Jpn. J. Vet. Res. 54(1) : 31-45, 2006

INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy (Ph.D) in Veterinary Medicine on March 24, 2006 to 17 recipients.

The titles of theses and other information are as follows :

Differential cellular expression of galectin, an endogeneous lectin, in the murine digestive tract and urogenital system

Junko Nio

Laboratory of Anatomy, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Galectin is an animal lectin, which recognizesβ-galactoside of glycoconjugates, and to date includes at least 15 members in mammals. They are classified into three groups based on their structural characteristics : proto type (galectin-1, -2, and -7), tandem repeat type (galectin-4 and -6), and chimeric type (galectin-3). Galectins are present in both the cytoplasm and nucleus, and also released extracellularly, regulating cell growth, differentiation, apoptosis, and cell migration. The present study revealed the cellular expression of predominant galectin subtypes (galectin-1, -2, -3, -4/6, and -7) in the digestive tract and urogenital system of mice by *in situ* hybridization and immunohistochemistry.

The murine digestive tract expressed five subtypes of galectin mRNAs (galectin-2, -3, -4/6, and galectin-7) in the epithelium with region-dependent and cell-specific patterns. In the glandular stomach, galectin-2 and galectin-4/6 were predominantly expressed from gastric pits to the neck of gastric glands, where mucous cells were the main cellular sources. The epithelium of the small intestine exhibited intense, maturation-associated expressions of galectin-2, -3, and galectin-4/6 mRNAs. Galectin-2 mRNA was intensely expressed from the crypt to the base of villi, whereas transcripts of galectin-3 gathered at villous tips. Signals for galectin-4/6 were most intense at the lower half of villi. Galectin-2 mRNA was also expressed in goblet cells of the small intestine but not in those of the large intestine. In the large intestine, galectin-4/6 predominated, and the upper half of crypts simultaneously contained transcripts of galectin-3. Stratified epithelium from the lip to the forestomach and of the anus intensely expressed galectin-7 with weak expressions of galectin-3. The maturation-dependent expression of subtypes provides an example of a periodical shift of galectin subtypes : the subtypes expressed by the same cell lineage changed from the proto type (galectin-2), through the tandem repeat type (galectin-4/6), to the chimeric type (galectin-3).

The major subtype expressed in the murine urinary system was galectin-3, which was expressed continuously from the kidney to the distal end of the urethra. Renal galectin-3 immunoreactivity was the strongest in the cortical collecting ducts, where principal cells were the sole cellular source. The transitional epithelium from the renal pelvis to the ure-

thra strongly expressed galectin-3 at the mRNA and protein levels. The adult urinary system showed intense and selective expressions of galectin-3 in epithelia of the uretic bud-and cloaca-derivatives.

In the ovary, galectin-1 and galectin-3 are predominant subtypes. Galectin-1 was diffusely expressed in the stroma, and the co-expression of galectin-1 and galectin-3 was found in the corpus luteum of non-pregnant and postpartum mice. However, the corpus luteum under gestation lacked any signals for galectin subtypes. The mRNA expression of galectin in the corpus luteum perfectly correlated with the mRNA expression of 20α-hydroxysteroid dehydrogenase, which is a degradation enzyme of progesterone. Therefore, galectin-1 and galectin-3 may function as negative regulators for the production and physiological activity of progesterone.

The present study revealed the differential cellular expression of galectin subtypes in the murine digestive tract and urogenital system ; however certain functions of the galectin in these tissues are under investigation. The identification of cell types expressing galectin subtypes in vivo will be important for a precise understanding of the function of galectins.

The original paper of this thesis appeared in *Histochem. Cell. Biol.*, 121 : 473-482 (2004) and *J. Histochem. Cytochem.*, 53 : 1323-1334 (2005).

Roles of mitochondrial uncoupling protein 1 in whole body energy metabolism : studies using gene-knockout mice.

Ken-ichi Inokuma

New Lead Pharmacology Laboratory, Institute for Medical Research, Wakunaga Pharmaceutical Co., Ltd.

Original papers of this thesis appeared in *Diabetes*, 54 : 1385-1391 (2005) and *Am. J. Physiol. Endocrinol. Metab.,* 290 : E1014-E1021 (2006).

Leptin in *Carnivora* : establishment of a specific ELISA method and assay of blood leptin of companion and feral animals.

Haruki, Shibata

Morinaga Institute of Biological Science, Inc.
Sachiura 2-1-16, Kanazawa-ku, Yokohama 236-0003, Japan

Leptin is one of the adipocytokines cloned as the *ob* gene product. It is a 16 kDa polypeptide and a key molecule for the regulation whole-body energy balance. It has been re-

ported in human and rodents that the circulating levels of leptin are positively correlated with body fat content, implying that blood leptin is a quantitative marker of adiposity reflecting long-term nutritional condition. Although specific immunoassay methods for human and rodent leptin have been established, a commercially available mutli-species radioimmunoassay (RIA) kit has been used for other species leptin. However, this kit may not be successfully applicable for leptin assay of some species, particularly those of *Carnivora,* because of a limited immunoreactivity of the antibody used in the kit. To overcome this problem and to assay blood leptin of the dog, cat and some other feral animals, in this study, I established a specific enzyme-linked immunosorbent assay (ELISA) method for these animal species, and confirmed its usefulness for the assessment of nutritional conditions.

First, I cloned a feline leptin cDNA using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of complementary DNA (cDNA) ends (RACE) methods. The full-length feline leptin cDNA was 2,935bp with a 501bp open reading frame encoding the precursor peptide of 167 amino acids including 21 residues of signal peptide. The sequence of a 146-amino acid mature leptin was 81.5-91.8% homologous to those of other species. RT-PCR analysis revealed that the leptin mRNA was expressed in adipose tissues but not in liver, heart, kidney, lung, pancreas, brain and skeletal muscle. These data show that feline leptin is highly homologous to leptin of other species including canine, and expressed in adipose tissue.

Next, I produced recombinant feline leptin as well as canine leptin in *Escherichia coli* transfected with respective leptin cDNAs. Both the recombinant leptins induced phosphorylation of the signal transducers and activators of transcription 3 and mitogen activated protein kinase in the cells expressing rat leptin receptor. Western blot analysis revealed that an anti-feline leptin antibody raised in rabbits reacted well to feline and human leptin, and less to rodents' leptin. An anti-canine leptin antibody reacted well to canine and feline leptin, but much less to human and rodents' leptin. ELISA methods specific to feline and canine leptins were developed using the antibodies and recombinant proteins of these two species. In these ELISA methods, the cross-reactivity to human, rat and mouse leptins was very low. The plasma leptin concentrations of 20 healthy dogs and 24 healthy cats were positively proportional to body fat content, respectively. These results indicate that my ELISA methods are much more useful for the assessment of body fat content in the dog and cat.

Finally, I tested the availability of my canine ELISA system for blood leptin assay of some feral animals, raccoon (*Procyon lotor*) and bear (*Ursus arctos, Ursus thibetanus*). When estimated by the ELISA method, but not by the RIA, serum leptin concentrations of 16 raccoons captured in various seasons showed clear seasonal variations, being high during autumn and low in spring and summer, being positively correlated with their body weight and body mass index. Thus, my canine leptin ELISA metod is applicable for not only the dog but also the raccoon and bear, and useful for the assessment of nutritional conditions in these animal species.

Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 63: 1115-1120 (2001), *J. Vet. Med. Sci.*, 65: 1207-1211 (2003) and *J. Exp. Zool.*, 303A: 527-533 (2005).

Development of kits for rapid diagnosis of influenza

Gui-Rong Bai

Laboratory of Microbiology, Department of Disease Control,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Original papers of this thesis appeared in *Microbiol. Immunol.*, 49 : 1063-1067 (2005), and *J. Vet. Med. Sci.*, 68 : 35-40 (2006)

Studies for the control of influenza : genetic and pathobiological analyses of highly pathogenic avian H5N1 viruses currently circulating in Asia and mechanism of viral genome incorporation into influenza A virions

Yukiko Muramoto

Laboratory of Microbiology, Department of Disease Control,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Original papers of this thesis appeared in *Microbiol. Immunol.*, 50 : 73-81 (2006) and *J. Virol.*, 80 : 2318-2325 (2006)

Development of anti-tick vaccines using serine protease inhibitors (SERPINs) from Ixodid ticks

Saiki Imamura

Laboratory of Infectious Diseases, Department of Disease Control,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

At present immunological protection of host against tick is the most practical and sustainable tick control method, which is more friend to natural environment compared to the current use of acaricides. Recently, focuses on the development of anti-tick vaccine are the identification, molecular cloning and *in vitro* production of recombinant protein, responsible for executing key roles such regulating physiology, modulation of host immune response and pathogen transmission via ticks. Among such molecules, serine protease inhibitors (serpins) have been thought as one of the most interesting vaccine candidates, because serpins are known to regulate important functions in the maintenance of homeostasis.

For these reasons, the study described in chapter 1 aimed at the cloning and characteri-

zation of serpin derived from tick that could be used as vaccine against tick. Though vaccination of rabbit with a single molecule resulted in high mortality of the ticks, the level of immunity induced by individual molecule appeared not to be able to sustain effective immunity for the protection of ticks under the field. Therefore, the study described in chapter 2 aimed at assessing the efficacy of a cocktail vaccine consisting of two serpins using cattle under a paddock experimental condition. Vaccination of cattle with the serpin cocktail induced protective immunity against ticks, resulted in the reduction in nymphal engorgement rates and the increase in adult mortality rates.

In conclusion, this work provided fundamental information to search and develop effective anti-tick vaccine by using genetic approaches. Furthermore, it could be important practical examples for realization of an anti-tick vaccine application.

Original papers of this thesis appeared in *Vaccine*, 23 : 1301-1311 (2005), and 24 : 2230-2237 (2006).

Studies on host immune responses affecting on viral propagation in bovine leukemia virus-infected animals

Tatsufumi Usui

Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

In this thesis, the epidemiological and immunological studies on the propagation of bovine leukemia virus (BLV) in infected animals were studied. The conclusions obtained by this study are summarized as follows.

Ⅰ) The prevalence of BLV in four farms, where enzootic bovine leukosis (EBL) had been reported, ranged from 9.2% to 37.5%. In a farm with 37.5% seropositivity, several cattle showed markedly high proviral load indicating the development of persistent lymphocytosis. The survey in this farm at 6 month later revealed that 13.3% of non-infected cattle became infected. These observations suggest a high risk of EBL and a high incidence of viral transmission in these farms.

Ⅱ) DNA vaccination trial was performed on sheep to determine whether vaccination with BLVtax DNA is effective against BLV infection. BLV titers determined by the syncytium formation assay and BLV proviral load detected by genomic PCR analysis showed higher levels of virus titers in control sheep than in Tax-vaccinated sheep. Higher levels of interferon (IFN)-γ and interleukin-2 mRNA expression have been demonstrated in vaccinated sheep after the challenge. These observations imply that Th 1 immunity is induced and BLV is eliminated in Tax-vaccinated sheep after the challenge.

Ⅲ) Interaction between the propagation of BLV and host immune responses during the early phase of infection were analyzed in sheep, experimentally infected with BLV. Sheep in which BLV did not propagate well showed augmented IFN-γ mRNA expression at 2 weeks after challenge, indicating that IFN-γ plays an important role in the elimination of BLV-infected cells.

Ⅳ) The expressions of tumor necrosis factor

(TNF)-α and TNF receptors, TNF-R1 and TNF-R2, in B-cells from BLV-infected sheep were studied. The expression of membrane-bound TNF-α in IgM^+cells from BLV-infected sheep with B-lymphocytosis, but not from BLV-infected sheep without lymphocytosis or uninfected sheep, was up-regulated by in vitro culture, indicating TNF-α expression could be related to the development of B-lymphocytosis in BLV-infected sheep.

Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 65 : 287-289 (2003), and 65 : 1201-1205 (2003).

Studies on the development of programmed cell death and drug resistance in *Trypanosoma brucei*

Akiko Tsuda

Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Trypanosoma brucei causes sleeping sickness in man, and Nagana in domestic animals, and is distributed within the area of tse-tse fly habitat in sub-Saharan Africa. Vaccine development for *T. brucei* has not progressed because the tactiful mechanism such as varing surface glycoproteins allows the parasites to evade host immune system. Furthermore, the sicknesses have re-emergenced, and chemotherapeutic failures due to drug resistance have been reported in recent years. However, molecular mechanisms of drug resistance are not well established.

In this thesis, it was described that programmed cell death (PCD)-like phenomenon was developed in *T. brucei* cultured under the high-density conditions, and the expression of trypanosome alternative oxidase (TAO) was associated with the development of PCD-like phenomenon and the drug resistances.

PCD-like phenomenon was developed in *T. brucei* cultured under the high-density conditions, but the deficiency in nutrients of the medium appeared to be unrelated to the development of PCD-like phenomenon. The development of PCD-like phenomenon was inhibited by the low-temperature stress concomitant with the up-regulation of TAO. On the other hand, the specific chemical inhibitor of TAO facilitated PCD-like phenomenon under the low-temperature condition, which implied that the expression of TAO would correlate with the development of PCD-like phenomenon. To ascertain the relativity between the expression of TAO and the development of PCD-like phenomenon, transgenic *T. brucei* over-expressing TAO was generated and the rate of PCD-like phenomenon under high-density culture conditions was compared to that of wild-type. As the result, the transgenic cells showed the resistance to the development of PCD-like phenomenon.

Furthermore, transgenic cells showed the resistance to suramin and antrycide comparing to wild-type. Transgenic *T. brucei* overexpressing TAO produced lower levels of reactive oxygen species (ROS) in the treatment of antrycide, which indicated that ROS would be involved in the resistance to antrycide. In contrast, the parasites treated with suramin showed the same amount of ROS.

More detailed elucidation of the interplay

between the development of PCD and drug resistance following TAO expression is needed, however, these results suggested that the development of PCD would be associated with drug resistance in *T. brucei*.

Original papers of this thesis appeared in *Parasitol. Int.*, 54 : 243-251 (2005), and 55 : 135-142 (2006).

Determination and characterization of biologically significant substances in salivary gland of the hard tick, *Haemaphysalis longicornis*

Chie Nakajima

Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Ticks produce various bioactive molecules in their salivary gland, and inject them into the host during blood feeding. These molecules are supposed to modulate hosts' physiological and immunological reactions, and may help in the transmission of pathogens of tick-borne-diseases. In the current study, the profile of the genes expressed in the salivary gland was analyzed by using the expressed sequenced tag (EST) method.

A cDNA library was constructed from the salivary glands of partially-fed adult female *Haemaphysalis longicornis*. Randomly selected clones were sequenced, and a total of 633 sequences were analyzed by bioinformatic programs. About 36% of the mRNA sequences showed significant similarity to known proteins in the non-redundant protein database by the NCBI blastx program whereas the remaining 64% had no similar sequences. Two thirds of the predicted proteins were annotated as housekeeping proteins. In addition, several protease inhibitors, anticoagulants, two metalloproteases and a potential immunosuppressive protein were identified.

Among the un-annotated protein sequences, a protein without cysteine residues in its sequence was identified. The sequence had a signal peptide for secretion, had low pI and the length was short, less than 100 amino acids. These characteristic features were similar to those of a thrombin inhibitor previously identified from *Haemaphysalis longicornis*, therefore the function of this un-annotated protein was investigated although the sequence had little similarity to the thrombin inhibitor.

The recombinant protein, named chimadanin, significantly prolonged the activated partial thromboplastin time and the prothrombin time of sheep plasma in a dose-dependent manner. Amidolytic activity of thrombin was also inhibited by chimadanin in a dose dependent manner, and thus chimadanin was thought to be a thrombin inhibitor. This newly identified thrombin inhibitor may play an important role during tick blood feeding.

There may still be a large number of novel bioactive protein coding sequences in the tick salivary gland EST database. These molecules have possibilities of becoming new candidate antigens for anti-tick or tick-borne disease vaccines. Identification of these molecules could provide some information for the development of new therapeutic drugs, such

as anticoagulants for thrombosis. They may also help our understanding of the host-parasite interactions.

Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 67 : 1127-1131 (2005), and (in press).

Genetic analysis and development of new diagnostic assays of Newcastle disease virus

Hang Minh Pham

Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Newcastle disease (ND) is a highly contagious viral infection of poultry caused by Newcastle disease virus (NDV), also designated as avian paramyxovirus serotype 1 (APMV1). Since several outbreaks of ND are currently reported worldwide, molecular epidemiology of NDV and development of new rapid diagnostic assays are important to prevent future ND outbreaks. For these reasons, in this thesis, phylogenetic analysis of NDV isolates in Japan was performed and three different diagnostic methods for NDV were established.

A phylogenetic tree construct based on the nucleotide sequence of the complete nucleocapsid protein (NP) gene of Japanese isolates revealed that the old (prior to 1970s) and the new (after 1980s) isolates could be classified into two major groups, i. e., a group comprising virulent strains, and the other group composed of avirulent strains. It was also found that PCR products from the NP genes of virulent strains were not cleaved by a restriction enzyme *Pst*I while those of avirulent strains were cleaved. Thus, the RT-PCR-restriction fragment polymorphism (RFLP) assay using *Pst*I can be applied for a simple primary screening test to rapidly identifying NDV isolates.

A novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), was applied for diagnosis of ND. By using two sets of specific primers targeting the fusion (F) protein gene, this LAMP assay rapidly amplified the target gene from clinical samples within 2h, requiring only a regular laboratory water bath or heat block for the reaction. This method was as sensitive and specific as nested PCR. These results clearly demonstrated that LAMP-based assay can be used for the rapid, simple, sensitive, and cost-effective diagnosis of NDV.

A highly sensitive and specific assay for rapidly differentiating NDV isolates based on real-time RT-PCR SYBR Green I melting-curve analysis of the F gene was developed. This assay was 100 times more sensitive than conventional RT-PCR, directly detected NDV from infected tissues, and eliminated the gel electrophoretic step using ethidium bromide for analyzing PCR product. The NDV isolates were rapidly differentiated within 1 h based on their distinctive melting temperatures (T_ms) : 89.23 ± 0.27℃ for velogenic strains, 90.17 ± 0.35℃ for pigeon mesogenic strains, and 91.25 ± 0.14℃ for lentogenic strains. This real-time PCR is a good screening test for the detection and differentiation of NDV isolates.

Although gene amplification methods can

not be substituted for conventional culture methods to isolate NDV, the combined use of more than one molecular diagnostic assay, each directed against a different target of the virus genome, is superior to the use of single assay. As shown by *in vitro* and *in vivo* experiments, three assays presented here provide rapid, safe, cost-effective, sensitive, and reliable tool for the detection and identification of NDV isolates. Each assay can be used depending on the facilities in the clinical laboratories and promises as a good routine tool for laboratories in the future.

Original papers of this thesis appeared in *Arch. Virol.*, 149 : 1559-1569 (2004), 150 : 2429-2438 (2005), and *J. Clin. Microbiol.*, 43 : 1646-1650 (2005).

Analysis of tumor suppressor gene *p53* in chicken lymphoblastoid tumor cell lines and field tumors

Michihiro Takagi

Department of Microbiology and Immunology, Faculty of Agriculture, Kobe University, Kobe 657-8501, Japan

It has been still largely unknown that the molecular mechanism(s) for the transformation in many human and animal tumors. This mechanism includes abnormalities in several cellular oncogenes or interaction of the products from the cellular oncogenes with the proteins produced by DNA or RNA tumor viruses. Marek's disease virus (MDV), avian leucosis virus (ALV) and reticuloendotheliosis virus (REV) are known as avian oncogenic viruses, but their mechanisms of the transformation have not been also understood. Since the tumor suppressor gene *p53* was discovered, several mutations and deletions in the *p53* gene and its gene product were detected in many kinds of human and animal tumors, showing that abnormalities in p53 could play an important role in the tumorigenesis. Thus, in this thesis, abnormalities in p53 were studied in lymphoblastoid cell lines established from tumors caused by MDV, ALV, and REV to clarify the roles of p53 in the transformation by these viruses.

Several mutations were identified in chicken lymphoblastoid cell lines and tumor samples from commercial flocks. These mutations, however, are not localized at the "hot spot", which has been reported in the site for transformation-activating mutations in human and mammalians. Interestingly, several deleted forms of the *p53* transcripts were present in chicken tumor cell lines, but frame shifts occurred in open reading frames of most of these transcripts. In addition, the p53 proteins with smaller molecular weights than native p53 were also detected in these cell lines. These deleted forms of the *p53* transcripts were expressed independent of the progression of cell cycle, and also constantly expressed during apoptosis. These deleted forms of the *p53* transcripts and their gene products may result from differential or alternative splicing induced by the interaction between viral and intracellular proteins during the RNA transcription or processing.

To study the roles of p53 in the oncogenic

potentials of tumor cells, the expression patterns of the *p53* transcripts was analyzed in sublines obtained from an MDV-transformed cell line which are different in their transplantabilities to chickens. The expression patterns of the *p53* transcripts and proteins were different among these sublines, suggesting that the deleted forms of the p53 could contribute to the variety of biological characteristics, such as transplantability and metastatic nature *in vivo* in these cell lines.

The deleted forms of p53 have been reported to regulate apoptosis or growth of the cells. In this study, it was found that the expression of the deleted forms of p53 is increased during the apoptosis induced by chemotherapeutic compounds but not when apoptosis was inhibited. This result showed that the quantitative and qualitative balance in the expression of the short form of p53 and native p53 could control the p53 activity in chicken tumor cell lines. In other words, the deleted forms of p53 may inactivate the activities of the wild type p53, and thus may contribute to the transformation by avian oncogenic viruses. Further study will be necessary to understand the functions of the deleted forms of p53.

Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 60：923-929 (1998), *ibid.* (in press), and *Leukemia Res.* (in press).

Development of *Taenia asiatica* in alternative intermediate and definitive hosts and coproantigen detection in the definitive hosts

Shiou-Ling Chang

Laboratory of Parasitology, Department of Disease Control,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

---

Original papers of this thesis appeared in *Parasitol. Res.*, 96：95-101 (2005) and *J. Helminthol.*, 80：in press (2006).

Analysis of the novel neuro-pathogenicity of fowl glioma-inducing avian leukosis virus

Takeshi Toyoda

Laboratory of Comparative Pathology, Department of Veterinary Clinical Sciences,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Fowl glioma-inducing virus (FGV), which belongs to avian leukosis virus subgroup A (ALV-A), causes multiple astrocytoma in chicken. Since ALV-A generally induces hematopoietic tumors, the elucidation of neuropathogenicity of FGV will be helpful in understanding the neuro-pathogenicity of retroviruses in mammals and avian species. The pre-

sent study describes the novel neuropathogenicity of FGV and the promoter activity of FGV-specific long terminal repeat (LTR).

Chapter Ⅰ. I examined multiple enlargements of peripheral nerves (PNs) in chickens infected with FGV. Lumbosacral plexus and brachial plexus were commonly affected and the PNs contained diffuse proliferation of spindle cells with characteristic onion bulb-like structures. The spindle cells were immunohistochemically negative for S-100α/β protein. Electron microscopy revealed that these cells had short bipolar processes, occasional pinocytotic vesicles and discontinuous basal laminae. These morphologic features are consistent with those of intraneural perineurioma. Furthermore, FGV-specific sequence was detected in the PNs of 8/11 birds by polymerase chain reaction. These results suggested that the multiple intraneural perineuriomas of chicken may be associated with FGV.

Chapter Ⅱ. I examined the cerebellar anomaly found in chickens infected with FGV *in ovo*. The affected cerebellum included diffuse depletion of granular cells of the internal granular layer, remnants of the external granular layer (EGL) and disorganization of the Purkinje cell layer (PL). In the infected embryos, formation of the cerebellar cortex was delayed and apoptotic granular cells were frequently observed in the EGL and molecular layer. Immunohistochemical studies revealed the presence of ALV common antigen in granular cells and disarrangement of Bergmann's fibers. These results suggested that the cerebellar anomaly is hypoplasia primarily resulting from the apoptosis of granular cells.

Chapter Ⅲ. The LTR sequence of ALV plays important roles in viral replication and tissue-specific oncogenesis. To examine the promoter activity of FGV LTR *in vivo*, transgenic mice expressing a transgene under the control of the LTR were generated and the tissue-specificity of the expression was analyzed. The mRNA expression of transgene was frequently detected in the cerebrum and cerebellum although the expression was also detected in other organs. These results suggested that the FGV LTR is capable of driving transgene expression *in vivo* particularly in the targeted organs of FGV pathogenicity although the LTR acts as a pan-specific promoter.

The present studies demonstrated that the FGV was associated with the cause of multiple intraneural perineurioma and the virus caused cerebellar hypoplasia in the *in ovo* infected chickens. In addition, the FGV LTR promoter was suggested to be associated with the neuro-pathogenicity of FGV.

The original papers of this thesis appeared in *Vet. Pathol*., 42 : 176-183 (2005) and *Vet. Pathol*., 43 : 294-301 (2006).

In vivo and in vitro analysis of neuropathogenicity of equine Herpesvirus-1

Rie Hasebe

Laboratory of Comparative Pathology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sappro 060-0818, Japan

Equine herpesvirus-1 (EHV-1) causes rhinopneumonitis, abortion, and encephalo-

myelitis in horses. A relevant model for studying EHV-1 encephalomyelitis has not been established whereas an adult BALB/c mouse model has many features similar to the natural infection in horses. Intracerebral inoculation of suckling mice with EHV-1 results in viral replication in the neuronal cells and causes encephalitis. However, neuropathogenicity of EHV-1 in mice remains largely unknown. In chapter 1, a neuroadapted variant of EHV-1 was generated by serial passages of the virus in suckling mouse brains. Histological examination was performed on 6-day-old mice infected with neuroadapted EHV-1 (NHH1) and its parental strain (HH1) by various routes. Both NHH1 and HH1 caused encephalitis in mice infected via intracerebral and intranasal route. Encephalomyelitis was observed in mice infected intraperitoneally with NHH1, but not with HH1. In chapter 2, the route of viral invasion in the CNS of suckling mice was determined after intraperitoneal inoculation with NHH1 and HH1. In NHH1 infected mice, viral dissemination to the CNS is occurred by cell-associated viremia, which results in encephalomyelitis. Neither viremia nor histological lesion was observed in mice infected with HH1. The characteristic lesions in mice infected with NHH1 and HH1, regardless of the route of virus inoculation, were degeneration and necrosis of the infected neuronal cells. Viral infection of CNS endothelial cells, which plays an important role in the pathogenesis of EHV-1 encephalomyelitis in horses, was not detected. These observations suggest that EHV-1 infection in the suckling mouse model is different from that in horses in the type of cell susceptible to infection.

In chapter 3, the primary cultured equine brain microvascular endothelial cells (EBMECs) were established as an *in vitro* model for studying EHV-1 endotheliotropism. To explore the basis for species difference regarding endotheliotropism of EHV-1, the susceptibility of EBMECs was compared with that of mouse brain microvascular endothelial cells (MBMECs) to EHV-1 infection. As expected from the susceptibility *in vivo*, EBMECs were highly susceptible to EHV-1 infection. On the other hand, MBMECs were resistant to EHV-1 infection. No viral particle was detected in the cytoplasm of MBMECs by ultrastructural study, whereas enveloped virions were found frequently in the cytoplasm of EBMECs. The EHV-1 IE gene was not expressed in infected MBMECs. These studies suggest that the difference in the degree of virus internalization is responsible for the difference in the susceptibility to EHV-1 infection between EBMECs and MBMECs.

In chapter 4, the mode of EHV-1 entry into EBMECs and two other susceptible cell types: rabbit kidney (RK13) cells, and equine dermis (E. Derm) cells was examined. Enveloped viral particles were detected in non-coated vesicles within the cytoplasm of all cell types by electron microscopy. Analysis with the use of confocal microscopy suggests that EHV-1 internalization into EBMECs and RK 13 cells is mediated by caveolae-dependent endocytosis and the entry into E. Derm cells occurs in caveolae-independent clathrin-independent manner. Treatment of cells with tyrosine kinase inhibitor largely prevented EHV-1 entry into EBMECs and RK13 cells, but did not block the entry into E. Derm. Viral entry into EBMECs and E. Derm was inhibited by lysosomotropic agents, but the entry into RK13 cells was not affected. These results suggest that EHV-1 makes use of different endocytic pathways in different cell types to establish productive infection. Further studies on the molecular mechanisms of EHV-1 endocytosis into EBMECs, especially concerning viral receptors, may facilitate an under-

standing of EHV-1 neuropathogenicity.

The original papers of this thesis appeared in *J. Comp. Pathol.*, 127：118-125 (2002), *J. Vet. Med. Sci.*, 64：907-912 (2002) and *Arch. Virol.*, 151：775-786 (2006).

Studies on the cytoplasmic morphology, maturational and developmental ability of bovine oocytes derived from small antral follicle

Masashi Nagano

Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

---

The original papers of this thesis appeared in *J. Vet. Med. Sci.*, 61：531-535 (1999), *Zygote* 14：53-61 (2006), and *Zygote* (in press).

Molecular pathobiology for renal tubular dysplasia in Japanese black cattle due to claudin-16 deficiency

Hiroshi Ohta

Laboratory of Molecular Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Claudins are the major constituents of tight junction (TJ) strands and participate in the cell-cell adhesion and permeability barrier in epithelial cell layers. Claudin-16 was identified as the product of the *CLDN16* gene responsible for familial hypomagnesemia with hypercalciuria and nephrocalcinosis in the human and renal tubular dysplasia with interstitial nephritis in Japanese black cattle. Although clinical phenotypes for these disorders have suggested that claudin-16 participates in paracellular transport of Mg^{2+} and Ca^{2+}, the molecular pathogenesis of claudin-16 deficiency remains unknown.

As the first step to clarify molecular pathobiology for claudin-16 deficiency, the expression and distribution of claudin-16 and several other major claudin subtypes, claudins 1-4 and 10, in bovine renal tubular segments were examined by immunofluorescence microscopy. Claudin-16 was exclusively distributed to the TJ in the tubular segment positive for Tamm-Horsfall glycoprotein, the thick ascending limb (TAL) of Henle's loop, and was found co-localized with claudins 3, 4, and 10, indicating that the total lack of claudin-16 in the TAL segment is the sole cause of renal tubular dysplasia in cattle and is likely to impair the tubular development during the prenatal stage.

To examine the prenatal pathogenesis in the claudin-16 deficiency, *CLDN16*$^{-/-}$ bovine fetuses at embryonic days 100, 180, and 270 were generated by embryo transfer, and the tubular formation and the expression of the tight junction proteins were examined. The *CLDN16*$^{-/-}$ fetuses showed no histological abnormality compared with normal *CLDN16*$^{+/+}$ fetuses even at 270 days of gestation. Claudins 3, 4, and 10 were constitutively distributed at the TJ of the TAL of Henle's loop in the kidneys of *CLDN16*$^{-/-}$ fetuses as in normal *CLDN16*$^{+/+}$ kidneys. Distributions of claudins 3, 4, and 10 were also found at the TAL regions in the kidney with aberrant tubular dysplasia from a 7-month-old *CLDN 16*$^{-/-}$ animal. These findings demonstrated that deficiency of claudin-16 did not cause apparent morphological abnormality in renal tubules during the period of renal development *in utero*. Thus, claudin-16 appears necessary for the maintenance of normal tubular architecture after birth presumably by its function of paracellular transport mechanism.

To examine the suitability of organotypic culture of metanephroi as an *in vitro* system to study paracellular transport under the regulation of specific genes, mouse metanephroi from embryos at day 12 of gestation were cultured and expression of claudins was compared with that in embryonic kidneys. During *in vitro* culture for 8 days, the metanephroi showed expression patterns very similar to those observed in embryonic kidneys in reverse transcription-polymerase chain reaction for claudins 1-4, 8, 10, 11, and 16. Immunofluorescence microscopy for claudins 1-4, 8, 10, and 16 showed localization of these claudins at the TJ with occludin and ZO-1 in some restricted tubular segments. These findings indicate that the metanephroi show developmental changes in the expression of the TJ protein claudins, representing those in embryonic kidneys, and thus suggest that the mouse metanephros is suitable to examine the functions of specific claudins.

In conclusion, the present study demonstrates that the total lack in the TAL segment of claudin-16 is the sole cause of inherited renal tubular dysplasia in cattle. This study evidently shows that the lack of claudin-16 dose not affect renal tubular development in the prenatal stage, indicating that claudin-16 is indispensable for the maintenance of tubular architecture because of it's paracellualr transport mechanism that remains to be clarified.

Original papers for this thesis appeared in part in *J. Vet. Med. Sci.*, 68 : 149-155 (2006) and *J. Vet. Med. Sci.*, 68 : 453-463 (2006).

PKCδ is involved in the operating of the superoxide production and the phagocytosis in bovine neutrophil and differentiated HL60 cells

Kenji Waki

Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

This experiment was performed to clarify the role of protein kinase C (PKC) δ in NADPH oxidase-dependent O_2^- production and actin polymerization followed by phago-

cytosis in neutrophils. Bovine neutrophils and human neutrophil-like differentiated HL60 (dHL60) cells were stimulated with serum-opsonized zymosan (OZ) and fMet-Leu-Phe (fMLP), respectively. Rottlerin, a specific inhibitor of PKCδ, attenuated the production of O_2^- from NADPH oxidase in both neutrophils and dHL60 cells. However, it did not inhibit the translocation of $p47^{phox}$ from the cytosol to the membrane in either type of cell or the phosphorylation of $p47^{phox}$ in dHL-60 cells. GF 109203X (GFX), an inhibitor of cPKC, attenuated not only the production of O_2^- but also the translocation of $p47^{phox}$ in both cells. Furthermore, rottlerin significantly attenuated the ingestion of opsonized particles and the formation of F-actin in OZ-stimulated neutrophils, whereas GFX did not affect those phagocytic processes. These results suggest that both PKCδ and cPKC regulate NADPH oxidase through different pathways, but only PKCδ regulates the phagocytic function in neutrophils.

The original paper of this thesis appeared in *Free Radic. Res.*, 37 : 665-671 (2003) and *ibid.*, 40 : 359-367 (2006)