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INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy (Ph. D) in Veterinary Medicine on December 25, 2003 to 8 recipients and March 25, 2004 to 9 recipients.

The titles of their and other information are as follows :

Bovine insulin-responsive glucose transporter (GLUT 4) :
cDNA cloning, tissue distribution, and postnatal development

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Facilitated transport of glucose across the plasma membrane is mediated by specific glucose transporters (GLUT). GLUT is a family of at least 10 isoforms, each of which functions in tissue- and age-specific manners. As an attempt to examine possible relations of GLUTs to ruminants' specific glucose metabolism, in the present study, I have cloned cDNA of bovine GLUT 4, and examined the GLUT 4 mRNA and protein expression patterns in bovine tissues. The author also determined the GLUT expression changes during postnatal development. The major findings were as follows :

1) Nucleotide sequence analysis of the cDNA clones revealed that bovine GLUT 4 cDNA was composed of 2,642 base pairs with a coding region for a 509 amino acid protein. The deduced amino acid sequence was 65% and 91-94% identical with bovine GLUT 1, and GLUT 4 of the rat, mouse and human, respectively. Although the amino acid sequence of the GLUT 4 COOH-terminal region is highly conserved among the species so far reported, asparagine (Asn) at position 508 of this region was replaced by histidine (His) in bovine GLUT 4. The tissue distribution of GLUT 4 was also examined by Northern blot analy-

sis using a probe prepared from the bovine cDNA. GLUT 4 mRNA was detected in skeletal muscle, heart, and adipose tissue, but not in the liver, kidney, lung, brain, or spleen.

2) The nucleotide sequence of cDNA coding the 38-amino acid of the COOH-terminal domain of GLUT 4 was determined in the sheep, goat and pig, and compared with that of bovine which has a unique amino acid conversion of Asn 508 to His. The deduced amino acid sequence was completely identical in the three species, and did not have the amino acid conversion at position 508. Thus, the conversion of Asn 508 to His is unique to bovine but not common to ruminants, and it is not the primary reason for the insulin resistance widely seen in ruminant species.

3) The postnatal change in the bovine GLUTs, the protein levels of GLUT 1 and GLUT 4 were measured by Western blot analysis of skeletal muscles, adipose tissue and brain of Holstein male calves aged from 0 to 12 months. The GLUT 1 protein content in brain, adipose tissues and skeletal muscles unchanged during the postnatal period. In contrast to GLUT 1, the GLUT 4 levels in skeletal muscle and subcutaneous adipose tissue decreased gradually, and at 12-month old,

they were about 40% of those seen at 0 - month old. These results are contrast to those in non-ruminant species, in which GLUT 4 in-

creases during postnatal development, and may be related to the insulin-resistance seen in adult ruminants.

Original papers of this thesis appeared in *J. Anim Sci.*, 75 : 182-188 (1997), *J. Vet. Med. Sci.*, 60 : 769-771 (1998) and 63 : 1071-1075 (2001).

Study of chromogranin A as a stress index in horses.

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Chromogranin A (CgA) is a highly acidic secretory protein, which is co-stored with catecholamines in the adrenal medulla, and co-released in response to splanchnic nerve stimulation. Furthermore, a prompt elevation of salivary CgA-like immunoreactivity (CgA-LI) was shown in psychosomatic stress in human. In this study, I tried to establish an assay system for equine CgA and estimate utility of CgA for a stress index in horses.

The first chapter dealt with the cloning and expression of equine CgA complementary deoxyribonucleic acid (cDNA). Analysis of the nucleotide sequence (total 1,828bp) showed that the equine CgA consists of 448 amino acids preceded by an 18-residue signal peptide. Comparison of the amino acid sequence of equine CgA with those of human, porcine, bovine, mouse, rat and frog CgA shows highly conservation of the NH₂- and COOH-terminal regions as well as the potential dibasic cleavage site. Data on nucleotide and amino acid sequences of equine CgA may be useful to investigate the expression, secretion and biological activity of CgA.

In the second chapter, a region-specific enzyme immunoassay (EIA) for equine CgA was developed using synthetic equine CgA

(335-365), based on the amino acid sequence revealed in the first chapter. The usefulness and specificity of the antiserum was checked by immunohistochemistry and western blotting. Standard displacement curve of the developed EIA system was paralleled with the dilution curves of equine plasma and saliva. The minimum detection limit of the assay was approximately 200 fmol/ml. Intra- and inter-assay reproducibility of plasma and saliva were 9.26-12.11% and 10.85-14.22%, respectively. These results indicate that the present EIA system may be of great use for the measurement of CgA in plasma and saliva.

In the third chapter, using the EIA system, we studied the CgA secretion from salivary glands, the circadian rhythm of plasma and saliva CgA concentration, and its changes in physical and psychological stress conditions as following.

- 1) CgA secretion from parotid and submandibular glands in an anaesthetized horse.
- 2) The circadian rhythm of the CgA concentration in plasma and saliva.
- 3) Plasma and saliva CgA responses to exercise.
- 4) Plasma and saliva CgA responses to

novel environmental stress.

5) Plasma and saliva CgA responses to sexual behavior of stallions.

These experiments indicate that the present EIA is greatly useful for the measurement of CgA levels in equine plasma and saliva. I found the unique changes of plasma and saliva CgA levels with the sympathoadrenal system activity under various stress conditions. The present EIA system is sensitive and reproducible, and has several advantages in comparison with the measurement of catecholamines. The handling of this assay

system is also easy and needs only popular laboratory instruments.

In conclusion, the measurement of plasma and saliva CgA with the EIA system developed in this study can be a useful tool for evaluation of physical and psychological stress in horses. The present study on stress response of the race horses is expected to allow elucidation of the exercise ability and to apply as a novel marker for evaluation of equine temperament and control of body condition.

Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 62 : 953-959 (2000), *Adv. Exp. Med. Biol.*, 482 : 137-142 (2000), *Cells Tissues Organs*, 172 : 29-36 (2002), *Jpn. J. Vet. Res.*, 49 : 3-17 and *Biomed Res.*, 22 : 261-264 (2001).

Regulation of the bovine membrane tethering protein p115 gene in mammary epithelial cells

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The membrane tethering protein p115 is a vesicular transport factor increasing traffic efficiency, in various types of vesicular transport, by tethering traffic vesicles to their acceptor membrane preceding the fusion of these membranes each other. The present study was designed to examine developmental changes of p115 and its mRNA in bovine mammary gland during the course of hormonally induced lactation, and to understand mechanisms of transcriptional regulation of the p115 gene in mammary epithelial cells.

The amounts of p115 and its mRNA in mammary glands from Holstein cows in which lactation was hormonally induced were measured. The p115 mRNA levels determined by Northern blotting, was the highest in the

developing stage. In contrast to mRNA, the p115 protein levels, determined by immunoblotting, was the highest during the lactating stages. Immunohistochemical examination on the mammary tissues revealed that p115 was predominantly localized in mammary epithelial cells. The increased level of p115 mRNA during the developing stage and the maintenance of p115 protein during lactation suggests that the synthesis of p115 is regulated during mammary development and differentiation, and that this protein is involved in lactation in cows.

As the first step to elucidate the molecular mechanisms of transcriptional regulation of the membrane tethering protein p115, a genomic DNA clone including a 5'-flanking

region of the p115 gene was isolated from a bovine genomic DNA library. Southern blot analysis showed that the p115 gene was single copy gene. Nine transcriptional initiation sites were determined by the CapSite Hunting method. In primary cultured bovine mammary epithelial (BME) and MCF-7 cells, luciferase reporter gene assays of deletion constructs of the 5'-flanking region suggested the region required for promoter activity of the bovine p115 gene. In the promoter region, a binding site for nuclear respiratory factor 1 (NRF-1) was present. In both BME and MCF-7 cells, mutation to impair the NRF-1 consensus sequence in the p115 promoter gave substantially lowered luciferase expression. An electrophoretic mobility shift assay and supershift assay showed that NRF-1 specifically bound to the NRF-1 consensus sequence in the p115 gene promoter. Estradiol-17 β , insulin or both stimulated the p115 promoter activity correlated with the cell proliferation

rate in MCF-7. These results indicate that the NRF-1 consensus sequence in the p115 promoter is important for promoter function and involves binding of NRF-1. Furthermore, the p115 promoter may be activated in association with proliferation of mammary epithelial cells.

In conclusion, during the course of hormonally induced lactation, it was suggested that transcription of the p115 gene is increased by activation of transcriptional regulatory factor NRF-1 in association with the mammary epithelial cell growth primed with estradiol-17 β . The increased p115 contents in the lactating mammary epithelial cells would be implicated in various vesicular transports related to lactation, although the mammary content of p115 protein is regulated not only by p115 mRNA levels but also by various other factors such as translation and degradation of the protein.

Original papers of this thesis appeared in *Journal of Endocrinology*, 166 : 319-327 (2000) and *Biochimica et Biophysica Acta*, 1629 : 60-72 (2003).

Characterization of cytopathogenic classical swine fever virus

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Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 63 : 751-758 (2001), *Arch. Virol.*, 148 : 297-310 (2003), and *J. Vet. Med. Sci.*, 66 : 161-167 (2004).

Studies on mechanisms of action of and resistance to quinolones in Gram-positive cocci

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Original papers of this thesis appeared in *Antimicrob. Agents Chemother.*, 41 : 2362-2366 (1997), *J. Antimicrob. Chemother.*, 44 : 533-536 (1999), and *Antimicrob. Agents Chemother.*, 46 : 1800-1804 (2002).

Polymorphisms and Antiviral Activity of the Mx and PKR in the Chicken

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Type I interferons (IFNs) are induced by virus infection of virtually all cell types, and then make host cells antiviral state by up-regulating transcription of a set of genes. Among the IFN-inducible genes responsible for the actions of IFN are the *Mx* and RNA-dependent protein kinase (PKR). The nucleotide sequence of chicken *Mx* cDNA was reported previously using the White Leghorn breed in Germany, but it showed no enhanced resistance to viruses. The analysis of PKR has been especially processed in mouse and human. However, there is no information about chicken PKR. In the present study, the characterization of the *Mx* gene in the chicken has been demonstrated. The author has cloned and determined the nucleotide sequences of chicken PKR cDNA in various chicken breeds. Furthermore, the author analyzed the antiviral activity of chicken *Mx* and PKR to RNA viruses with transfected BALB 3T3 cell lines.

In part 1 of this study, the nucleotide sequences of chicken *Mx* cDNA were determined in many breeds. A total of 25 nucleotide

substitutions, of which 14 were deduced to cause amino acid exchanges, were detected, suggesting that the chicken *Mx* gene is very polymorphic. Transfected cell clones expressing chicken *Mx* mRNA were established after the *Mx* cDNA was constructed with an expression vector and introduced into mouse 3T3 cells. Using these transfected cells, *Mx* genes from some breeds were demonstrated to confer positive antiviral responses to influenza virus and vesicular stomatitis virus (VSV). On the basis of the comparison among the antiviral activities associated with many *Mx* variations, a specific amino acid substitution at position of 631 (Ser to Asn) was considered to determine the antivirally positive or negative *Mx* gene. Thus, a single amino acid substitution influences the antiviral activity of *Mx* in the chicken.

In part 2 of this study, the author has cloned and determined the nucleotide sequences of chicken PKR cDNA in various chicken breeds. Chicken PKR was a 550-amino-acid protein as deduced from the cDNA

open reading frame (ORF), and there were specific domains (two double-stranded RNA binding domains (DRBDs) and numerous kinase subdomains) characterized in RNA binding proteins and kinase families. Furthermore, it was suggested that chicken PKR is polymorphic. Transfected cell clones expressing chicken PKR mRNA were demonstrated to confer positive antiviral responses to vesicular stomatitis virus, except for Koshamo type-3 (KS-3). KS-3 PKR, which has an amino acid substitution at position 507 (Arg to Gln), showed amphibious antiviral

responses. This specific amino acid substitution was considered to determine the antiviral function of chicken PKR in addition to essential domains as DRBDs and kinase subdomains.

The present study has clearly confirmed that there are polymorphisms in chicken Mx and PKR, and the variation of antiviral function. These results would provide information for correlation of the polymorphisms or mutations in the Mx and PKR with the antiviral functions.

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Studies on the cause of the well multiplication of *Babesia gibsoni* in canine reticulocyte

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Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 62 : 737-741 (2000), *Am. J. Vet. Res.*, 61 : 1520-1524 (2000), *Exp. Parasitol.*, 102 : 164-169 (2002) and *J. Parasitol.*, 89 : 1142-1146 (2003).

Early Maturation of Oligodendrocytes and Hyperthyroidism in Hypomyelinated Black Tremor Hamster

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Myelination is regulated by complicated interactions between oligodendrocytes and axons in the CNS. Oligodendrocytes play key roles in CNS myelination, however, other cellular and/or humoral factors like thyroid hormone participating the proliferation and dif-

ferentiation of oligodendrocyte are essential for myelinogenesis. Oligodendrocytes can be divided into three subtypes : light, medium and dark, based on ultrastructural characteristics. The light cells are actively dividing, immature young cells, and the medium cells are

involved in myelination, and the dark cells are old cells after myelination. The black tremor hamster is originated from an inbred colony of APG strain of Syrian hamsters and the generation is regulated by single autosomal recessive gene, designated as 'bt'. The main features of myelin disorders in this mutant hamster has been described as diffuse CNS myelin deficiency. There were thin myelin sheaths in all neuronal axons and the small axons less than 1 μm in diameter were unmyelinated in the white matter of the spinal cord. The number of glial cells was in the normal range and the peripheral nerve fibers were normally myelinated. This mutant hamster has an insertional mutation of the Attractin gene. The mutant animals (black tremor hamster, zitter rat and mahogany mouse) show darkened coat color and diffuse hypomyelination with vacuolation in the CNS, while the biochemical components of the myelin are normal.

In part1, oligodendrocytes and myelin in the *corpus callosum* of black tremor and normal hamsters (young animals, 3-14 weeks old) were ultrastructurally examined to determine myelination index (ratio of myelin thickness/diameter of axon), percentage of naked axons and proportions of oligodendroglial subtypes (light, medium and dark). The relative numbers of actively mitotic light/medium oligodendrocytes and the post-mitotic dark oligodendrocytes are thought to reflect the myelination. The mutant hamsters were remarkably hypomyelinated with a low myelination index (0.02-0.05, in contrast with 0.11-0.20 in age-matched normal controls) and a high proportion of naked axons (54-82%, in contrast with 19-37% in the normal controls). The proportion of oligodendrocytes subtypes in mutant hamsters were compatible with those of age-matched normal controls at 3 weeks of age. Thereafter dark cell population

in mutant hamsters sharply increased from 7 weeks of age. These results suggested that the myelinogenetic activity of oligodendrocytes in the mutants younger than 3 weeks of age were low in compare with those in age-matched control, and then oligodendrocytes of the mutant hamsters matured earlier than those of age-matched controls, since the proportions of dark inactive oligodendrocytes at 7 and 14 weeks of ages were higher than those of the control hamsters.

In part2, the same ultrastructural investigations as part 1 concerning myelination index, percentage of naked axons and proportions of oligodendroglial subtypes was performed in the aged mutant (older than 1.5 years of age) and normal animals. Severe hypomyelination (myelination index of 0.09 in mutants in contrast with 0.22 in normal animals), high ration of naked axons (48% in mutants in contrast with 10% in normal animals), and high proportions of dark cell (65% in mutants in contrast with 30% in normal animals) were maintained in the aged mutant hamsters. These results indicated that the hypomyelination with large number of naked axon in mutant hamster could not be recovered over their lives because of the paucity of actively myelinating oligodendrocytes.

In addition, serum thyroid hormones concentrations of the young and aged, normal and mutant hamsters were examined. The concentrations in the mutants were higher for more than 2-fold (T_3) or 3-fold (T_4) of normal animals at 6 weeks of age. In the aged mutant animals, serum concentrations of T_4 were higher than those of normal controls with statistical significance. It was concluded that the sustained high serum thyroid hormones might play a role in the early maturation of oligodendrocytes and hypomyelination in the black tremor hamster.

Original papers of this thesis appeared in *J. Toxicol. Pathol.*, 16 : 209-213 (2003), and *Jpn. J. Vet. Res.*, 51 : 113-120 (2003)

Two types of K⁺ channels in salivary acinar cells : their possible role in fluid secretion

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Original papers of this thesis appeared in *J. Physiol.*, 547.1 : 255-269 (2003), and *Am. J. Physiol. Cell Physiol.*, 286 : C635-646 (2004).

Anti-tick effects of monoclonal antibodies against the midgut of *Haemaphysalis longicornis*

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An ixodid tick, *Haemaphysalis longicornis* is a bovine ectoparasite that causes economic losses in Asia, mainly as a vector of Theileriosis and Babesiosis, due to the high costs required to control the parasite. Currently, the principal tick control method is by application of acaricides. Acaricidal control is problematic because of residues in foods and the potential for tick to develop resistance to the acaricides, reducing their effectiveness. Tick vaccines inducing host protective immunity have been developed to resolve these problems. Ticks have two kinds of vaccine candidate antigen ; exposed antigen which is secreted from salivary gland and exposed to the host immune-system, and concealed antigen which is present in internal organs such as midgut, and thus, does not naturally induce a host immune-response. Previous workers defined a

protective antigen, Bm86, purified from the gut of semi-engorged adult female *Boophilus microplus* ticks. Ticks that had fed on recombinant Bm86-immunized cattle, had extensive damage. Bm86-based vaccine have shown a reduction in the transmission of babesiosis in vaccinated herds.

In this study, we focused on concealed antigen of the midgut of *Haemaphysalis longicornis*. We produced and characterized monoclonal antibodies (mAbs) against *H. longicornis* midgut proteins as the first step toward defining vaccine antigens. A total of eight hybridomas secreting mAbs were selected, and their isotypes were either IgG1, IgG2a or IgG2b. By immunoblots using midgut antigens, all three mAb isotypes uniformly reacted with a 76kDa protein present in the adult tick midgut. Immuno-stained sections of

the midgut showed that membrane and internal region of digestive cells were specifically stained with all of the mAbs. The different mAbs isotypes showed a similar staining pattern. In addition, immunogold staining revealed that the protein reacted with these mAb is a membrane surface protein. The effect of mAb on tick feeding activity was assessed by using mice producing ascites of mAbs. Adult ticks which were fed on two mice producing mAb (IgG1 isotype) showed a distinct red coloration. This change was observed in 3 out of 7 adult ticks on each mouse at 4-6 days after attachment. However, this mAbs did not have any effect on the feeding ability of nymphs. In the midgut sections of adult ticks which showed the red coloration, the destruction of midgut digestive cells was observed determined by toluidine blue staining. Lysis of digestive cells in the midgut causes leakage of host cells into the hemocoel, infiltration of host leukocytes into reproductive tissue and muscle leading to pathological tissue changes. Molecular cloning of the gene en-

coding for this 76 kDa protein and precise characterization are now under investigation.

Anti-tick effects of the mAbs and differences in the magnitude of effects among the isotypes were examined by performing passive immunization experiments. The results of this test against adult ticks with the mAbs at high dose, showed significant effects such as a reduction in engorgement, capacity to reproduce, and hatching. Both the IgG1 and 2b isotypes elicited a reduction in engorgement and egg weight, and especially the number of hatched larvae. On passive immunization at a low dose, no reduction in engorgement or egg weight was observed as compared to controls. However, remarkable reductions in the number of hatched larvae were observed. These results suggest that the anti-tick effects of the mAbs work independent of complement, because no differences of mAbs showed anti-tick effects. It is thought that these mAbs binding to 76 kDa protein caused the physiological changes in midgut, resulting in the destruction of cells and reduced tick feeding.

The original paper of this thesis appeared in *Vet. Parasitol.*, 115 : 355-363 (2003) and *Jpn. J. Vet. Res.*, 50 : 157-163 (2003).

Studies on the mechanism of persistent infection of hantavirus in mice

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In nature, hantaviruses are maintained in persistently infected asymptomatic rodent reservoirs despite the presence of neutralizing antibodies. Humans become infected with hantaviruses from the rodent reservoirs. Mechanisms of persistent hantavirus infection are poorly understood. On the other hand,

it is known that when newborn BALB/c mice are inoculated with a sublethal dose of HTNV, which is maintained in *Apodemus agrarius* in nature, most of mice develop persistent infection. Investigation of the mouse model of persistent HTNV infection should provide information regarding the mechanism of persis-

tent hantavirus infection in natural rodent reservoirs. In addition, the persistently HTNV-infected mouse has potential as a model of persistent human viral infection. Therefore, the mechanism of persistent HTNV infection in mice was studied in this thesis.

First, to investigate the relationship between HTNV-specific CD 8⁺ T-cell responses and HTNV persistence, a method for counting HTNV-specific CD 8⁺ T cells by flow cytometry was established. Next, newborn mice were inoculated within 24 h after birth with sublethal dose of HTNV to produce persistent HTNV infection in mice. All mice were persistently infected with HTNV until at least 30 days after virus inoculation, and had no virus-specific CD 8⁺ T cells. Subsequently, the virus was eliminated from some of the mice, depending on the appearance of functional HTNV-specific CD 8⁺ cells. Neutralizing antibodies were detected in all mice, regardless of the presence or absence of virus. In the acute phase, which occurs within 30 days of infection, IFN- γ (producing HTNV-specific CD 8⁺ T cells were detected on day 15 after virus inoculation. However, TNF- α production and cytotoxic activity of these specific CD 8⁺ T cells were impaired and HTNV was not removed. Almost all of these specific CD 8⁺ T cells disappeared by day 18. These suggest that the lack of a functional virus-specific CD 8⁺ T-cell response is important for HTNV persistence in mice.

As contrasted with HTNV-infected newborn mice, HTNV infection was transient and many HTNV-specific CD 8⁺ T cells were induced by infection in adult mice. In the second chapter, the author addressed age of the shift from newborn-to adult-type HTNV-specific

CD 8⁺ T-cell responses to understand HTNV-specific CD 8⁺ T-cell responses in detail. Mice were inoculated with HTNV at 0, 3, 7, 14, or 35 days after birth. HTNV-specific CD 8⁺ T cells were measured on day 30 after HTNV inoculation. Most mice over 7 days old have the ability to induce functional HTNV-specific CD 8⁺ T-cell responses that are indistinguishable from the responses of adult mice. A strong correlation between HTNV persistence and a lack of HTNV-specific CD 8⁺ T cells was also observed in the chapter 2.

However, the author was unable to obtain unambiguous evidence that CD 8⁺ T cells contributed to the clearance of HTNV, due to difficulties in modulating the immune responses of newborn mice. Therefore, a new model of HTNV persistence was established using SCID mice with the adoptive transfer of spleen cells from immunocompetent mice. Accordingly, it has been proven that CD 8⁺ T cells contribute to the clearance of HTNV in mice. In addition, disseminated HTNV infection prior to the initiation of immune responses appears to be important for virus persistence.

Thus, it seems that HTNV-specific CD 8⁺ T cells play a key role in HTNV persistence in both the newborn mouse model and the SCID mouse model. The results obtained in this thesis suggest that persistent hantavirus infections in natural rodent reservoirs are due to escape from CD 8⁺ T-cell immune surveillance. Since it is possible to manipulate immune responses using SCID mice with adoptive transfer of spleen cells, this model will be applied as a useful system for study of various infectious disease.

Genetic and antigenic characterization of the Amur virus, a distinct hantavirus serotype carried by *Apodemus peninsulae*

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Hantaviruses are distributed widely in the world depending on the geographic distribution of their reservoir rodent species. The number of the newly discovered hantaviruses have been increasing rapidly. Many hantaviruses are pathogenic for humans and cause either hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS). Far East Russia is a HFRS-endemic area where 100-200 severe cases are reported annually. A few studies reported presence of hantaviruses in rodents and patients in the area. Amur (AMR) and Far East (FE) are two genotypes found in HFRS patients in Far East Russia recently.

In this study, genetic characterization of hantaviruses carried by *Apodemus (A) peninsulae* was performed. Further, genetic and antigenic properties of hantavirus-related viruses exist in China were also determined. First, in order to characterize hantaviruses in Far East Russia, an epizootiological study was carried out in a suburb of Vladivostok, Russia. Antibodies to hantaviruses were detected in *A. agrarius*, *A. peninsulae*, and *C. rufocanus*. The entire S and partial M segments of hantaviruses (Solovey sequences) were determined from lungs of antibody positive *A. peninsulae*. At the same time, partial M segments of hantaviruses were identified and the nucleotide sequences were determined (Primorye sequences) from two seropositive, fatal cases of HFRS in the Primorye region of Russia. Sequence and phylogenetic analysis showed that Solovey and Primorye sequences were

closely related to each other (98.2% in nucleotide) and to AMR sequences found in HFRS patients in Far East Russia (91.3%-98.3% in nucleotide). Based on these data, the causative agent of fatal HFRS cases in Primorye was the virus found in *A. peninsulae* (Solovey sequences). Taken together, it was suggested that *A. peninsulae* is the reservoir animal for AMR virus. This hantavirus and related HFRS cases may be widely distributed since *A. peninsulae* inhabits over a vast area including Far East Russia, China, Korea, and Japan.

Next, Chinese hantavirus isolates related to AMR and FE genotypes were characterized genetically and antigenically. Based on the phylogenetic analysis of the partial M segments two Chinese human isolates, H 5 and B 78 were used to represent AMR genotype while one Chinese isolate, Bao 14 from *A. agrarius* was taken to represent FE genotype. For genetic characterization the entire S and M genes of strains of AMR and FE genotypes were sequenced. Sequence analysis showed that AMR genotype was distinct from HTN76-118, the prototype HTN virus compared to the diversities of FE genotype with HTN 76-118. The M gene of the AMR genotype had more diversity at 8% in amino acid level with the other HTN viruses. In contrast, FE genotype had only 2% diversity in amino acid level with the prototype of HTN virus. In addition, 10 amino acid substitutions unique to AMR genotype viruses (signature amino acids) were also found. Further, strains of AMR

genotype formed a distinct branch from the HTN clade in both phylogenetic trees based on S and M genes. Antigenic analysis with a panel of monoclonal antibodies showed a quite similar antigenicities among the prototype HTN, FE, and AMR except for the non-binding of G 1 -b specific 2 D 5 monoclonal antibody with the strains of AMR genotype. However, cross-neutralization analysis revealed that AMR genotype viruses were antigenically different from the prototype HTN vi-

rus with an eight-fold titer difference. These data suggest that AMR is a distinct virus in the genus *Hantavirus* while FE is a subtype of the prototype HTN virus.

This study shows that a new pathogenic *hantavirus* is harbored by *A. peninsulae*. In addition, genetic and antigenic properties of the AMR and FE genotypes are shown in more detail that the AMR is a distinct virus that belongs to the genus *Hantavirus*, while FE is a subtype of HTN virus.

The original papers of thesis appeared in *Emerg. Inf. Dis.*, 8 : 768-776 (2002), *Virus Res.*, 101 : 127-134 (2004), and *Jpn. J. Ver. Res.*, 51 : 143-149 (2004).

Genomic analysis of hereditary myopathy of diaphragmatic muscles in Holstein-Friesian cattle

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Linkage analysis is a very powerful method to detect a causative gene in recessive disease. However, we tend to find false-positive regions in the whole genome even with typing many microsatellite markers as shown in Chapter I. The reason is that number of samples is not sufficient. Enough number of both affected and normal samples can derive significant differences of allele frequencies of microsatellite markers between two groups. Even after the whole genome sequence in human has been published, they have not yet identified the causative gene in autosomal dominant distal myopathy of human after they mapped the locus on human chromosome 14 [20]. They might find a false-positive region by linkage analysis with insufficient number of samples. I resolved this problem by adding more eight normal sam-

ples in the hereditary myopathy of diaphragmatic muscles (HMDM) pedigree as shown in Chapter II and found a causative deletion of heat-shock protein 70 (Hsp70) gene as shown in Chapter III.

A deletion of one of the duplicated inducible Hsp 70 genes resulted in markedly decreased Hsp 70 protein levels, leading to HMDM. Similar results were obtained by other investigators in one of the duplicated genes, *hsp 70.1*, a murine homolog of heat-shock 70-kilodalton protein 1 B, knockout mice in which the remaining Hsp 70 gene was intact. Despite those *hsp 70.1*^{-/-} mice were viable with no obvious abnormalities, HSPA 1 B deficient cattle showed fiber degeneration with central core-like structure in their diaphragmatic muscle. Moreover, I found that in normal diaphragmatic muscle Hsp 70 protein

bound several proteins involved in energy metabolism including glycogen phosphorylase while that protein accumulated in the central core-like structures in affected cattle. Hsp 70 has been known to suppress aggregates of toxic proteins. My discovery shows evidence to the notion that Hsp70 also prevents aggregation of normal proteins supposed to be functional for organisms.

The reason that Hsp70 deficiency induces protein aggregation mainly in diaphragmatic muscle remains to be clarified. Grosz et al. reported that Hsp 70 family consists of at least three members in cattle [11]. Other Hsp 70 members might fail to compensate Hsp 70 deficiency in HMDM diaphragmatic muscle. In-

terestingly, hsp 70.1^{-/-}-mice are no longer protected against tumor necrosis factor lethality after heat shock [23], indicating no compensation of hsp70.1 deficiency by other hsp70 members.

The relation between central core-like structures and muscle fiber degeneration requires further investigation. It could be confirmed by checking whether central core-like structures are diminished by expression of Hsp 70 in diaphragmatic muscle cells from HMDM-affected cattle. Although central core-like structures might indirectly cause fiber degeneration, impaired protein folding due to Hsp 70 deficiency appears to underlie the pathogenesis of HMDM-type myopathy.

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The molecular mechanisms operating phagocyte NADPH oxidase activation.

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Phagocytes such as neutrophils and macrophage play an indispensable role in the first line of host defenses against invading pathogens. The release of reactive oxygen species derived from NADPH oxidase is recognized as the main mechanism in bactericidal responses of phagocytes. On the other hand, because the inappropriate activation of the NADPH oxidase and overproduction of reactive oxygen species can inflict harm on host tissues, the activation of NADPH oxidase has to be tightly regulated. Previous studies have established that the activation of NADPH oxidase requires two distinct processes : p47^{phox} phosphorylation and Rac activation. Although

various molecules are suggested to be involved in the signaling pathways leading to NADPH oxidase activation, the molecular mechanisms operating its activation have not fully been elucidated. To clarify the signal transduction pathways leading to NADPH oxidase activation, especially p47^{phox} phosphorylation and Rac activation, I focused on two kinases, p38mitogen-activated protein kinase (p38MAPK) and phosphoinositide 3-kinase (PI 3 K), and examined their roles in its activation.

NADPH oxidase activation was observed after stimulated with serum-opsonized zymosan (OZ) in bovine neutrophils. This activation

was attenuated by p38 MAPK inhibitor, SB 203580, in a dose-dependent manner. The translocation of p47^{phox} and Rac from cytosol to membrane was also induced by OZ stimulation and SB 203580 completely blocked the translocation of Rac, but only partially inhibited that of p47^{phox}. This result suggested that p38MAPK was more responsible for the regulation of Rac than p47^{phox} to control NADPH oxidase activation. In fact, OZ-induced Rac activation, a prerequisite for the translocation, was clearly prevented by SB203580. In addition, this OZ-induced Rac activation was also attenuated by PI 3 K inhibitors, wortmannin and LY294002. The effect of SB203580 on PI 3 K activation and the effect of wortmannin on p38MAPK activation were next examined to address the relationship between p38MAPK and PI 3 K in the signaling pathways of neutrophils. While SB203580 showed no effect on the PI 3 K activation, OZ-stimulated activation of p38MAPK was abolished by wortmannin, demonstrating that PI 3 K is the upstream regulator of p38 MAPK.

I next evaluated the effect of PI 3 K inhibitors on NADPH oxidase activation stimulated with a chemotactic agent, formyl-Met-Leu-Phe (fMLP) in HL-60 cells differentiated to a neutrophil-like phenotype (dHL-60 cells). PI 3 K inhibitors dose-dependently reduced fMLP-stimulated NADPH oxidase activation in dHL-60 cells. Pharmacological inhibition of PI 3 K also attenuated both p47^{phox} translocation and phosphorylation after exposure to fMLP, suggesting that PI 3 K was involved in

the signaling pathways leading to not only Rac activation but also p47^{phox} phosphorylation. Since Akt is known as a typical Ser/Thr kinase in the downstream of PI 3 K, I inferred that Akt might be a p47^{phox} kinase regulated by PI 3 K in dHL-60 cells and examined this hypothesis. Although fMLP elicited the Akt activation in a PI 3 K-dependent manner, an Akt inhibitor had no effect on the oxidase activity triggered by fMLP. Furthermore, in vitro kinase assay revealed that protein kinase C (PKC) clearly phosphorylated p47^{phox} proteins, but no phosphorylation of these proteins by Akt was observed. Contradictory to my hypothesis, these results indicated that Akt was little related to p47^{phox} phosphorylation and NADH oxidase activation. Interestingly, the activation of cPKC and PKC δ was observed after fMLP stimulation and their activation was shown to be dependent on PI 3 K. Moreover, PI 3 K inhibitors reduced the activation of phospholipase C γ 2 (PLC γ 2) without affecting tyrosine phosphorylation on it.

These results in this study indicated the presence of novel molecular mechanisms operating NADPH oxidase activation that are the PI 3 K-p38 MAPK-Rac pathway for Rac activation and the PI 3 K-PLC γ 2-cPKC/PKC δ -p47^{phox} pathway for p47^{phox} phosphorylation. In other words, this study showed that PI 3 K plays an important role in two essential intracellular events for NADPH oxidase activation, *i.e.* Rac activation and p47^{phox} phosphorylation, through the different mechanisms.

Development of an age estimation technique utilizing dental incremental lines of sika deer (*Cervus nippon*)

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This study was designed to develop a technique that could estimate age in months or even in days utilizing minute dental incremental lines of sika deer. In a fundamental study, incremental lines were observed and their periodicity investigated using a chronological labeling method; with neonatal lines that are formed at birth being referenced. Then day-age estimation was attempted with individuals of known ages using the results obtained from the fundamental studies.

When the minute incremental lines were observed in decalcified specimens stained by Bodian's silver technique, it was suggested that dentin incremental lines reflect daily periodicity. However daily increments were not distinct near the pulp cavity and neonatal line could not be identified in decalcified cross sections of deciduous teeth.

We therefore prepared undecalcified longitudinal sections from first molars (permanent tooth), and examined the incremental lines in enamel and dentin with a polarized light and fluorescence microscope. Enamel increments occurred at a mean interval of 10.6 (SD=1.5) μm , and were formed each day. Dentin increments occurred at a mean interval of 17.3 (SD=1.8) μm , and were formed almost every second day. Due to the longer interval of its increments and the longer formation time, dentin appears more suitable than enamel for age estimation in sika deer.

The neonatal line, also, could be observed in longitudinal ground sections. It was recognized as a prominent dark line in enamel un-

der transmitted light; and as a border line of different hues in enamel and dentin under polarized light. The line intersects with the enamel-dentin junction at approximately the one-third cervical portion of the first molar. The presence of the neonatal line made it possible to distinguish pre-natal from post-natal enamel and dentin, therefore identification of neonatal lines using these features will be useful for age estimation and reconstruction of individual life histories of wild animals.

The day-age estimation was tried in practice in age-known animals, and the estimation errors were examined. The regression analysis of day-age (Y) and the number of dentin increments (X) gave a formula as follow: $Y = 2.295X$. The periodicity of dentin incremental lines observed under polarized light (2.3 days) was very close agreement with the coefficient of the formula, so their consistency was suggested. The buccal area showed the smallest error among the count areas and age estimation could be conducted in the area in 8 of the 13 individuals (62%). Estimation error was within almost one month, from -15 days to 19 days.

In conclusion, the age estimation in months is possible using minute dentin incremental lines in sika deer fawns. Because fawns are the most numerous age class of a deer population, their population dynamics strongly affect the dynamics of the whole population. Therefore the monthly-age of fawns may available to understand the factors of mortality and should contribute to the

management of wild populations.

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Studies on reproductive ecology of mating and parturition seasons in Hokkaido sika deer (*Cervus nippon yesoensis*)

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Timing of parturition is one of the important factors for investigation into life history strategy of cervid species, because the timing of parturition probably affects individual reproductive success, especially for populations inhabiting temperate and arctic environments. In the present study, possible factors affecting conception date and gestation period were examined using captive sika deer to reveal sources for variations in the parturition date. Furthermore, effects of birth date on survival and growth of sika deer fawns were also elucidated. The following results were obtained :

1. Females repeated ovulation without copulation during the early mating season, which were confirmed by fecal progesterone profiles. Most females conceived at the first copulation. Thus, some females had repeated ovulation without copulation several times, creating a 3 - 4 week variation in the timing of conception. But a few females conceived very late in the mating season after the repetition of ovulation and copulation.
2. Two corpora lutea (CLs) were found in females collected during early mating season, though sika deer basically deliver a single fawn. The timing of formation of the two CLs seemed different each other. Both CLs synthesize pregnenolone and progesterone from cholesterol using P450 scc and 3 β SD in early mating season as same as during pregnancy.
3. The timing of conception was related to lactational status and all non-lactating females conceived before the median conception date. Young females were more likely to conceive after the median date of the conception than older females. The relationship between body weight and gestation period differed according to winter severity. I suggest that females in poor body condition compensate for poor fetal growth by lengthening their gestation period after a severe winter.
4. The effect of birth dates on the body weight of fawns remained until late winter. Hence, late-born fawns would be more vulnerable in the first winter than early-born ones. However, there was no effect of birth date on the body weight on October 1 when they were one year of age. Once fawns survive their first winter, the effects of birth date on body weight and reproductive success are considered little.

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Conservation biological study of the Japanese sable *Martes zibellina brachyura* in Hokkaido

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Biological characteristics of the Japanese sable, *Martes zibellina brachyura*, which is categorized as DD subspecies in the IUCN red data list were revealed. The Japanese sable is distributed in northern and eastern Hokkaido, while the Japanese marten is distributed in western and southern Hokkaido. These two species share small area around Sapporo.

Genetically, the Japanese sables and the introduced Japanese martens in northern Japan were closely related. Comparative analysis in the 521-524bp DNA sequence from the cytochrome *b* /transfer RNA and control region of the mitochondrial genome revealed that intraspecific differences in sequences of the Japanese sable and the Japanese marten (3.8-15.0% and 1.9-16.4%, respectively) were similar to interspecific differences between these two species (5.8-16.6%). Comparison of sequence data exhibited nine haplotypes, which clustered into two groups (Clusters-A and-B). Cluster-A included two haplotypes of the Japanese marten and two haplotypes of the Japanese sable, whereas clusterB included three haplotypes of the Japanese marten and two haplotypes of the Japanese sable. Results of this study lead three possible explanations. Firstly, past hybridization between the Japanese sable and the Japanese marten might have occurred. Secondary, these two species might have simi-

lar heteroplasmy of mt DNA. Thirdly, these haplotypes might have come from nuclear genome.

The Japanese sable proved to be omnivorous, taking various food items including mammals, insects, plants, birds, reptiles, amphibians, fish and crustaceans from the analysis of 193 feces and 20 stomachs. Mammals were the commonest food items throughout the year, with voles *Clethrionomys* spp.

(frequency of occurrence 56.5%), Siberian chipmunks *Tamias sibiricus* (19.3%) and wood mice *Apodemus* spp. (14.6%). Insects appeared mainly in summer (48.8%) and less often in other seasons (9.3% on average). Plant materials, chiefly fruits, were found mainly in autumn (45.7%) and winter (68.4%) but were rare in spring (5.1%) and summer (1.3%). Maintaining natural habitats, which provide various food resources, is important for conservation of the Japanese sable.

Nucleotide sequences of cytochrome *b* / tRNA/D-loop region on mitochondrial DNA of mustelids feces were compared to identify species. PCR amplification of target sequence for 47 (24.9%) feces and species identification of five feces (2.6%) out of 189 feces, collected at several study sites in Hokkaido, were successful. The low success rate of identification appeared to be due to failure of PCR amplifi-

cation by inhibitors in feces. It was suggested for not only identify mustelids species, but that the method used in this study was useful also analyzing their genetic relationships.

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