Distribution of ecotropic retrovirus receptor protein in rat brains detected by immunohistochemistry

Sayaka Takase-Yoden and Rihito Watanabe

Institute of Life Science, Soka University, Tangi-cho 1-236, Hachioji, Tokyo 192-8577, Japan

Friend murine leukaemia virus (FrMLV) FrC6 clone A8 causes spongiform degeneration in the central nervous system (CNS) of newborn but not 3-week-old rats. To assess whether expression of the ecotropic MLV receptor (CAT-1) in the CNS correlates with the pathogenicity of the A8 virus, we generated an anti-CAT-1 antibody raised against a synthetic peptide that corresponds to the carboxyl-terminal amino acid sequence of CAT-1. In the CNS of newborn and 3 to 4-week-old rats, a strong immunoreactivity against the antibody was detected in most of the endothelial cells. However, almost no expression of CAT-1 was detected in the CNS of 21-week-old rats. In newborn rats, many parenchymal cells in the brain as well as the vascular wall expressed CAT-1 antigen. These findings suggest that retrovirus receptor-bearing glial cells contribute to the neuropathogenesis of MLV, including clone A8, which induces spongiosis in rats only when inoculated into newborns.

An important determinant of infection with ecotropic MLV in rodents is the expression of the receptor, which is known to be an Na+-independent transporter for cationic amino acids (CAT-1) (Albritton et al., 1989; Kim et al., 1991; Wang et al., 1991). The CAT-1 contains 14 hydrophobic potential membrane-spanning sequences. The carboxyl terminus of CAT-1 is proposed to be in the cytosol (Albritton et al., 1989; Kavanaugh et al., 1994). The third extracellular loop of CAT-1 contains the virus-binding site (Albritton et al., 1993; Yoshimoto et al., 1993) and two N-linked glycosylated sites that are glycosylated intracellularly (Kim & Cunningham, 1993). The amino acid sequence near the two glycosylated sites in loop 3 of the receptor derived from rat glial cells, F10 (F10-cat-1, originally designated as F10-ecoR) is different from that of the receptor derived from NIH 3T3 cells (Takase-Yoden & Watanabe, 1999). The transcript of the cat-1 gene was

The spongiform neurodegeneration caused by a Friend murine leukaemia virus (FrMLV) variant, FrC6 virus clone A8, is generated when the virus is inoculated into newborn rats, but not when it is inoculated into 3-week-old rats (Watanabe & Takase-Yoden, 1995; Takase-Yoden & Watanabe, 1997). Age-dependency of neuropathogenicity is observed for other neuropathogenic MLV (Czub et al., 1991; Kai et al., 1996). Czub et al. (1991) reported that age-dependent resistance to neuropathogenic disease is a consequence of the restriction of virus replication within the central nervous system (CNS) due to the developmental state of the system. Furthermore, this developmental resistance of the CNS to infection can be bypassed by direct introduction of virus-infected microglia into the brain (Lynch et al., 1995).

Fig. 1. Detection of CAT-1 protein in cultured cells after introduction of the cat-1 gene by radio immunoprecipitation using the anti-CAT-1 antibody. SIRC, normal SIRC cells; F10-cat-1/SIRC, SIRC cells after introduction of the cat-1 gene derived from F10 cells; NIH-cat-1/SIRC, SIRC cells after introduction of the cat-1 gene derived from NIH 3T3 cells; vector/SIRC, SIRC cells after introduction of the expression vector, pcDNA3.1. Asterisks indicate the position of the CAT-1 protein on SDS-polyacrylamide gels (Kim & Cunningham, 1993).
detected in a wide variety of tissues in rodents, but not in liver (Kim et al., 1991; Wu et al., 1994; Aulak et al., 1996; MacLeod, 1996; Ito & Groudine, 1997). In the brain, the distribution of the cat-1 transcript has been investigated (Stoll et al., 1993; Braissant et al., 1999), but the distribution of the product of the gene has not. In this study, we present the distribution of the CAT-1 protein in rat brain as determined by immunohistochemical analyses.

Serum against a synthetic peptide corresponding to a sequence of 15 residues in the carboxyl terminus of CAT-1 (NH$_2$ AGQAKTPDNSLDQCK COOH) was purified by affinity chromatography using agarose gel coupled with the synthetic peptide. To examine the specificity of the antibody, a radio immunoprecipitation assay was performed using SIRC cells, resistant to ecotropic virus infection, F10-cat-1/SIRC cells and NIH-cat-1/SIRC cells, into which were introduced the F10-cat-1 gene and the receptor gene derived from NIH 3T3 cells (NIH-cat-1), respectively (Takase-Yoden & Watanabe, 1999; Ikeda et al., 2000). The cells labelled with [$^{35}$S]methionine and [$^{35}$S]cysteine were lysed in radio immunoprecipitation assay buffer (Ikeda et al., 1995). The anti-CAT-1 antibody was added to the lysate diluted with NET-gel buffer (Sambrook et al., 1989). The proteins precipitated with protein G–Sepharose beads were analysed by 15% SDS–PAGE and autoradiography with a BAS 2000 Bio-Image Analyser (Fuji Photo Film). The image was scanned into Adobe Photoshop (version 5.0 LE), and Microsoft Power Point (version 8.0) was used for the final layout of the figures. Proteins ranging from 70 to 85 kDa were abundantly precipitated from the lysates of F10-cat-1/SIRC cells and NIH-cat-1/SIRC cells (Fig. 1). The molecular mass of these proteins is identical to that of the mature CAT-1 of NIH 3T3 cells (70 kDa) determined by Kim & Cunningham (1993). A 53 kDa protein, almost identical in size to unglycosylated CAT-1 (55 kDa), was also precipitated from the both cells. An additional 60 to 70 kDa protein in NIH-cat-1/SIRC cells is identical in size to the CAT-1 with one of two sites glycosylated (Kim & Cunningham, 1993). The 158 kDa protein precipitated from NIH-cat-1/SIRC cells is identical in size to the dimeric CAT-1 protein (Masuda et al., 1999). A large amount of CAT-1 protein is expressed in NIH-cat-1/SIRC cells compared with F10-cat-1/SIRC cells; the protein was glycosylated to a moderate extent and the oligomerized protein may have accumulated. Amino acid substitutions near two glycosylated sites between F10-CAT-1 and NIH-CAT-1 might affect the potential for glycosylation.

For immunohistochemical analyses, the tissues and the cultured cells were fixed with 4% paraformaldehyde buffered with 0.12 M phosphate (pH 7.3). The CAT-1 antigen was detected by the indirect streptavidin–biotin peroxidase method. Images were photographed using colour reversal films and scanned using POLASCAN 35 Ultra (Polaroid) into Adobe Photoshop (version 5.0 J). Microsoft Power Point (version 8.0) was used for the final layout of the figures. NIH 3T3 cells showed specific immunoreactivity to the anti-CAT-1 antibody (Fig. 2a, b). The F10-cat-1/SIRC cells reacted to the antibody (Fig. 2c), while normal SIRC cells, which are not susceptible to A8 virus (Takase-Yoden & Watanabe, 1999), did not (Fig. 2d). We carried out immunohistochemical analyses of the rat brain using frozen sections and the anti-CAT-1 antibody. Immunoreactivity to the anti-CAT-1 antibody was observed in the cerebrum, cerebellum and spinal cord of newborn rats. In these regions, most endothelial cells showed

---

**Fig. 2.** Immunohistochemical staining of the CAT-1 protein in cultured cells. NIH 3T3 cells (a), F10-cat-1 gene-containing SIRC cells (c) and normal SIRC cells (d) were immunostained with the anti-CAT-1 antibody. Normal rabbit IgG used instead of the primary antibody served as a control of NIH 3T3 cells (b). Bars, 100 µm.
strong immunoreactivity to the antibody (Fig. 3a, b). In addition, some of the glial cells also showed immunoreactivity to the antibody (Fig. 3c). There was a reduction in the number of glial cells stained and the intensity of immunostaining in the 25-day-old rats although the expression of CAT-1 antigen in the vascular wall was comparable with that in the newborn rats (Fig. 3d). As is shown in Fig. 3(c), the strong deposition of brown polymerized 3,3’-diaminobenzidine tetrahydrochloride after the immunohistochemical procedure in the cytoplasm, including the perikaryon that was observed in the parenchymal cells clearly distinct from the vascular wall in the brain of newborn rat, had disappeared in the 25-day-old rats. In the brain of 21-week-old rats, even the endothelial expression of CAT-1 antigen was diminished to a faint trace of brown on a few vascular walls (Fig. 3e). The blood vessels in the choroid plexus were devoid of CAT-1 expression (Fig. 3d, j, arrowhead). The level of CAT-1 expression was not remarkable in any organs other than the CNS. Only a fuzzy deposition of immunolabelled peroxidase products was observed in a few cells in the spleen, thymus and liver of newborn rats (Fig. 3f, g). In these tissues, the vascular wall exhibited negative immunostaining for anti-CAT-1 antibody (Fig. 3f, g). In order to ensure the specificity of the antibody, the sera were absorbed with the synthetic CAT-1 peptide. The immunoreactivity of the absorbed anti-CAT-1 serum to the brain of 25-day-old rats disappeared (Fig. 3j, k), although the immunoreactivity of the anti-GFAP (glial acidic fibrillary protein) serum absorbed with the synthetic CAT-1 peptide remained (Fig. 3l, m).

For detection of viral antigen, anti-Rauscher MLV gp70 antibody (Quality Biotech Incorporated Resource Laboratory) and paraffin sections were used. The viral antigen in the CNS of 6-week-old rats infected with the A8 virus at birth distributed in the same manner as the CAT-1 antigen in newborn normal rats. In spite of a ubiquitous spread of viral antigen along the vascular wall in the brain of infected rats, the spongiform degeneration induced by A8 infection did not for the most part accompany the staining (Fig. 3h). In contrast, near the early lesions of spongiotic formation, microglial-like structures were often detected by immunostaining with anti-gp70 antibody (Fig. 3i).

Immunohistochemical analyses showed that endothelial cells and glial cells in the brains of newborn rats expressed the CAT-1 protein. In the brains of rats infected with A8 virus at birth, viral antigens were mainly detected in the endothelial cells, though some glial cells also expressed the antigen. These findings indicate that the distribution of receptor protein in the rat brain was well correlated with the cell tropism of the A8 virus. When A8 virus is injected into 3-week-old rats, it is recovered from brain and thymus, but encephalopathy is not observed (Takase-Yoden & Watanabe, 1997). In the present study, we showed that the in vivo expression of CAT-1 in the CNS was age-dependent. In the CNS of newborn and 3 to 4-week-old rats, a strong immunoreactivity against the anti-CAT-1 antibody was detected in most of the endothelial cells. However, almost no expression of CAT-1 was detected in the CNS of 21-week-old rats. Furthermore, in newborn rats, many parenchymal cells in the brain also expressed CAT-1 antigen.

Those retrovirus receptor-bearing glial cells, which appear in newborns, might well contribute to the pathogenesis of the neuropathogenic variants of MLV, including A8, because virus infection of endothelial cells does not appear to induce spongiform degeneration directly. The discrepancy between the distribution of viral antigen in endothelial cells and that of the spongiotic lesions in the CNS became more obvious when we examined the distribution of viral antigen in the spinal cord of infected animals and compared the A8 virus with the PVC211 virus. A neuropathogenic variant of FrMLV, PVC211 (Kai & Furyta, 1984; Masuda et al., 1992), causes more severe pathological change in the spinal cord than A8 (Takase-Yoden & Watanabe, 1997). In the spinal cord of PVC211-infected rats, we found many infected microglia, while the A8 infection induced fewer lesions in the spinal cord with little or no infection of microglia (unpublished results) although the virus titre from the spinal cord and the antigen expression in endothelial cells are comparable for the two viruses (Kai & Furuta, 1984; Takase-Yoden & Watanabe, 1997). A correlation between microglial infection and the distribution of regions undergoing spongiform degeneration in the brains of rats infected with similar neuropathogenic viruses has been reported (Baszler & Zachary, 1990, 1991; Lynch et al., 1991; Czub et al., 1995). These observations suggest that infection of glial cells in newborns plays an important role in induction of spongiform neurodegeneration and age-dependent expression of the receptor protein on glial cells could explain the age-dependency of the neuropathogenicity.

The distribution of the transcript of the gene encoding CAT-1 has been investigated by in situ hybridization, Northern hybridization, and RNase protection analyses. In this study, we discerned the existence of the CAT-1 protein in tissues by immunohistochemistry. The strong expression of CAT-1 in endothelial cells of the brain is consistent with the finding by Stoll et al. (1993) that the cat-1 mRNA content of capillaries averaged 38 times that of whole brain, when measured by RNase protection assay. But, there was a discrepancy between the distribution of the transcript of the cat-1 gene and that of the CAT-1 protein. The transcript of the cat-1 gene is not detected in the liver of adult rodents (Kim et al., 1991; Wu et al., 1994; Aulak et al., 1996; MacLeod, 1996; Ito & Groudine, 1997). In addition, no cationic amino acid transporter activity or ecotropic MLV infection is observed in mouse hepatocytes (Jaenisch & Hoffman, 1979; White & Christensen, 1982; Kim et al., 1991). Whereas, in the present study, some cells of the livers of newborn rats showed faint immunoreactivity to the anti-CAT-1 antibody. Foetal liver and regenerating liver cells express the gene and are susceptible to retrovirus infection (Wu et al., 1994; Clapp et al., 1991; Hatzoglou et al., 1990), and the expression of this gene is enhanced in rapidly proliferating
Fig. 3. Immunohistochemistry for CAT-1 protein in normal rats (a–g), and the viral antigen gp70 in the brains of A8 virus-infected rats (h, i). Distribution of the CAT-1 protein in normal brain (a–e) obtained from newborn (a, b, c), 25-day-old (d) and 21-week-old (e) rats was detected by use of the anti-CAT-1 antibody. In the newborn rat, most of the vascular wall is CAT-1 positive (arrow in a), which is clear at a higher magnification (b). Beside the blood vessels, CAT-1-positive glial-like cells were observed (arrowhead in c). The arrowheads in (d) and (j) indicate the area of choroid plexus where CAT-1 antigen was not detected in the vascular walls. In the brain parenchyma (arrow in d) of the 25-day-old rat, the intensity of staining in the vascular wall was comparable to that in the newborn. In the brain of the 21-week-old rat, CAT-1 expression is negligible (e), but the vascular wall shows a faint expression of CAT-1 antigen (arrow in e). In the liver (f) and the thymus (g) of newborn normal rats, few cells are immunostained (arrowheads). In the brain of A8-infected rats, the viral antigen is detected in the vascular wall (arrow in h) and glial cells (arrowhead in i) as well. After absorption of the anti-CAT-1 serum with the cat-1 synthetic peptide, the immunoreactivity of the serum to the brain of 25-day-old rats disappeared (k), although the anti-GFAP serum, absorbed with the cat-1 synthetic peptide, was still immunoreactive to the brain of 25-day-old rats (m). Before absorption with the cat-1 peptide, both the anti-CAT-1 serum (j) and anti-GFAP serum (l) were immunoreactive to the brain of 25-day-old rats. The arrowheads in (j)–(m) indicate the area of choroid plexus. Bars, 200 µm.
cells of haematopoietic origin (Yoshimoto et al., 1992). Therefore, the cells expressing the CAT-1 protein in the liver of rats might be proliferating cells. In most tissues, transcript of the cat-1 gene is detected (Kim et al., 1991; Wu et al., 1994; Aulak et al., 1996; MacLeod, 1996; Ito & Groudine, 1997). In whole brain, the transcript of the cat-1 gene is present both in neurons and astrocytes, and probably in oligodendrocytes, based on in situ hybridization (Braissant et al., 1999). But we did not find the CAT-1 antigen in heart, lung, intestine or kidney, or in neurons (data not shown). Although we confirmed the specificity of the anti-CAT-1 antibody by immunoreactivity to cat-1 gene-introducing cells and loss of the immunoreactivity of the antibody after absorption by the cat-1 peptide, the antibody may not detect low-level expression of CAT-1 protein. The possibility also remains that our antibody failed to recognize the conformational change or modification of the carboxyl terminus, the portion of the CAT-1 protein that the antibody recognizes.

The authors thank Katsumi Goto for performing histological studies. This work was supported in part by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science, and a Health Science Research Grant, Research on Brain Science from the Ministry of Health and Welfare.

References


Received 14 December 2000; Accepted 11 April 2001