NOTE *Clinical Pathology*

Generation of Monoclonal Antibody against Canine Neural-Cell Adhesion Molecule

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ABSTRACT. A monoclonal antibody, K9BYU, was generated using *Escherichia coli* recombinant extracellular domain of canine neural-cell adhesion molecule (N-CAM) as an antigen. Immunoreactivity of K9BYU to insect cell recombinant canine N-CAM was demonstrated by Western blotting using Sf9 insect cells transfected with the canine N-CAM gene. In Western blotting against canine brain tissue, K9BYU detected three isoforms of N-CAM that correspond to three major isoforms of human and mouse N-CAM (N-CAM-120, -140, and -180). From these results, K9BYU was considered to be a useful tool for research of canine N-CAM.
KEY WORDS: canine, monoclonal antibody, neural-cell adhesion molecule.

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Neural cell adhesion molecule (N-CAM), also known as CD56, is a cell surface glycoprotein belonging to the immunoglobulin superfamily and mediates homophilic and heterophilic cell-cell interactions [9]. N-CAM is expressed as three major isoforms generated by alternative splicing of a single gene product with identical extracellular domains: two transmembrane isoforms of 140-kDa (N-CAM-140) and 180-kDa (N-CAM-180), and a glycosylphosphatidylinositol-anchored 120-kDa isoform (N-CAM-120) [10]. All of the three N-CAM isoforms are abundantly expressed in the brain, where neurons express N-CAM-140 and -180 and glial cells express N-CAM-120 and -140 [7]. These isoforms have been shown to play important roles for cell proliferation and migration, axonal outgrowth, fasciculation, and synaptic plasticity [11]. An isoform of N-CAM-140 is also found on the surface of natural killer (NK) cells [2], rendering the molecule as an important cell surface marker to distinguish NK cells from T and B cells in circulating blood [6].

Despite the importance of N-CAM for the function of neuron/glial cells as well as identifying NK cells in peripheral blood, N-CAM has been poorly investigated in dogs due to a lack of antibody for canine N-CAM. In the present study, we thus developed an antibody for canine N-CAM.

To generate a monoclonal antibody that reacts to all isoforms of canine N-CAM, the recombinant extracellular domain of canine N-CAM was used as an antigen. For preparation of the recombinant protein, total RNA was extracted from canine brain tissue, according to the guidelines of Nippon Veterinary and Life Science University. After extraction, RNA was subjected to reverse transcription polymerase chain reaction (RT-PCR) using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and *LA Taq* DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) to amplify the nucleotide sequence of the extracellular domain of canine N-CAM. For the amplification, the following primer set that was designed based on the nucleotide sequence of canine N-CAM-140 (GenBank accession number: AY860627) was used: 5' primer: 5'-ACCGCTCGAGGTGGATATTGTC-3' and 3' primer: 5'-ACCGCTCGAGCTATGTAGGGCTGCC-3', containing *Xho*I site at the 5'-end of both primers. After 25 cycles of amplification, the PCR product was digested by *Xho*I restriction enzyme (TaKaRa Bio Inc.) and cloned into the His-tag sequence containing the pET14b *Escherichia coli* (*E. coli*) expression vector (Novagen, Damstadt, Germany). The vector was introduced into *E. coli* BL21 (Novagen), and crude protein was extracted from the *E. coli* using BugBuster Protein Extraction Reagent (Novagen). After the protein was solubilized in denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, pH 8.0), N-terminally His-tagged extracellular domain of canine N-CAM (His-dNCAM-ECD) was purified from the protein extract with a Ni-NTA agarose column (Qiagen, Hilden, Germany) and finally refolded by dialysis against phosphate buffer saline.

Generation of mouse monoclonal anti-canine N-CAM antibody was performed by a conventional method [3] using His-dNCAM-ECD as an antigen. Briefly, BALB/c mice were immunized with 125 μg of His-dNCAM-ECD emulsified with an equal volume of Freund's adjuvant (BD Bioscience, Franklin Lake, NJ, U.S.A.) with 3 subcutaneous injections every 2 weeks. Spleen lymphocytes of the mice were collected and fused with a non-secreting mouse myeloma cell line (P3-X63-Ag8-U1) by polyethylene glycol (Sigma-Aldrich, St. Louis, MO, U. S. A.). For screening of the hybridomas producing an antibody for canine N-CAM, an enzyme-linked immunosorbent assay using His-

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dNCAM-ECD and the culture supernatant of the cells was performed. One hybridoma cell-clone whose culture supernatant reacts to His-dNCAM-ECD was selected, and the monoclonal antibody produced was purified from the culture supernatant using protein G agarose; this monoclonal antibody was named K9BYU.

The immunoreactivity of K9BYU to canine N-CAM was examined by Western blotting using Sf9 insect cells that express V5/His-tagged canine N-CAM-140 on their surface (V5/His-cNCAM-Sf9). For examination of the cross-reactivity of K9BYU with feline N-CAM, Sf9 cells expressing V5/His-tagged feline N-CAM-140 (V5/His-fNCAM-Sf9) were also subjected to the same Western blotting. For preparation of the cells, plasmid vectors pIB/V5-His containing the full-length sequence of canine or feline N-CAM-140 fused C-terminally with the V5/His-tag were transiently transfected into Sf9 cells using Cellfectin Reagent (Invitrogen). Each of the plasmid-transfected cells was lysed in cell-lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, pH 7.9). The lysates were applied to a 7.5% sodium dodecyl sulfate-polyacrylamide gel (4.5 μg protein/lane), transferred onto a polyvinylidene fluoride membrane (Roche Diagnostics, Indianapolis, IN, U.S.A.), and blotted with K9BYU or mouse monoclonal anti-V5 antibody (Invitrogen). The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cappel, Aurora, OH, U.S.A.) and immunoreactive bands were visualized with a LumiGLO chemiluminescent substrate system (KPL, Gaithersburg, MD, U.S.A.).

K9BYU produced a band of 140 kDa by Western blotting against V5/His-cNCAM-Sf9 (Fig. 1, lane 1). A band of the same size was also detected using anti-V5 antibody against V5/His-cNCAM-Sf9 (Fig. 1, lane 3), suggesting that K9BYU recognizes the recombinant canine N-CAM expressed by Sf9 cells. Conversely, K9BYU failed to produce a band against V5/His-fNCAM-Sf9 (Fig. 1, lane 2), although anti-V5 antibody produced a band against the cells at the size of 140 kDa (Fig. 1, lane 4), suggesting a lack of K9BYU cross-reactivity to feline N-CAM.

Using the monoclonal antibody K9BYU, we next performed Western blotting against lysate prepared from canine brain tissue that would express all three major isoforms of N-CAM (N-CAM-120, -140, and -180). Cell lysate prepared from peripheral blood mononuclear cells (PBMC), in which N-CAM-140-expressing NK cells should exist, was also examined by the same assay. Additionally, PBMC cultured in the presence of phytohemagglutinin (PHA; Sigma-Aldrich) for 3 days was subjected to the assay, because up-regulation of N-CAM mRNA expression has been reported in canine PBMC cultured with PHA [1]. After homogenization of brain tissue and PBMC samples in cell-lysis buffer, the lysates were subjected to Western blotting using K9BYU (4.5 μg protein/lane for brain tissue and 11 μg protein/lane for PBMC corresponding to 1.8×10^6 cells).

K9BYU produced three discrete immunoreactive bands in canine brain tissue (Fig. 2, lane 1) similar to the results of

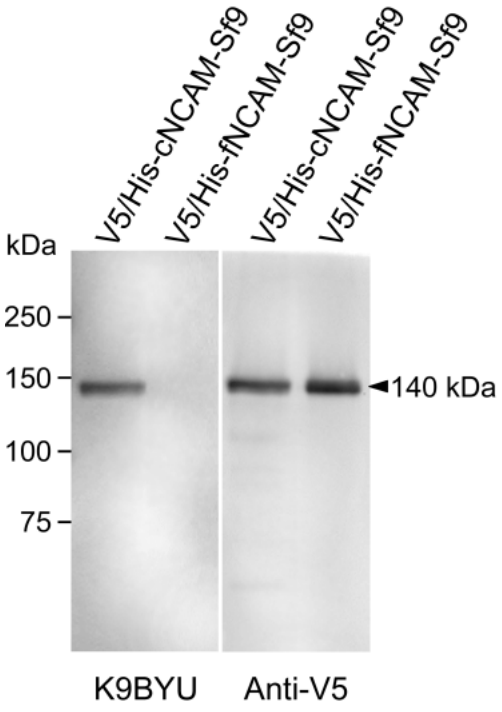

Fig. 1. K9BYU recognizes insect cell recombinant canine N-CAM by Western blotting. Cell lysates prepared from Sf9 insect cells that express V5/His-tagged canine N-CAM-140 (V5/His-cNCAM-Sf9) and V5/His-tagged feline N-CAM-140 (V5/His-fNCAM-Sf9) were subjected to Western blotting using K9BYU or anti-V5 antibody. An immunoreactive band was detected at the size of 140 kDa against V5/His-cNCAM-Sf9 (lane 1) but not V5/His-fNCAM-Sf9 (lane 2) using K9BYU. Using anti-V5 antibody, a band at the size of 140 kDa was detected against V5/His-cNCAM-Sf9 (lane 3) and V5/His-fNCAM-Sf9 (lane 4). Each lane was applied with 4.5 μg protein of the cell lysates.

Western blotting against mouse and human brain tissues using anti-pan-NCAM antibody [4, 5]. Two of the three bands were detected at the sizes of 120 and 140 kDa, suggesting that K9BYU recognizes the canine counterparts of mouse/human N-CAM-120 and -140. The uppermost band was detected at the size of 220 kDa. Although the size of N-CAM-180 has been described as 180 kDa, its size in excess of 180 kDa has been demonstrated by Western blotting in mouse brain tissue [5] and a human astrocyte cell line [8] due to post-transcriptional modification, including glycosylation. Therefore, it was considered that K9BYU detected canine N-CAM-180 with similar modification.

In contrast to brain tissue, no band was detected in PBMC (Fig. 2, lane 2) and those cultured with PHA (Fig. 2, lane 3), although a larger amount of protein compared to that of brain tissue was used for the assay. We have previously demonstrated that expression of canine N-CAM is undetectable in PBMC at the level of mRNA, possibly due to the low

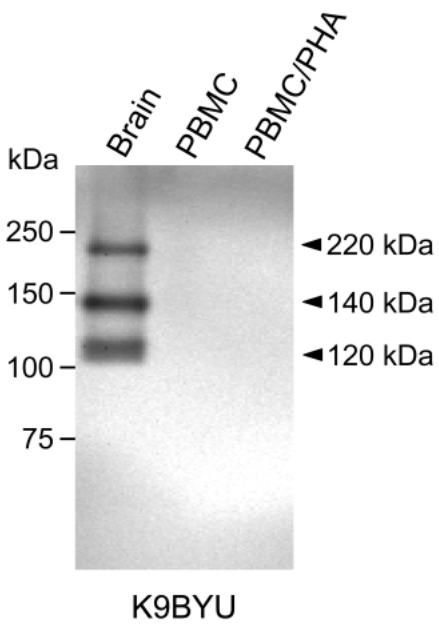

Fig. 2. K9BYU recognizes three isoforms of N-CAM in canine brain tissue. Lysates prepared from brain tissue, peripheral blood mononuclear cells (PBMC), and PBMC cultured with PHA were subjected to Western blotting using K9BYU (4.5 μg protein /lane for brain tissue and 11 μg protein/lane for PBMC). Three immunoreactive bands with sizes of 120, 140, and 220 kDa were detected against brain tissue (lane 1). No band was detected against PBMC (lane 2) and PBMC cultured with PHA (lane 3).

numbers of NK cells in PBMC [1]. Although that expression became detectable after culturing in the presence of PHA, it was still much lower than that in brain tissue. The frequency of NK cells in PBMC may be lower than the detectable level with K9BYU, even cultured with PHA.

K9BYU was also examined for detection of canine N-CAM with flow cytometric analysis using canine N-CAM-expressing Sf9 cells and with immunohistochemistry using formalin-fixed and frozen sections of canine brain. However, K9BYU failed to detect positive signal in these assays (data not shown), suggesting that the antibody recognizes only denatured N-CAM protein.

In the present study, K9BYU apparently detected three major isoforms of canine N-CAM by Western blotting. The monoclonal antibody K9BYU could contribute to the investigation of canine N-CAM.

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