse serum.

## Results

In the cerebellar hemispheres of both control and CVD rat on P3, vimentin-positive Bergmann glia were observed in the developing cerebellar cortices; the distribution of the glia in the cortices was located randomly without any integrated pattern (Fig. 1). On P5, the number of the Bergmann glia was dramatically increased, and they were located mainly in the Purkinje cell layer along with the settlement of the Purkinje cells (Fig. 2).

Our studies on the histogenesis of the cerebellar dysplasia in the CVD rat [11, 12] indicated that, from P5, external granule cells (EGCs) began to aggregate around the blood vessels, leading to the dysplastic abnormalities in the cerebellar lamination. At this point, dislocation of the Bergmann glia was observed in the lesions with abnormally aggregated EGCs. Deeply positioned Bergmann glia were also noted in the cerebellar hemispheres. Around P10, perivascular EGCs gradually increased in number, and penetrated deeply into the white matter of the cerebellum, resulting in separation of the normal Purkinje cell and internal granule cell layers. During this period, rearranging Bergmann glia were seen around the penetrating EGCs (Fig. 3) and the processes of the Bergmann glia were directed toward the EGCs. After P20, deeply dislocated Bergmann glia were observed in the foci of the molecular layer invagination.

In the cerebellopontine junctions, a completely dysplastic cerebellum developed in the CVD mutant [9]. Pre-

**Fig. 1** P3, CVD rat. Vimentin-positive glia scattered in the cerebellar cortex (*P* postnatal day, *CVD* cerebellar vermis defect), × 243 ▶

**Fig. 2** P8, normal arrangement of the cerebellar cortex of CVD rat. Vimentin-positive Bergmann glia are settled in Purkinje cell layer; × 243

**Fig. 3** P13, CVD rat. Bergmann glia in the lesion of EGC invagination. Rearranging Bergmann glia are seen *(arrows)*. *Inset* Higher magnification of rearranging Bergmann glia (*EGC* external granule cells; *GFAP* glial fibrillary acidic protein). GFAP immunohistochemistry, × 243, *inset* × 388)

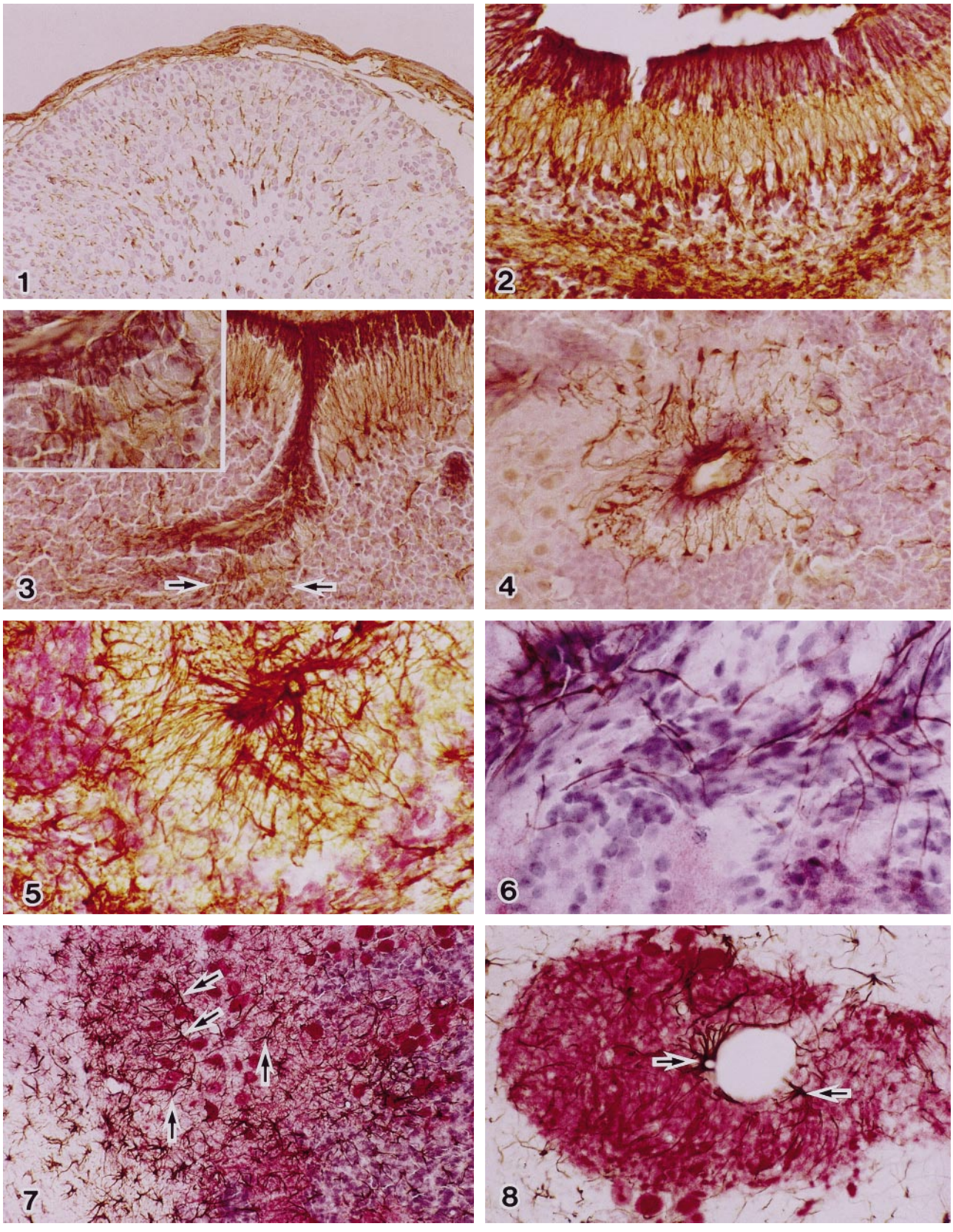
**Fig. 4** P15, cerebellopontine junction in the CVD rat. Perivascularly formed cerebellar cortex. Bergmann-like glia are seen around the vessel. Vimentin immunohistochemistry, × 270

**Fig. 5** P20, cerebellopontine junction in the CVD rat. Many Bergmann-like glia are observed. Their processes sometimes have a wavy arrangement and are disoriented. GFAP immunohistochemistry, × 243

**Fig. 6** P18, cerebellopontine junction in the CVD rat. Among penetrating EGCs, long processes of Bergmann-like glia are seen. GFAP immunohistochemistry, × 375

**Fig. 7** P18, cerebellopontine junction in the CVD rat. Bergmann-like glia *(arrows)* are noted in a cluster of Purkinje cells without EGCs. Their orientation is completely disturbed. Double-labeling immunohistochemistry of GFAP *(brown)* and calbindin *(red)* for Purkinje cells, × 122

**Fig. 8** P40, cerebellopontine junction in the CVD rat. Indistinct alignment of Bergmann-like glia and normally shaped astrocytes *(arrows)* co-exist. Double-labeling immunohistochemistry of GFAP *(brown)* and calbindin *(red)* for Purkinje cells, × 243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viously, we showed [12] that the Purkinje cells and EGCs penetrated into the pons, and that EGCs aggregated markedly around the vessels, resulting in a haphazardly arranged cerebellum. On P3, a few vimentin- or GFAP-positive astrocytes were seen in the pontine tissue, while the Purkinje cells and EGCs had already invaded the pons. No obvious Bergmann glial fibers were detected in the pons until P5. From P10 on, a small number of EGCs started to aggregate around the blood vessels. In such regions Bergmann-like glia with a few long processes extending to the vessels or in other directions were observed. Around P14, the number of EGCs in the pons was drastically increased, and many perivascularly located EGCs and Purkinje cells were seen. Coinciding with the perivascular settlement of EGCs and Purkinje cells, the Bergmann glia increased in number and were frequently observed perivascularly arranged in the cerebellar cortices (Fig. 4). However, the processes of Bergmann glia sometimes showed a wavy arrangement and were occasionally disoriented as compared to those seen in the normal cerebellar hemispheres (Fig. 5). Among the penetrating EGCs, haphazardly arranged long glial processes were seen whose morphology resembled that of the Bergmann glia (Fig. 6). Some Purkinje cells were randomly distributed in the areas without perivascular arrangement of EGCs. In such areas, a small number of the Bergmann-like glia were observed in the clusters of Purkinje cells, but their orientation was completely disturbed (Fig. 7). At P18, perivascularly aggregated EGCs were reduced in number and the arrangement of the Bergmann glia gradually became indistinct. Obscure alignments of the Bergmann-like glia and normally shaped astrocytes co-existed in the perivascularly formed molecular layers in the pons of the CVD rat (Fig. 8).

## Discussion

Disorganized cerebellar cortices have been described in human cerebellar cortical dysplasia [23], Walker's lissencephaly, and Neu-Laxova syndrome [2, 14, 15, 19]. In human cerebellar dysplasia, GFAP-positive glia are integral components of the dysplastic lesions and, thus, the cells may play an important role in the formation of dysplasia [23]. In the meander tail mutant mouse, cerebellar disorganization develops in the anterior lobe of cerebellum, in which the Bergmann glial processes are virtually absent [17, 20]. These findings suggest a close relationship between neurons and glia in the development of the cerebellum. CVD rats show perivascular aggregation of EGCs during postnatal cerebellar development; similarly, an abnormal behavior in EGCs is observed in human Walker's lissencephaly, referred to as "cortical rings" [14]. Thus, the CVD mutant may provide a unique animal model to study the pathogenesis of cerebellar dysplasia due to genetic insults. In the present studies we examined the developmental dynamics of Bergmann glia in the formation of cerebellar dysplasia.

In the cerebellar hemispheres of the CVD rat, the Bergmann glia retained their normal position and showed no obvious abnormality in their morphology until P5 when EGCs were normally located beneath the pia mater. In appearance of rearranging Bergmann glia coincided with the time and site of EGC penetration into the cerebellar parenchyma. Thus, it is most likely that dislocation of the Bergmann glia is the consequence of environmental alterations due to the abnormal behavior of EGCs. Our previous studies on EGC migration using bromodeoxyuridine (BrdU) as a tracer [13] indicated that perivascularly aggregated EGCs differentiated around the vessels, resulting in dysplastic lamination and occasional perivascular lamination in the CVD rat. It is speculated that the rearrangement of Bergmann glia may be related to the aberrant migration of the EGCs.

In those studies, many BrdU-labeled cells penetrated into the pons, leading to the development of a heterotopic and dysplastic cerebellum in the CVD rat [12, 13]. Furthermore, only haphazardly arranged GFAP-positive glial fibers were found in the cerebellopontine junctional areas in the CVD rat [12]. However, the present double-labeling immunohistochemistry revealed that GFAP-positive Bergmann glia appeared in the pons after penetration of calbindin-positive Purkinje cells and EGCs into the pons. In in vitro studies on the genesis of the mouse cerebellum two types of glial cells were identified by immunohistochemistry for glial filament protein [7]; one type possessed long processes with two to three neurons associated with these processes, giving an appearance of Bergmann glia; the other had markedly shorter arms which were nestled with several dozen neuronal cells, and resembled astrocytes of the granule cell layer. Further, time-lapse video microscopy revealed extensive cell migration along the arms of Bergmann-like glia, whereas no cells migrated along the arms of astrocyte-like astroglia. These findings indicate striking morphological and functional differences between the two types of glia. In the CVD rat, it is intriguing to note that the Bergmann-like glia were induced in the pons through the contact to the heterotopically penetrating Purkinje cells and EGCs.

In some clusters of the Purkinje cells without settlement of EGCs in the CVD pons, a small number of Bergmann-like glia were occasionally observed and their alignment was completely disturbed. Otherwise, even in the pons, Bergmann-like glia extended their processes toward the EGCs around the blood vessels. These findings suggests that morphological changes of the Bergmann-like glia depend on their contact with the Purkinje cells, but the direction of their processes may be determined by EGC settlement.

In conclusion, the present study investigated the developmental dynamics of the Bergmann glia and revealed that the glia may play an important role in EGC migration and differentiation in the development of cerebellar dysplasia in the CVD mutant. The earliest morphological abnormality in the CVD rat is cerebellar peduncle hypoplasia that is already found at embryonic day 16 [12]. At this stage, there are no obvious Bergmann glia in the cerebellum and cerebellopontine junctions. Thus, it remains to be elucidated whether the mutant gene in the CVD rat target

glial cells or not. Further understanding of the molecular mechanisms involved in the development of cerebellar dysplasia may be gained using this mutant rat.

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