Relationship of angiogenesis and microglial activation to seizure-induced neuronal death in the cerebral cortex of Shetland Sheepdogs with familial epilepsy

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Objective—To determine whether angiogenesis and microglial activation were related to seizure-induced neuronal death in the cerebral cortex of Shetland Sheepdogs with familial epilepsy.

Animals—Cadavers of 10 Shetland Sheepdogs from the same family (6 dogs with seizures and 4 dogs without seizures) and 4 age-matched unrelated Shetland Sheepdogs.

Procedures—Samples of brain tissues were collected after euthanasia and then fixed in neutral phosphate-buffered 10% formalin and routinely embedded in paraffin. The fixed samples were sectioned for H&E staining and immunohistochemical analysis.

Results—Evidence of seizure-induced neuronal death was detected exclusively in samples of cerebral cortical tissue from the dogs with familial epilepsy in which seizures had been observed. The seizure-induced neuronal death was restricted to tissues from the cingulate cortex and sulci surrounding the cerebral cortex. In almost the same locations as where seizure-induced neuronal death was identified, microvessels appeared longer and more tortuous and the number of microvessels was greater than in the dogs without seizures and control dogs. Occasionally, the microvessels were surrounded by oval to flat cells, which had positive immunohistochemical results for von Willebrand factor. Immunohistochemical results for neurons and glial cells (astrocytes and microglia) were positive for vascular endothelial growth factor, and microglia positive for ionized calcium–binding adapter molecule 1 were activated (ie, had swollen cell bodies and long processes) in almost all the same locations as where seizure-induced neuronal death was detected. Double-label immunofluorescence techniques revealed that the activated microglia had positive results for tumor necrosis factor-α, interleukin-6, and vascular endothelial growth factor receptor 1. These findings were not observed in the cerebrum of dogs without seizures, whether the dogs were from the same family as those with epilepsy or were unrelated to them.


Epileptics have chronic recurrent seizures, and epilepsy is sometimes associated with brain dysfunction (eg, memory loss, language impairment, and olfactory dysfunction).1–3 The seizures reportedly induce brain damage, including neuronal death in human epilepsy and epilepsy models in other animals.4,5 Seizure-induced neuronal death is believed to be responsible for the brain dysfunction7 and contributes to the induction of subsequent seizures.5,6 However, the mechanism of seizure-induced neuronal death remains unknown, and an appropriate treatment for prevention of seizure-induced neuronal death has not been identified.
Angiogenesis is associated with an increase in local blood flow and is purportedly neuroprotective. However, VEGF, which is an important angiogenic factor, has neurodegenerative effects such as induction of neuroinflammation as produced by activated glial cells and breakdown of the blood-brain barrier. Activated microglia are a major source of proinflammatory cytokines, such as TNF-α and IL-6. These cytokines are associated with neuronal damage in several neurologic disorders, including epilepsy, Alzheimer's disease, and amyotrophic lateral sclerosis.

In a colony of Shetland Sheepdogs with familial epilepsy, all dogs had abnormal EEG activity, but some dogs had seizures and others did not. The purpose of the study reported here was to examine whether angiogenesis and microglial activation were related to seizure-induced neuronal death in the cerebral cortex of these dogs.

Materials and Methods

Animals—Ten epileptic Shetland Sheepdogs from the same family and 4 age-matched unrelated Shetland Sheepdogs were used in this study. The related dogs were classified into 2 groups: 6 dogs with a history of recurrent seizures (age range, 20 months to 5 years) and 4 dogs without seizure history (age range, 21 months to 4.4 years). The 4 unrelated dogs, which were used as a control group, ranged in age from 2.3 to 4.6 years and were privately owned dogs that had died from nonneurologic disorders such as tumors or chronic pneumonia. A conventional vaccination schedule had been followed, and a monthly heartworm preventive treatment had been administered. Appropriate veterinary care had been provided when a generalized seizure occurred. For the study, dogs with familial epilepsy were euthanized by administration of pentobarbital. This study was performed with the approval of the Animal Research Committee, in accordance with the guidelines for animal experimentation of the Faculty of Agriculture, Tottori University.

Figure 1—Representative photomicrographs of tissue specimens from the cerebral cortex (cingulate cortex) of a Shetland Sheepdog with familial epilepsy that had seizures (A) and one from the same family but with no seizures (B). Neurons with ischemic-like change (shrunken eosinophilic cytoplasm and loss of nuclei; arrows) are evident in the specimen from the dog that had seizures, whereas no remarkable changes were observed in the specimen from the other dog. H&E stain; bar = 50 μm.
rabbit IgG as secondary antibody for 1 hour at room temperature (approx 20°C). Next, sections were incubated with an anti-mouse primary antibody and then incubated with fluorescent red dye-conjugated donkey anti-mouse IgG as secondary antibody for 1 hour at room temperature. Sections were mounted in plain 80% Tris-buffered glycerol. Histochemical evaluation was performed with a confocal imaging system.

Nonspecific staining was confirmed by replacing the primary antibody with PBS solution.

Statistical analysis—Statistical analyses were performed to compare the severity of neuronal death, the number of microvessels, and the number of IL-6–positive cells in tissue specimens from the cingulate cortex of all dogs. For analysis of the severity of neuronal death, an H&E-stained section obtained at the coronal level of corpus mamillare was used and 5 fields of the cingulate cortex and cortex around the sulci (there were no overlaps) were randomly displayed at 200X magnification via light microscopy for each dog with seizures. Healthy neurons (defined as those with a large nucleus with a prominent nuclear body and large cytoplasm with Nissl bodies) and dead neurons (ischemic-like change in neuronal specimens, with shrunken eosinophilic cytoplasm and loss of nuclei) were counted, and the mean percentage of dead neurons was calculated.

For analysis of the number of microvessels, a tissue section immunohistochemically stained with von Willebrand factor antibody (at the coronal level of corpus mamillare) was used, and 10 fields of the cingulate cortex (there were no overlaps) were randomly displayed at 400X magnification for each dog (ie, the dogs with seizures, dogs without seizures, and unrelated control dogs). The number of microvessels was counted. The mean number of microvessels was calculated for each dog and each group and compared via the Student t test (normality of data distribution was confirmed with the Kolmogorov-Smirnov test, and equality of variances was checked with the F test).

For analysis of the relationship between the severity of neuronal death and the number of IL-6–positive cells, the severity of neuronal death was defined as the number of neurons with signs of ischemic-like change.
at 400X magnification. For cerebral tissues from the dogs with seizures, 10 fields of each severity of neuronal death were randomly selected for evaluation at 400X magnification and IL-6-positive cells were counted. The mean number of IL-6-positive cells was calculated for each severity of neuronal death and compared via the Student t test after normality of data distribution was assessed. Summary statistics are reported as mean ± SD.

Results

Animals—The 6 dogs with familial epilepsy in which seizures had been evident had had generalized seizures since they were 1 to 1.5 years of age. The ictal periods ranged from 1 to 4 minutes, and seizure frequency varied from 1 seizure/wk to 1 seizure/6 mo, although the dogs were not watched continuously. Seizures were often observed at night. All dogs with seizures had been continuously monitored by EEG. With onset of seizures, as the seizures became more severe (longer lasting and more frequent), the paroxysmal discharges spread to other regions (parietal and occipital lobe). On the basis of EEG findings, these dogs were considered to have had frontal lobe epilepsy. More details about the clinical history of this family of dogs are described elsewhere. In the 4 related dogs lacking clinical epileptic signs, which had also undergone EEG, the paroxysmal discharges (spikes and sharp waves) had been localized in the frontal lobe, particularly the internal area.

Histologic and immunohistochemical evaluation—The 6 dogs with a history of recurrent seizures had histologic evidence of neuronal death (ischemic-like change in neuronal specimens, with shrunken eosinophilic cytoplasm and loss of nuclei) and astrocytosis in the cerebrum (Figure 1). Clinically and histologically, no remarkable changes were detected in brain tissues from the 4 related dogs without seizures or the unrelated control dogs.

The neurons with signs of ischemic-like change were isolated to the cingulate cortex (mean ± SD percentage of neurons with ischemic-like change, 84.2 ± 18.9%) and sulci surrounding the cerebral cortex (mean percentage of neurons with ischemic-like change, 34.4 ± 6.8%). No apoptotic bodies were evident. In the related dogs with seizures, microvessels appeared longer and more tortuous than those of the related dogs without seizures in and around the regions of neuronal death (Figure 2). In some instances, the microvessels were surrounded by oval to flat cells, which had positive immunohistochemical results for von Willebrand factor. In tissue specimens from the cingulate cortex of the related dogs with seizures, the mean number of microvessels was significantly greater (82.9 ± 5.9) than in related dogs without seizures (62.5 ± 2.4) or control dogs (61.3 ± 4.6), whereas no significant differences were found between the related dogs without seizures and the control dogs (Figure 3).

In tissue specimens from the related dogs with seizures, glial cells and neurons in the cingulate cortex and sulci surrounding the cerebral cortex had positive immunohistochemical results for VEGF; whereas...
VEGF-positive cells were not found in the cerebrum of the related dogs without seizures or control dogs (Figure 4). The double-label immunofluorescence method revealed that the VEGF-positive glial cells consisted of Iba-1-positive microglia and GFAP-positive astrocytes (Figure 5).
Greater numbers of microglia with positive results for Iba-1 were evident in the cingulate cortex and sulci surrounding the cerebral cortex in the related dogs with versus without seizures. In those dogs with seizures, Iba-1–positive microglia had a morphologically activated shape (ie, swollen cell bodies and longer processes), which was not observed in tissues from the related dogs without seizures or the control dogs (Figure 6).

The double-label immunofluorescence method similarly revealed that the activated microglia had positive results for VEGFR-1, TNF-α, and IL-6. The locations of VEGF–positive cells and activated microglia were similar to those of neurons with ischemic-like change. In addition, VEGF–positive cells and activated microglia were also observed in the areas surrounding neurons with ischemic-like change (Figure 7).

In the related dogs with seizures, positive staining for IL-6 was evident not only in microglia but also in neurons (except neurons with ischemic-like change) and GFAP-positive astrocytes in the cingulate cortex and sulci surrounding the cerebral cortex (Figure 8). Such positive staining was not identified in tissue specimens from the cerebrum of the other 2 groups of dogs. Statistical analysis revealed that the number of IL-6–positive cells significantly increased with increasing severity of neuronal death. In regions with 5 to 9 neurons with evidence of ischemic-like change, a mean of 21.7 ± 2.3 IL-6–positive cells was observed; in regions with 10 to 19 neurons with ischemic-like change, the mean was 32.8 ± 3.1, and in regions with >19 neurons with ischemic-like change, the mean was 48.9 ± 2.7. Staining for VEGFR-1 and TNF-α was not detected in GFAP-positive astrocytes.

Discussion

In the present study, histologic and immunohistochemical examinations of brain tissue specimens from Shetland Sheepdogs with familial epilepsy manifested in seizures revealed evidence of neuronal death, angiogenesis, and microglial activation that was isolated to the cingulate cortex and sulci surrounding the cerebral cortex. The neuronal death was believed to represent seizure-induced neuronal death because such change was not detected in dogs from the same family without seizures. The seizure-induced neuronal death appeared to be necrosis because no apoptotic cell bodies were detected.

Production of VEGF in neurons and glial cells, long and tortuous microvessels, proliferation of von Willem...
brand factor–positive vascular endothelial cells, and an increase in the number of microvessels are reportedly related to angiogenesis.\textsuperscript{10,13} Morphological changes in microglia (swollen cell bodies and long processes) and proinflammatory cytokine production suggest microglial activation.\textsuperscript{11} In our study, proinflammatory cytokine production was immunohistochemically detected in the cerebral cortical area of dogs with seizures. Immunolabeling of TNF-α was detected in activated microglia. Tumor necrosis factor-α can induce neurotoxic effects by downregulation of astrocytic glutamate transporter 1\textsuperscript{12} (an important transporter for astrocytic glutamate uptake from the synaptic cleft\textsuperscript{21,22}), and the induction of excess glutamate release due to microglial activation in an autocrine manner has been described.\textsuperscript{23}

A previous study\textsuperscript{19} demonstrated a high concentration of glutamate in the CSF in the cerebral cortex of dogs with familial epilepsy with versus without seizures, a decrease in positive immunohistochemical results for glutamate transporter 1, and excess glutamate immunoreactivity in almost the same distribution as that of neuronal death and activated microglia.\textsuperscript{24} Glutamate is an excitatory neurotransmitter, and excess glutamate has neurotoxic effects referred to as excitoneurotoxicity.\textsuperscript{25} Tumor necrosis factor-α, the production of which was induced in the activated microglia in the present study, could cause glutamate metabolism dysfunction, leading to neuronal death.

Immunolabeling of IL-6–positive cells was detected in activated microglia and also in neurons and astrocytes in the cerebral cortex of the Shetland sheepdogs with seizures. Neuronal hyperexcitability reportedly induces neuronal production of IL-6.\textsuperscript{26} IL-6 is believed to have direct and indirect neuroprotective effects against neuronal hyperexcitability.\textsuperscript{27-30} However, in cerebral cortical tissue specimens from the dogs with seizures, IL-6–positive cells were observed in almost the same distribution as that of neuronal death, and the increase in the number of IL-6–positive cells was related to the severity of neuronal death. The increased number of IL-6–positive cells reflected an increase in the number of IL-6–positive glial cells because neurons with ischemic-like change had no evidence of IL-6.

Overproduction of IL-6 in glial cells has been reported to cause neuronal death.\textsuperscript{31} In the cerebral cortex of dogs with seizures, production of IL-6 in glial cells may cause local overproduction of IL-6, resulting in neuronal death. Thus, microglial activation with proinflammatory cytokine production was believed to be responsible for the seizure-induced neuronal death in the cerebral cortex of the study dogs with seizures.

Neurons, astrocytes, and microglia in cerebral cortical tissue specimens from dogs with seizures had positive immunohistochemical results for VEGF. Production of VEGF in neurons and astrocytes can be induced by seizures in humans with epilepsy and in models of epilepsy in animals, and VEGF is believed to contribute to angiogenesis for neuroprotection.\textsuperscript{10,13} However, in our study, the distribution of the VEGF-positive cells was consistent with that of the activated microglia and the activated microglia were immunohistochemically positive for VEGFR-1. Vascular endothelial growth factor activates microglia via VEGFR-1 and induces an inflammatory response.\textsuperscript{14,33} In addition, production of TNF-α and IL-6 is induced by VEGF via VEGFR-1 in macrophages and monocytes.\textsuperscript{32}

Circulating monocytes are believed to enter the brain during embryonic development and differentiate into microglia, displaying many cell surface antigens found in macrophages.\textsuperscript{33} In the cerebral cortex of dogs with seizures, VEGF produced in neurons and astrocytes could activate microglia and induce overproduction of TNF-α and IL-6 in microglia. Moreover, evidence suggests that microglia are not only responsive to but also sources of VEGF.\textsuperscript{34} The activated microglia in the present study, which were immunohistochemically positive for VEGF, may induce further microglial activation in the cerebral cortex of dogs with seizures.

The angiogenesis and microglial activation detected were believed to be related to seizure-induced neuronal death. However, it could not be determined whether angiogenesis and microglial activation were a cause of seizure-induced neuronal death or sequelae to that death in the study dogs. The VEGF-positive cells and activated microglia were detected not only in the areas of neuronal death but also in the surrounding areas. This pattern suggested that angiogenesis and microglial activation can accelerate seizure-induced neuronal death and expand the lesions. Vascular endothelial growth factor–induced microglial activation with proinflammatory cytokine production is believed to play a critical role in neuronal death. Additional investigation is required into mechanisms of microglial activation and activated microglial damage of neurons in an epileptic brain after a seizure. Such investigations may lead to novel therapeutic approaches for controlling the progression of epilepsy.

**References**


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### Appendix

Primary antibodies and techniques used for immunohistochemical staining of brain tissues.

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<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
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<td>GFAP&lt;sup&gt;+&lt;/sup&gt;</td>
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