



HOKKAIDO UNIVERSITY

Title	INFORMATION: Theses for the Doctor of Veterinary Medicine (equivalent to Ph.D.)
Citation	Japanese Journal of Veterinary Research, 53(1-2), 47-69
Issue Date	2005-08
Doc URL	https://hdl.handle.net/2115/883
Type	departmental bulletin paper
File Information	47-69.pdf



INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy (Ph.D) in Veterinary Medicine on March 25, 2005 to 20 recipients and June 30, 2005 to 1 recipient.

The titles of theses and other information are as follows :

Characterization of the genetically obese diabetic Wistar fatty rat as a "Syndrome X" model and the therapeutic effect of an insulin sensitizer

Masami Suzuki

*Pharmacology Research Laboratories II ,
Takeda Chemical Industries Ltd, Osaka, 532-8686, Japan*

"Syndrome X" is a well known condition associated with hyperinsulinemia, glucose intolerance, insulin resistance, hyperlipidemia, hypertension, and visceral obesity, and is a pivotal risk factor for development of atherosclerosis. The Wistar fatty rat has been established as an animal model of obese type 2 diabetes mellitus, showing visceral obesity, insulin resistance, glucose intolerance, hyperlipidemia, and hyperinsulinemia. However, vascular complications in this model animal, including nephropathy and hypertension, remain to be studied. In the present study, to clarify the usefulness of the Wistar fatty rat as a model of "Syndrome X", I examined the development of nephropathy and hypertension in this rat strain. I also examined the therapeutic effects of an insulin sensitizer, pioglitazone · HCl, on vascular complications.

First, I examined the age-dependent development of nephropathy and its relationship to plasma parameters. In male Wistar fatty rats, the plasma levels of glucose, triglyceride and insulin began to increase at 8 weeks of age, followed by increases in the urinary excretion of albumin, protein and N-acetyl-β-glucosaminidase (NAG) . Histological examination revealed renal lesions such as expansion

of the glomerular mesangial area, slight thickening of the glomerular capillary basement membrane, formation of hyaline casts, and atrophy of the renal tubules. These changes are similar to those in diabetic patients with early-stage nephropathy. Female Wistar fatty rats and male Zucker fatty rats were also obese, hypertriglyceridemic and hyperinsulinemic, but showed much less obvious changes in the parameters of nephropathy than male Wistar fatty rats. These rats are less hyperglycemic than male Wistar fatty rats, suggesting that hyperglycemia plays an important role in the development of nephropathy. When male Wistar fatty rats were administered pioglitazone · HCl orally for 12 weeks beginning at 5 weeks of age, the age-dependent increases in plasma glucose, triglyceride and insulin were markedly suppressed. Treatment with pioglitazone · HCl also suppressed urinary excretion of albumin, protein and NAG as well as the development of renal glomerular lesions.

I then examined the development of hypertension in Wistar fatty rats. Their systolic blood pressure was slightly but significantly higher compared with lean control rats at over 22 weeks of age. The administration of

pioglitazone·HCl decreased the systolic blood pressure to normal levels, and increased insulin sensitivity as assessed by the glycemic response to exogenous insulin. Hypotensive effects of pioglitazone·HCl were also observed in Wistar Kyoto rats, which show hepatic insulin resistance and hypertension, given drinking water containing 10% fructose for 6 weeks. Both rat models showed a good correlation between systolic blood pressure and the level of plasma insulin, suggesting that insulin resistance is a causal factor for hypertension.

Finally, I examined the therapeutic effect of another insulin sensitizer, biguanide metformin, which has an insulin-sensitizing mechanism different from that of pioglitazone·HCl, in male Wistar fatty rats. Plasma glucose was significantly decreased by treatment with pioglitazone·HCl or metformin alone, and decreased additively by combination of the two drugs. Pioglitazone·HCl, but not metformin, also decreased the plasma levels of

triglyceride and total ketone bodies. The glycogen content of skeletal muscle was not influenced by pioglitazone·HCl or metformin alone, but increased by combined treatment. These results suggest that the combination of pioglitazone·HCl and metformin is more effective for treating the metabolic abnormalities in this animal model.

Thus, male Wistar fatty rats develop nephropathy and hypertension, as well as insulin resistance and hyperlipidemia, all of which were typical features of "Syndrome X", and these disorders can be effectively normalized by the insulin sensitizer, pioglitazone·HCl. Therefore I conclude that the Wistar fatty rat is useful as a model of "Syndrome X" for investigating the pathophysiological mechanism of diabetes mellitus and atherosclerosis closely associated with insulin resistance, and for studying and evaluating possible therapeutic agents for these metabolic disorders.

Original papers of this thesis appeared in *Jpn. J. Pharmacol.*, 74 : 297-302 (1997), and *Clin. Exp. Pharmacol. Physiol.* 29 : 269-274 (2002).

Studies on the prevalence of leptospirosis in animals in Mongolia

Namsrajav Odontsetseg

*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 *Vet. Rec.*, 157 : 120-121 (2005), and *Vet. Rec.*, (2005) (in press), and *Jpn. J. Vet. Res.*, 52 : 151-162 (2005).

Studies on pathogenicity of avian H5 and H9 influenza viruses in chicken and duck

Noriko Kishida

*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 *Arch. Virol.*, 149 : 2095-2104 (2004), and *Arch. Virol.*, 150 : 1383-1392 (2005).

Morphogenesis of influenza and Ebola virus particles

Takeshi Noda

*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 *J. Virol.*, 76 : 4855-4865 (2002), and *J. Vet. Med. Sci.*, 67 : 325-328 (2005).

Molecular cloning and quantification of llama and camel cytokines

Raadan ODBILEG

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

Camels and llamas are members of the *Camelidae* family, *Tylopoda* suborder, *Artiodactyla* order, Mammalian class. Cytokines have been explored in a wide variety of infectious diseases and the important roles of cytokines in the regulation of immune and inflammatory responses are now clearly understood. However, there is no information available about cytokine genes of *Camelidae*. Thus, the purpose of this study was to identify the cytokine cDNAs of *Camelidae* (llama and camel) by cloning and sequence analysis, and

to develop a real-time PCR method to quantify the expression of cytokine mRNAs in the members of the *Camelidae* family. This method was used to monitor the cytokine mRNA levels in camels vaccinated with *B. abortus* S19.

cDNA synthesis was performed with purified total RNA derived from ConcanavalinA-stimulated llama PBMCs. To identify cytokine genes, we used primers designed based on bovine cytokine genes (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-13, IL-12p35, IL-

12p40, IFN- γ and TNF α) previously reported. cDNAs encoding for Th1, Th2 and inflammatory cytokines were cloned, sequenced and compared with those of other mammalian species. Homology analyses of nucleotide and deduced amino acid sequences of llama Th1, Th2 and inflammatory cytokines and phylogenetic analysis based on their nucleotide sequences indicated the close relationship in these cytokine cDNAs between llama and eutherian mammalian order *Artiodactyla* (pig, cattle) and *Perissodactyla* (horse). The cloning, sequence and phylogenetic analyses of llama Th1, Th2 and inflammatory cytokines will be useful data for cell-mediated and humoral immunity and may become implicated

in the regulation of many immune responses and outcomes of diseases in *Camelidae*. In the next experiment, I developed a new method by which llama cytokine mRNAs can be quantified using real-time PCR and then examined the cytokine responses in 7 healthy camels vaccinated with commercial *B. abortus* S19 live vaccine.

The methodology of the real-time PCR described here was easily and successfully applied for the quantification of several cytokine cDNAs from llama and camel. Analysis of the cytokine response to *B. abortus* S19 vaccine showed high mRNA levels of IFN- γ and IL-6 but low levels of TNF α and IL-10 in vaccinated camels.

Original papers of this thesis appeared in *Vet. Immunol. Immunopathol.*, 102 : 93-102 (2004), *Vet. Immunol. Immunopathol.*, 104 : 145-153 (2005) and *J. Vet. Med. Sci.*, 67 : 195-198 (2005).

Genetic factors of drug resistance and diversity in *Trypanosoma evansi*

Witola William Harold

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

The emergence of resistance against major drugs for the control of trypanosomiasis is now a serious and increasing problem. However, drug resistance molecular mechanisms in trypanosomes are largely unknown. This study aimed at identification and functional validation of genes mediating resistance against diminazene aceturate (berenil, a major trypanocide) in *Trypanosoma evansi*.

A gene, *TevAT1*, which encodes a P2 aminopurine permease was cloned and functionally characterized. The first ever engineered transgenic cell line for induction of RNAi in *T. evansi* was generated. Induction of RNAi of the *TevAT1* gene in *T. evansi* con-

ferred highly significant resistance to berenil, validating that loss of function of the *TevAT1* gene is largely responsible for the resistance. Because different resistance mechanisms can complement each other and give rise to high level resistance, in this study a novel gene, *TeDR40*, up-regulated in berenil-resistant *T. evansi* was cloned by differential display technique. Expression analysis of *TeDR40* indicated that it is at least 1,000-fold up-regulated in berenil-resistant *T. evansi* and encodes a 40kDa protein. The first ever engineered *T. evansi* cell line for gene overexpression was generated for inducible overexpression of the *TeDR40* gene in wild-type

berenil-sensitive *T. evansi*. Subsequent over-expression of TeDR40 gene conferred significant resistance to berenil in wild-type *T. evansi* validating that TeDR40 gene plays a significant role in mediating the resistance.

Transferrin receptors are now important drug permeases in cancer cells, but they have not yet been exploited in trypanosomes. However, with the presumed diverse nature of transferrin receptor genes in different species of trypanosomes, prior genetic characterization of their diversity would help in understanding how efficiently they can be utilized as drug permeases. Further, because the transferrin receptor plays a crucial role in the survival of trypanosomes in the host through efficient uptake of iron, genetic diversity in the transferrin receptor genes could play a crucial role in the pathogenicity and host range of a particular trypanosome species and/or strain.

In this study, different transferrin recep-

tor genes (ESAG6 genes) from different isolates of *T. evansi* were cloned and genetically characterized. It was established that intra- and inter-species genetic variability exists in the transferrin receptor genes regions involved in transferrin binding. Further, it was found that, *T. evansi* possesses the widest range of ESAG6 variants among all the trypanosome species characterized so far. It was also observed that different strains of *T. evansi* possess different variants, though at least two conserved ESAG6 variants were established among all the different seven strains analyzed. The widely diverse transferrin genes in *T. evansi* is consistent with its ability to infect and cause disease in a very broad range of mammalian hosts. The diversity in the transferrin receptors among different strains of *T. evansi* could be important in mediating the pathogenicity of different strains.

Original papers of this thesis appeared in *Exp. Parasitol.* 107 : 47-57 (2004) , *Acta Trop.* 93 : 63-73 (2005) and *Parasitology* (2005), (in press).

Genetic analysis of jumbled spine and ribs (*Jsr*) mutation affecting the vertebral development in mice

Shinya Okano

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

Original papers of this thesis appeared in *Biochem. Genet.*, 40 : 311-322 (2002) and *Jpn. J. Vet. Res.*, 52 : 145-149 (2005).

Genetic analysis of the meiotic metaphase-specific apoptosis
and the heat stress resistance of spermatocytes in the MRL/MpJ mouse

Yuka Namiki

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

The MRL/MpJ strain shows several unique phenotypes, including rapid wound healing, inherent collagen disease. Especially, it has been found two characteristics in testis of the MRL/MpJ mouse, metaphase-specific apoptosis (MSA) and heat stress resistance. The author has determined the causal gene for the MSA phenotype in the MRL/MpJ mouse. Furthermore, the author analyzed genetic factor(s) for the heat stress resistance in the testis of the MRL/MpJ mouse.

In Part 1 of this study, the genetic mode of the MSA was determined in the MRL/MpJ mouse using 555 backcross progenies. This phenotype is controlled by a single autosomal recessive locus. Thus, the locus was tentatively termed *msa*. The author has shown that a mutation of the *Exo1* gene is responsible for it. Interestingly a T-to-A transition 2 bp upstream of the branch site in intron 8 results in several types of abnormal splicing of the mRNA to produce an altered or premature Exo1 protein.

In Part 2 of this study, the author has performed genetic analysis of heat stress resistance of spermatocytes in the MRL/MpJ mouse. Thus, quantitative trait loci (QTL) analysis was performed using 98 microsatellite markers. The weight ratio of the cryptorchid testis to the intact testis (testis weight ratio) and the Sertoli cell index were used for quantitative traits. The result indicated that a major QTL was mapped to 100-cM region in Chr 1, where the *Exo1* gene locates. This fact suggests that a mutation of the *Exo1* gene is, in turn, responsible for the heat stress resistance of spermatocytes.

The present study propose that a mutation of the *Exo1* is responsible for both MSA and heat stress resistance of spermatocytes in the MRL/MpJ mouse. Thus, the MRL/MpJ mouse is a useful animal model in the research of molecular mechanisms for not only spermatogenesis but also for apoptosis after the DNA damage.

Studies on the functionality of alk(en)yl thiosulfates derived from garlic and onion

Hye-Sook Chang

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

The use of natural substances has become more widespread over the past few years, driven undoubtedly by the belief that natural substances have fewer side effects than do pharmaceuticals, and by their availability to the public without prescriptions or visits to health providers. In recent years, extensive research has focused on the beneficial and medicinal properties of garlic and onion. In particular, the use of these agents in the treatment and prevention of neoplasm and cardiovascular disease is an area of considerable investigation and interest.

It is known that garlic and onion are oxidatively toxic to erythrocytes resulting in hemolytic anemia in domestic animals, particularly in dogs, however, there is no evidence that the consumption of garlic and onion causes hemolytic anemia in humans. Two alk(en)yl thiosulfates, sodium 2-propenyl thiosulfate (2PTS) and sodium *n*-propyl thiosulfate (NPTS), are natural constituents of garlic and onion respectively, and were identified originally as causative agents of garlic-and onion-induced hemolytic anemia in dogs. The purpose of this study was to investigate the functionality of two alk(en)yl thiosulfates, 2PTS and NPTS, by elucidating whether these compounds have any beneficial effects as reported in many studies concerning garlic and onion.

To elucidate the beneficial functions of 2PTS and NPTS, I investigated the antitumor effects of these compounds in the first section. They were shown to inhibit the proliferation of three human tumorigenic cell lines,

WiDr, 293 and HL-60, *in vitro*, in a dose-dependent manner. Overall, NPTS seemed to have weak activity for inhibiting cell growth compared with 2PTS, though not in WiDr cells, which were sensitive to both compounds. 2PTS and NPTS caused oxidative damage to HL-60 cells and induced apoptosis. The extent of apoptosis was approximately proportional to that of the oxidative damage and also to that of the cytotoxicity caused by these compounds. These results suggest that the alk(en)yl thiosulfates have an antitumor effect through the induction of apoptosis initiated by oxidative stress.

In the second section, I examined the anti-aggregatory effect of 2PTS and NPTS on platelet aggregation in dogs and humans *in vitro*. These compounds were shown to significantly inhibit adenosine 5'-diphosphate (ADP)-induced platelet aggregation at 0.01 mM ($P < 0.01$) in canine platelets and at 0.001-0.1 mM ($P < 0.05$) in human platelets. On the contrary, the extent of aggregation tended to return to the control level at 1 mM of alk(en)yl thiosulfates in both canine and human platelets. To clarify the mechanism of this modulatory influence of alk(en)yl thiosulfates on the aggregation of platelets, I investigated the effects of 2PTS on cyclooxygenase (COX) activity and the reduced glutathione (GSH) concentration in canine platelets. Platelet COX activity was inhibited by 2PTS in a dose-dependent manner up to 0.1 mM, but tended to return to the control level at 1 mM. In contrast, the platelet GSH concentration decreased in a dose-dependent manner

after treatment with 2PTS and a significant decrease was observed at 0.1 mM ($P < 0.05$) and 1 mM ($P < 0.001$). Furthermore, the activity of purified COX-1 was directly inhibited by addition of GSH in a dose-dependent manner. From these results, I conclude that alk(en)yl thiosulfates have an anti-aggregatory effect on both canine and human platelets *in vitro* and could contribute to the prevention of cardiovascular diseases. This inhibition of platelet aggregation occurs as a result of inhibition of COX activity and these compounds may have a modulatory effect on the aggregation by affecting the concentration of GSH in platelets which ultimately explains the safety of garlic and onion as functional foods.

In the last section, I investigated the promoting effects of 2PTS and NPTS on the generation of superoxide (O_2^-) by peripheral polymorphonuclear leukocytes (PMNs) in dogs and humans *in vitro*. Both compounds increased O_2^- generation significantly ($P < 0.005$ at 0.1 and 1 mM 2PTS, $P < 0.05$ at 1 mM NPTS) and reduced its reaction time signifi-

cantly ($P < 0.05$ at 1 mM 2PTS and between 0.01 and 1 mM NPTS) in canine PMNs stimulated by phorbol 12-myristate 13-acetate, compared with the control without alk(en)yl thiosulfates. However, both O_2^- generation and its reaction time tended to return to the control level at 10 mM of the alk(en)yl thiosulfates. Although 2PTS and NPTS did not exert any significant effect on the O_2^- generation in human PMNs, 2PTS reduced its reaction time significantly ($P < 0.05$) at 1 and 10 mM compared with the control, showing that 2PTS accelerated O_2^- generation in human PMNs. From these results, I conclude that alk(en)yl thiosulfates have the potential to promote immune functions by enhancing superoxide generation in PMNs.

In the present study, I showed that 2PTS and NPTS have beneficial functions such as an antitumor effect, an anti-aggregatory effect on platelet aggregation and a promoting effect on immune functions. It is our hope that the beneficial effects of 2PTS and NPTS will contribute to the improvement of human health and well-being.

A study of chondrogenic differentiation of bovine bone marrow mesenchymal stem cells

Darko Bosnakovski

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

The bone marrow harbors a population of Mesenchymal stem cells (MSCs) that possess the potential to differentiate into bone, cartilage, fat, and along other tissue pathways. MSCs from various species have been studied, and all of them demonstrated some species specific characteristics. Despite the bovine experimental model being widely used in ex-

periments *in vivo* and *in vitro*, only a limited amount of information regarding bovine MSCs is available. MSCs to undergo chondrogenic differentiation, factors that support strong cell-cell interaction (pellet culture system), growth factors (TGF β 1), and an environment that maintains spherical morphology (polymer and collagen type II gel) are nec-

essary.

In the first study, the capability of fibroblast-like cells, isolated from bovine bone marrow, are capable of *in vitro* extensive multiplication and multilineage differentiation that makes them a relevant and invaluable model in the field of stem cell research. Bovine MSCs were isolated using cell ability to attach to the plastic bottom of the flask and rapidly to proliferate. Cells from other lineages presented in bone marrow aspirate were removed by changing the culture media. First passage-cell culture under phase-contrast microscope and on FACS analyses appeared to be homogeneous. Multilineage differentiation (osteogenesis, chondrogenesis, and adipogenesis) were induced using a combination of previously reported protocols for other species. The level of differentiation was evaluated by histological examination, Western blotting, and by analyzing the expression of tissue-specific genes by a qRT-PCR technique and Northern blotting. Following osteoinduction, the isolated fibroblast-like cells transformed into cuboidal cells and formed ALP positive colonies; during differentiation, these colonies transformed into mineralized nodules. In addition, osteogenesis was followed by osteocalcin and collagen type I mRNA expression. Chondrogenesis was confirmed by the demonstration of collagen type II, aggrecan, and sox9 mRNA expression and by detecting synthesized collagen type II in the cells stimulated by TGF β 1 in monolayer culture. After being cultured in an adipogenesis-inducing medium for 18 days, the MSCs responded by the accumulation of lipid vacuoles and expression of adipocyte-specific genes, PPAR γ 2 and aP2.

In the second study, chondrogenic differentiation of bovine bone marrow MSCs in pellet cultural system was induced. Beside the effect of the potent chondrogenic bioactive fac-

tors TGF β 1 on differentiation, which was demonstrated in the first study, in the present trials, the influence of strong cell to cell interaction on the MSC chondrogenesis was analyzed. For inducing chondrogenesis, MSCs were cultured in pellet culture system in a chemically defined medium supplemented with 0 and 10 ng/ml of TGF β 1. In both experimental groups (0 and 10 ng/ml TGF β 1) after 7 days of culturing, metachromatic alcian blue staining and immunoreactivity for collagen type II were detected in the centre of the pellets. The positive stained area for chondrocytic markers increased with culturing time, and almost whole pellets were positively stained after 20 days culturing. *In situ* hybridization demonstrated strong expressions of collagen type II and aggrecan mRNA in the round cells located at the centre region of pellets and at densely organized areas. On the other hand, collagen type I mRNA was strongly expressed in the superficial layer of the pellets. After 20 days of pellet culture, expression of collagen type II mRNA in the cells which were not treated by TGF β 1 was 1.7 folds higher compared with that treated by TGF β 1. Independent, spontaneous chondrogenesis of bovine MSCs in pellet culture occurred without addition of any external bioactive stimulators, namely factors from TGF beta family, which were previously considered necessary.

Third study aimed at analyzing the MSC chondrogenic response during culture in different types of ECM with a focus on the influence of collagen type II on MSC chondrogenesis. Bovine MSCs were cultured in monolayer manner as well as in alginate and collagen type I and II hydrogel, in both serum free medium and medium supplemented with TGF β 1 media. Chondrogenic differentiation was detected after 3 days of culture in 3-D hydrogels, by examining the presence of GAG and newly

synthesized collagen type II in the ECM. Differentiation was most prominent in cells cultured in collagen type II hydrogel, and it increased in a time-dependent manner. The expression levels of the of chondrocyte specific genes *sox9*, collagen type II, aggrecan and COMP were measured by qRT-PCR, and genes distribution in the hydrogel beads were localized by *in situ* hybridization. All genes were upregulated by the presence of collagen, particularly type II, in the ECM. On the other hand, the expression of collagen type I mRNA, which is a chondrocyte de-differentiation marker, was suppressed. Additionally, the chondrogenic influence of TGF β 1 on MSCs cultured in collagen-incorporated ECM was analyzed. TGF β 1 and dexamethasone treatment in the presence of collagen type I provided more favorable conditions for expressing the osteogenic phenotype whereas the

presence of collagen type II supported the expression of the chondrogenic phenotype. This study demonstrated that collagen type II alone has the potential to induce and maintain, and prior interaction with TGF β 1 dramatically enhances MSC chondrogenesis.

In conclusion, this study showed that ; i) cells from bovine bone marrow stroma have potential for extensive proliferation and multilineage differentiation, referring them as MSCs ; ii) bovine MSCs cultured in pellet culture system demonstrated specific species character to undergo spontaneous chondrogenic differentiation without addition of any external added bioactive stimulators ; iii) collagen type II, which is physiological component of articular cartilage has potential to induce and maintain, and prior interaction with TGF β 1 dramatically to increased MSC chondrogenesis.

Original papers of this thesis appeared in *Exp. Hematol.*, 32 : 502-509 (2004) and *Cell Tissue Res.* 319 : 243-253 (2005)

Demonstration of neural transmission of highly pathogenic avian influenza a virus

Kazuya Matsuda

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

Mice infected intranasally with H5 highly pathogenic influenza A viruses (HPAIVs) developed brain stem encephalitides, predominantly localized to the vagal medullary centers, following respiratory infections, whereas mice showed no pathologic lesions when the viruses were inoculated by intravenous route. From these findings, viral spread to the central nervous system (CNS) through the peripheral nerves, especially via the vagus nerves, was postulated. However, transneural

propagation of influenza virus has not be directly demonstrated and the machinery of the transmission remains to be elucidated.

In part I, I introduced the H5 HPAIV intranasally to unilaterally vagectomized mice and investigated the distribution of viral antigens, genomes and histological changes over time. The vagectomy resulted in appearance of viral antigen two days later in the vagal ganglion of vagectomized side than in the ganglion of non-vagectomized side. This was

resulted from that the virus could not reach the ganglion due to a discontinuity of the vagus nerve, and indicated that the virus spreaded from the respiratory tract to the vagal ganglia through the vagus nerves.

In part2, I studied the transneuronal properties of the H5 HPAIV using primarily cultured peripheral neurons. In chapter1, I investigated axonal transport of the HPAIV in a compartmented culture system, in which neuronal cell bodies and axons could be cultured compartmentedly. For positive and negative controls, we used pseudorabies virus (PRV) which could transport in the axons depending on the microtubules, and low-pathogenicity avian influenza virus which could not show any neuropathogenicity by intranasal infection in mice. Each viral antigen was detected in the neuronal cell bodies 12 hours after viral inoculation to the compartmented axons. This

was the first demonstration of the axonal transport of influenza viruses *in vitro*. From the difference in the infection patterns in the neurons between the HPAIV and PRV, difference in the transport machinery was suggested between them. Therefore, in chapter2, I investigated the effects of selective cytoskeleton perturbation on the neuronal infection of the HPAIV. For a positive control, I used the PRV. Nocodazole, cytochalasin D and acrylamide were used for interference of microtubules, microfilaments and intermediate filaments, respectively. From this experiment, neuronal infection of the PRV was significantly suppressed by microtubule perturbation, but that of HPAIV was not significantly affected. This indicated that the HPAIV spread in the neurons by different mechanism from the known transport machinery of neurotropic viruses.

The original papers of this thesis appeared in *Vet. Pathol.*, 41, 101-107 (2004) and *J. Gen. Virol.*, 86, 1131-1139 (2005).

Analysis of pathogenicity and molecular biological features of glioma-inducing avian leukosis virus

Yukiko Tomioka

*Laboratory of Animal Experiment for Disease Model,
Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan*

So-called fowl glioma is characterized by multiple nodular gliomatous growths associated with disseminated nonsuppurative encephalitis. Recently, the first case of fowl glioma in Japan has been described in a Japanese bantam (*Gallus gallus domesticus*) kept in a zoological garden. Avian leukosis virus (ALV) was isolated from the brain of the bantam as the causal agent. The purpose of the present study was to clarify the pathogenicity

and molecular biological features of the glioma-inducing ALV.

Chapter I. A resistance-inducing factor test indicated that the isolated ALV was classified into a subgroup A. The distribution and pathogenicity were investigated in C/O specific-pathogen-free (SPF) chickens infected *in ovo* with this virus. Histologically, the characteristic nodules with nonsuppurative encephalitis were reproduced in the infected

birds. Nonsuppurative myocarditis with matrix inclusions and atypical myocytes were also noted. The ALV antigens, proviral DNA and viral RNA were detected in various general organs as well as the central nervous system (CNS) and heart. These results suggested that this virus induced neoplastic lesions in CNS, despite the broad tissue tropism throughout the body.

Chapter II. We determined the complete nucleotide sequence of the glioma-inducing virus and performed a functional analysis of the long terminal repeat (LTR) to find a clue about the unique oncogenicity in the CNS. The full-length sequence was consistent with a genetic organization typical of ALV lacking viral oncogenes. The coding sequences were well conserved with those of ALV, but the 3' noncoding regions including LTR were most related to those of replication-defective sarcoma viruses. These findings and the analysis of the sequence of 3' untranslated region may suggest that the virus had a recombinant organization derived from these ALVs and avian sarcoma viruses. The LTR had a few deletions and several point mutations compared to that of other ALVs. The promoter activities of the LTRs of glioma-inducing ALV and ALV-A standard strain, RAV-1, were equivalent in

chick embryo fibroblasts, while that of glioma-inducing ALV was significantly lower than that of RAV-1 in human astrocytic cells. These subtle differences of the promoter activity of the LTR and/or the recombination may be related to the induction of glial neoplasm.

Chapter III. Proviral integration site, namely, involved proto-oncogenes influence neoplastic phenotype in ALV infectious diseases. Immunohistochemically, proto-oncogene products expressed in the brain lesions were examined. The astrocytes constituting the lesions consistently showed overexpression of K-Ras from the early stage of the disease. The results suggested that activation of proto-oncogene *K-ras* plays an important role in the pathogenesis of fowl glioma. Proviral integration into the *c-myc* locus was not detected in the lesion by laser-microdissection method, although some intralesional astrocytes showed weakly positive for c-Myc.

The present studies clarified that the isolated virus mainly induced astrocytic neoplasm in the CNS despite the broad tissue tropism and suggested that the CNS-specific oncogenicity was associated with viral recombination and/or activation of *K-ras* proto-oncogene.

The original papers of this thesis appeared in *Avian Pathol.*, 32 : 617-624 (2003) and *J. Gen. Virol.*, 85 : 647-652 (2004)

Molecular mechanisms of autoimmunity in IQI/Jic mice, a model for Sjögren's syndrome

Kensuke Takada

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

Sjögren's syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration into the salivary and lacrimal glands. In this study, I analyzed IQI/Jic mice, recently identified as an animal model for SS, to understand the molecular mechanisms underlying the autoimmune condition in SS.

In addition to primary lesions of dacryoadenitis and sialoadenitis, patients with SS occasionally exhibit various glandular and extra-glandular manifestations, and it is generally assumed that autoimmune reactions against antigens commonly expressed in target organs are associated with the pathogenesis. Systemic histological analysis of IQI/Jic mice at various ages demonstrated that IQI/Jic mice spontaneously develop inflammatory lesions infiltrated with CD4⁺ T cells and B cells not only in the lacrimal and salivary glands but also in multiple organs including the lung, pancreas and kidney at advanced ages. Among these organs, the salivary glands were initially affected, and the lesions appeared to gradually spread to other target organs with aging. Consequently, I next identified an autoantigen associated with the systemic autoimmunity in IQI/Jic mice. Polypeptides recognized by autoantibodies in sera from IQI/Jic mice but not in those from non-autoimmune control strains were identified as tissue kallikrein (Klk)-1 and -13, and they were crossreactive with the serum autoantibodies. Klk-13, but not Klk-1, caused a proliferative response of splenic T cells from IQI/Jic mice. In addition, Klk-13 was commonly ex-

pressed in various target organs in IQI/Jic mice, and in the salivary glands, its expression was markedly increased concomitantly with the development of inflammatory lesions. Taken together, Klk-13 was thought to act as an autoantigen and to play a pivotal role in the etiology of disease progression from salivary gland-specific to systemic disorder in IQI/Jic mice.

Then, I investigated on the mechanism underlying the loss of self-tolerance in IQI/Jic mice. Interleukin (IL)-2 is a cytokine produced by activated T cells and requisite for the maintenance of self-tolerance besides its action to promote the proliferation of T cells. Interestingly, during my examination of T cell immune response to autoantigens in IQI/Jic mice, I noted that splenocytes from IQI/Jic mice proliferated much less than did the cells from non-autoimmune control mice upon stimulation with concanavalin A, a T cell-specific mitogen. This proliferative abnormality was explained by the finding that splenic and lymph node T cells from IQI/Jic mice were partially defective to produce IL-2 with their activation, which was originally due to impaired transcription of IL-2 gene. One of the self-tolerance mechanisms through IL-2 has been described to develop and maintain CD4⁺CD25⁺ regulatory T cells (T_{reg}). To elucidate whether T_{reg} are disrupted and it contributes to the development of autoimmune lesions in IQI/Jic mice, I next performed neonatal thymectomy on day 3 after birth (D3Tx) in IQI/Jic mice, which is known to eliminate T_{reg}

from the peripheral T cell repertoire and lead to the activation of autoreactive CD4⁺CD25⁻ T cells in rodents. Histological analysis revealed that the dacryoadenitis, but not sialoadenitis, was accelerated after D3Tx, indicating that T_{reg} existed and preserved immunoregulatory function in IQI/Jic mice.

Collectively, in IQI/Jic mice, dysfunction

of activated T cells to produce optimal levels of IL-2 could abrogate the self-tolerance mechanisms not through T_{reg} and cause persistent activation of T cells reactive with autoantigens. Moreover, autoimmunity against one of the autoantigens, Klk-13, might be crucial in the etiology of disease progression from salivary gland-specific to systemic disorder.

Original papers of this thesis appeared in *Rheumatology*, 43 : 858-862 (2004), *J. Biol. Chem.*, 280 : 3982-3988 (2005), and *J. Vet. Med. Sci.* (in press).

Influence of the mutations of envelope protein amino acids on the biological characteristics of tick-borne encephalitis virus

Akiko Goto

*Laboratory of Public Health, Department of Environmental Veterinary Sciences,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*

Tick-borne encephalitis (TBE) virus, which is a member of the genus *Flavivirus* in the family *Flaviviridae*, causes fatal encephalitis in humans. TBE virus is transmitted by tick bite and maintained in zoonotic transmission cycle between ticks and mammals.

TBE virus has two viral membrane proteins, glycoprotein E and protein M. Protein E mediates virus entry into the cell *via* receptor-mediated endocytosis, and it carries the major antigenic epitopes that promote a protective immune response. The M protein is synthesized as the precursor protein prM. It has been suggested that prM holds E in an inactive conformation to prevent low-pH rearrangements during transportation through the acidic compartments of the trans-Golgi network. Several studies have established that the amino acid changes in critical determinants of the envelope glycoprotein are sufficient to cause loss of neuroinvasiveness. In this study, I reveal the relation among the

characteristic changes of TBE virus envelope proteins and the attenuation and secretion mechanisms of the TBE virus.

First, I derived the BHK-21 cell culture-adapted TBE virus mutant and compared the pathogenicity of the mutant (Oshima Cl-1) and parental (Oshima 5-10) virus in mouse model. The neurovirulence of mutant in mice was identical to that of the parent. However, the level of neuroinvasiveness was higher for parent than for mutant. The degrees of viremia and virus titers in the spleen were lower in mice that were inoculated s.c. with the mutant than in mice that received the parent. Unlike the parent, the mutant was rarely detected in the brains of s.c. inoculated mice. Genetic analysis revealed that the mutant had single amino acid substitutions in each of the E and NS 5 proteins compared with the parent. Furthermore, while infection of the mutant to BHK-21 cells was inhibited by GAGs, this was not the case for parent. In summary,

the BHK - 21 - cell-adapted mutant virus showed reduced neuroinvasiveness in mice due to low-level induction of viremia before the development of neutralizing antibodies. The attenuation process involved a single amino acid change in the E protein, which may have resulted in the rapid clearance of the virus due to its high affinity for negatively charged molecules *in vivo*.

Next, I investigated the function of glycosylation of TBE virus prM and E proteins in the secretion of TBE viral particles. Each prM and E protein has one N-linked glycan. Constructs that express prM and E proteins of TBE virus have been shown to produce virus-like particles (VLPs), of which surface properties are similar to those of infectious viruses. I expressed glycosylation-mutated prM and E proteins to compare the secretion levels and biological properties of the VLPs. In the prM protein glycosylation-deficient mutant, the level of secreted E protein was reduced to 60% of the wild-type level. On the other hand, in

the E or prM-E protein glycosylation-deficient mutant, the level of secreted E protein was reduced to 10% of the wild-type level. Furthermore, in the E glycosylation-added mutant, the level of secreted E protein was four-fold higher than that of the wild-type. However, in the E glycosylation-translocated mutant, E protein secretion was reduced to only 10% of the wild-type level. These data suggest that the glycan associated with the N-linked glycosylation site at position 154 in the E protein plays an important role in VLP secretion.

In this thesis, I revealed that the amino acid changes in prM and E proteins influenced the secretion and infectivity of TBE virus particles, and led to the attenuation and changes of biological characteristics of TBE virus. These results suggest that the amino acid changes can be used as markers for effective production of attenuated virus in the cell culture without changing its antigenicity. Thus, my results are useful for the development of the vaccines and the diagnosis.

Epidemiological study of hantavirus infection in Japan and Russia and isolation of the virus from *Clethrionomys rufocanus*

Nandadeva Lokugamage

*Laboratory of Public Health, Department of Environmental Veterinary Sciences,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*

Many hantaviruses are pathogenic to humans and are distributed worldwide depending on the geographic distribution of their reservoir rodent species. The hantavirus infection causes a great impact on the public as a significant health problem in Asia. However, Japan has no reported case of HFRS for more than 20 years. The epidemiological and epizootiological information on hantavirus infection has not been fully investigated in Japan.

Therefore, this study was conducted to see the hantavirus infection in rodents and humans in Japan.

Epizootiological surveys conducted in the 4 major islands of Japan showed that seropositive *A. speciosus* and *R. norvegicus* inhabit Shimane and Toyama, respectively. Further, it was also showed that there were infected *C. rufocanus* in forests of Nemuro, Ebetsu, and Tobetsu of Hokkaido. In addition, seroposi-

tive *R. norvegicus*, *R. rattus* and *C. rufocanus* were also found in semi-urban settings such as ports in Hokkaido and the airport at Chitose. Presence of anti-hantavirus antibodies in these rodents were identified by IFA and the findings were consistent with the results of WB analysis. The partial S segment (152-305 nucleotides) from one seropositive *R. rattus* found Hakodate was amplified and sequenced. The amplicon had 98.7% nucleotide identity with the SR-11, the prototype strain of SEOV. Seropositive *A. speciosus* found in Toyama and Shimane had 4- to 8-fold higher IFA antibody titers to HTNV than those of to SEOV. Further, some of the seropositive sera from *A. speciosus* neutralized HTNV but not SEOV. This suggested that *A. speciosus* might have been infected with hantaviruses that are closely related to HTNV. Finding of seropositive *R. rattus*, *R. norvegicus*, and *C. rufocanus* in seaport and at the Chitose airport raises alert on public health as these seropositive rodents could infect people in the area, travelers and quarantine office employees. Epidemiology of human hantavirus infection was also examined in at-risk population. Sera from the personnel of the Japan Ground Self-defense Force on Hokkaido were screened for anti-hantaviral antibodies by IFA. One individual (0.48%) was found to be positive for anti-SEOV antibody. Therefore, SEOV infections in humans may be misdiagnosed though no official HFRS patients have been reported for about 20 years. More attention should be paid to SEOV infection in Japan.

In order to develop an efficient method for isolation of PUUV and PUU-related virus, newborn rat, mice, and MGs were inoculated with the Sotkamo strain of PUUV. Delayed onset of antibody response and early appearance of the virus in high titers in most of the

organs of the MGs implied that the virus could be more easily recovered from MGs. Further, it was found that PUUV replicated well in the brain and lungs of PUUV infected MGs. The study was further extended by inoculation of newborn MGs with lung homogenates of naturally infected *C. rufocanus*. The virus sequences were detected in lungs of the inoculated MGs. Therefore, inoculation of samples into MGs is an efficient method for isolation of PUUV and PUU-related viruses from wild rodent samples.

Since an efficient method of virus isolation has been established, attempts were made to isolate the virus from seropositive *C. rufocanus* found in Tobetsu, Japan and Yuzhino-Sakhalinsk, Russia. The virus carried by *C. rufocanus* in Yuzhino-Sakhalinsk was successfully isolated through inoculation into newborn MGs and subsequent passage through cultured Vero E 6 cells. The S segment of isolated virus from *C. rufocanus* in Sakhalin (#99) was sequenced partially (568-654 nucleotide) and compared with the other hantavirus. The sequences between isolated virus and the virus in the original *C. rufocanus* lung tissue were identical. It was revealed that the viruses carried by *C. rufocanus* in Sakhalin and Tobetsu were the most closely related. Further, *C. rufocanus* derived viruses were closer to Sotkamo strain of PUUV from *C. glareolus*, in Europe. Since PUUV in Europe seem to have been evolving from the Glacial Eras to present with the reservoir animal, *C. glareolus*, PUU-related virus in *C. rufocanus* may also have long evolutionary history (5). The viruses in *C. rufocanus* from different geographical origin in Japan, Sakhalin, and Far East Russia may be important sources to analyze the hantavirus evolution in more detail.

Studies on the diagnostic methods and pathogenicity of the West Nile virus

Kazuya Shirato

*Laboratory of Public Health, Department of Environmental Veterinary Sciences,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*

In this thesis, development of genetic diagnostic methods of WN virus and studies of the pathogenicity of WN virus using mice infection model were described.

First, a genetic diagnostic method of WN virus by RT-PCR RFLP analysis was developed. RT-PCR RFLP methods is simple, cost-effective, and useful in the detection of genetic mutations in restriction enzyme recognition sequences. This method was able to discriminate WN and JE viruses, and also distinguish NY strain from other WN virus strains. Next, a genetic diagnostic method of WN virus by TaqMan PCR assay was described. The real-time PCR is more sensitive assay than RT-PCR, and useful in the assay which needs high sensitivity, such as human samples. A highly conserved sequence in the C protein coding region was selected as a probe, and the specificity of assay was assured by the specific primer sets. This method was able to detect and distinguish WN and JE viruses. As the results, the author considered that the genetic diagnostic methods for WN virus detection in JE virus endemic region were established.

Next, the expression of CC (RANTES/CCL5, MIP-1 α /CCL3, MIP-1 β /CCL4) and CXC (IP-10/CXCL10, BLC/CXCL13, BMAC/CXCL14) chemokines in WN encephalitis was examined using murine infection model of lethal NY strain and non-lethal Eg 101 strain. The mRNA of the CC chemokines, RANTES, MIP-1 α , MIP-1 β , and IP-10 was highly up-regulated in the brain of NY strain-infected

mice. By contrast, BLC mRNA was not detected in either group of mice, and BMAC mRNA was highly up-regulated in late stage of infection with the non-lethal Eg 101 strain relative to levels in NY strain-infected mice. These results suggested that these chemokines played immunological protective or immunopathological role in WN virus infection. Finally, the relationship of envelope protein glycosylation of WN virus and virus neuroinvasiveness was examined using plaque-purified variants from NY strains. Two NY strains were plaque-purified and four variants that had different amino acid sequences at the N-linked glycosylation site in the E protein sequence were isolated. The E protein was glycosylated in only two of these variants. Six-week-old BALB/c mice were infected subcutaneously with these variants. Mice infected with E glycosylated viruses developed lethal infection, whereas mice infected with non-glycosylated viruses showed low mortality. In contrast, intracerebral infection of mice with glycosylated virus or non-glycosylated virus resulted in lethal infection. These results suggested that E protein glycosylation is a molecular determinant of neuroinvasiveness in NY strain of WN virus. These findings, along with further evaluation of the mechanism of the pathogenesis in NY strain infection, are important for the development of specific therapies and new vaccines against WN encephalitis.

Redox regulation of radiation-induced death receptor expression in leukemia and adenocarcinoma cell lines

Taku Hamasu

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

The death receptors Fas and DR5 are known to express not only in immune cells but also in various tumor cells, and Fas ligand, agonistic Fas antibody (CH11) and TRAIL are able to induce apoptosis in tumor cells with diverse origins. Ionizing radiation was reported to up-regulate these death receptors in tumor cell lines. Therefore, combined treatment of tumor cells with radiation and these ligands is recently considered to have therapeutic potential against solid tumors. The present study was performed to examine whether radiation-induced up-regulation of death receptors was influenced by hypoxia condition in leukemia cells and controlled by redox regulation in various tumor cells with various Tp53 status.

In first experiment, to elucidate radiobiological effects of hypoxia on X-ray-induced apoptosis and Fas expression, leukemia cell line MOLT-4 was treated under four set of conditions: 1) both X irradiation and incubation under normoxia, 2) X irradiation under hypoxia and subsequent incubation under normoxia, 3) X irradiation under normoxia and subsequent incubation under hypoxia, and 4) both X irradiation and incubation under hypoxia, and the induction of apoptosis was examined. About 28-33% apoptosis was observed in cells treated under conditions 1 and 2, but this value was significantly reduced to around 18-20% in cells treated under conditions 3 and 4, suggesting that post-irradiation hypoxic incubation rather than hypoxic irradiation mainly caused the reduc-

tion of apoptosis. The activation and expression of apoptosis-signal-related molecules such as SAPK/JNK, Fas and caspase-3 were also suppressed by hypoxic incubation. Effects of hypoxic incubation were canceled when cells were treated under conditions 3 and 4 with an oxygen-mimicking hypoxic cell radiosensitizer doranidazol, whereas the addition of N-acetyl-L-cysteine (NAC) reduced the induction of apoptosis, suggested existence of redox regulation in this radiation-induced expression Fas and apoptosis. The aim of the next study was to determine whether X irradiation enhances induction of apoptosis by redox regulation in Tp53 wild type and Tp53-mutated tumor cell lines treated with CH11 and TRAIL. In gastric cancer cell lines MKN45 and MKN28, lung cancer cell line A549 and prostate cancer cell line DU145, X irradiation was demonstrated to significantly up-regulate the expression of functional death receptors, Fas and DR5 and that subsequent treatments with CH11 and TRAIL activated caspase 3, followed by the induction of apoptosis. The post-irradiation treatment of the cells with NAC abolished the up-regulation of the expression of Fas and DR5 on the plasma membrane. NAC also attenuated the induction of apoptosis by agonistic molecules.

In summary, the present data showed that the hypoxia treatment reduced the redox-regulated increase in expression of Fas and apoptosis in leukemia MOLT-4 cell exposed to radiation and the treatment with doranidazol abolished this hypoxia-induced down-

regulation of Fas expression and apoptosis. Furthermore, in four human carcinoma cell lines, the increase in the induction of apoptosis by the combined treatments of X irradiation and CH11 or TRAIL occurred through the

change of intracellular redox state independent of Tp53 status. These observations are important in development of a novel radiosensitization technique using death receptor agonists in radio resistance solid tumors.

The original papers of this thesis appeared in *J. Radiat. Res.*, 46 : 103-110 (2005) and *Apoptosis*, 10 : 557-568 (2005).

Effect on di-(2-ethylhexyl) phthalate (DEHP) on testis and pituitary
— A toxicological study —

Hyung-sub Kim

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

Di-(2-ethylhexyl) phthalate (DEHP), plasticizer, is an endocrine disrupter which causes testicular atrophy. The objective of this study is to investigate the mechanism of DEHP-induced testicular atrophy and the effect on tamoxifen-induced apoptosis in pituitary.

In this study, exposure to DEHP (100 or 1000mg/kg, 5 days) to 4 week old male rats did not affect the activity of 17 β -hydroxysteroid dehydrogenase, the rate-limiting enzyme of testosterone (T) biosynthesis. In rat testis treated with DEHP, I observed a significant induction of T6 β -hydroxylation and 16 α -hydroxylation activity mediated by cytochrome P450 (CYP) 3A2 and CYP2C11, respectively. In addition, mRNA expression of aromatase, responsible for estrogen synthesis, was significantly decreased. These results suggest that DEHP disturbs normal steroidogenesis by regulating CYP-dependent enzyme activities in the rat testis. DEHP alters the lipid composition of rat testis, yet the mechanism is unclear. Here, I examine the effect of DEHP on the synthesis and metabolism of

arachidonic acid (AA), a precursor of eicosanoids. DEHP administration caused a significant reduction in activity of cPLA₂. DEHP increased the expression of 12-LOX in rat testis, whereas COX-2 expression was not altered. CYP4A1, a product of a PPAR α -regulated gene, was markedly increased in the testis by DEHP administration. Taken together, DEHP suppresses cPLA₂ activity and induces the AA-metabolizing enzymes such as 12-LOX and CYP4A1, resulting in the reduction of AA level. These data suggest that altered AA metabolic cascades might be related to the decrease of T concentration in DEHP-induced testicular atrophy. Although DEHP is a well-known endocrine disrupting chemical, reports regarding toxic effects of DEHP on pituitary which regulates gonadal function of testis have been extremely limited. The aim is to examine the inhibitory effect of DEHP on tamoxifen-induced apoptosis in GH3 pituitary cells. Tamoxifen, an estrogen receptor antagonist, has been known as an anti-tumor drug through inducing apoptosis. In the cytotoxicity assay, the reduced cell viability in 0.1 μ M

tamoxifen-treated GH3 cells was recovered by 250 μ M DEHP for 4 days. Apoptotic morphological change such as nuclear fragmentation induced by tamoxifen was suppressed by DEHP treatment. Flow cytometry analysis revealed that apoptotic cell population in tamoxifen-treated cells significantly decreased by DEHP. Suppression of apoptosis by DEHP was verified by Western blot analy-

sis for PARP cleavage. These results indicated that DEHP suppressed tamoxifen-induced apoptosis in pituitary cells. In conclusion, DEHP affected steroidogenesis and AA metabolism in the testis of prepubertal rats. In addition, DEHP might counteract the therapeutic effect of tamoxifen for pituitary tumors.

The original papers of this thesis appeared in *Archives of Toxicology*, 77 : 446-51 (2003) and *Journal of Veterinary Medical Sciences*, 66 : 1119-1124 (2004).

Impact of AhR and PPAR interaction on CYP 450 isoforms

Zein Shaban Ibrahim El-Ezeary

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

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates a spectrum of toxic and biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. Peroxisome proliferator activated receptor alpha (PPAR α) is activated by fibrates and phthalate esters, and involved in the maintenance of lipid and glucose homeostasis. I have investigated the hypothesis the negative cross-talks between AhR and a member of the nuclear receptors, the PPAR α , and its impact on the cytochrome P 450 (CYP) enzymes.

In the first part of this study, I investigated the effect of a PPAR α ligand on the AhR target genes CYP1A1/CYP1A2 expression and activities. The results showed that PPAR α ligand treatment is able to down regulate AhR target genes expression and activities. However, to this end, it was not clear whether this effect is PPAR α dependent or not and so I used HepG2 human cells having

low levels of PPAR α . When HepG2 cells were treated with either SudanIII (S.III) and/or clofibric acid (CA), no suppressive effect on S.III-induced CYP1A1 protein expression due to CA was found. HepG2 cells were transiently transfected with increasing concentrations of PPAR α mammalian expression vector, and were exposed to the same treatment. CA co-treatment with S.III decreased AhR protein and S.III-induced CYP1A1 protein expression with increasing dose of PPAR α transfected into HepG2 cells.

In the second part of this study, I investigated the effect of AhR ligand S.III on the PPAR α target gene expression. Treatment of Wistar rats with S.III was found to down regulate the basal and CA-induced PPAR α target gene-dependent CYP4A protein expression. In HepG2 cells, the expressions of PPAR α and RXR α protein were decreased by S.III treatment in a dose dependent manner.

Finally, in the third part of this study, I

investigated whether the negative cross-talk I have proposed between AhR and PPAR α has any impact on other CYP enzymes that may be modulated in part by these two receptors. Treatment of male Wistar rats with a PPAR α ligand, CA, induced CYP2B1/2 and CYP3A proteins, activities and the mRNA expression of CYP2B1, CYP2B2, CYP3A1, and CYP3A2, and suppressed CYP2C11 protein expression, enzyme activities and mRNA expression. AhR ligand S.III treatment decreased basal and CA-induced CYP2B, CYP3A and CYP2C11 pro-

tein, activities and mRNA expression.

It is concluded that there is an inhibitory effect of PPAR α ligand on AhR functions which is PPAR α dependent and an inhibitory effect of AhR ligand on PPAR α function. Moreover it indicates the presence of mutual effects between AhR and PPAR α on CYP2B and CYP3A and additive inhibitory effects on CYP2C11 in the livers of male rats. Finally it indicates the presence of a negative cross-talks between PPAR α and AhR.

Original papers of this thesis appeared in *Arch. Toxicol.*, 78 : 496-507 (2004), *J. Vet. Med. Sci.*, 66 : 1377-1386 (2004), *Xenobiotica*, 35 : 51-68 (2005).

Polymorphism of diazepam metabolism in rats

Konomu Saito

*Laboratory of Toxicology, Department of Environmental Veterinary Sciences,
Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818. Japan*

I examined the strain differences of position specific diazepam metabolism in Sprague-Dawley (SD), Brown-Norway (BN), Dark-Agouti (DA) and W strains of rats.

I observed intra-strain variations in the metabolism of diazepam in Wistar (W) rats. I found that the variations are dimorphic and about 17% of male W rats (extensive metabolizers, EM) showed 2 times higher diazepam metabolic activities in their liver microsomes than the rest of animals (poor metabolizers, PM) at the low substrate concentrations. No significant differences were observed in activities of neither diazepam 3-hydroxylation nor N-desmethylation between EM and PM rats, while activity of diazepam *p*-hydroxylation was markedly higher in EM rats, indicating that this reaction is responsible for the

polymorphism of diazepam metabolism in W rats. I examined the expression levels of CYP2D1, which was reported to catalyze diazepam *p*-hydroxylation in W rats to find no differences in the expression levels of CYP2D1 between EM and PM rats. The kinetic study on diazepam metabolism in male W rats revealed that EM rats had markedly higher *V_{max}* and smaller *K_m* in diazepam *p*-hydroxylation than those of PM rats. The current results infer polymorphic expression of diazepam *p*-hydroxylating enzyme with lower *K_m* than CYP2D1 in EM W rats.

Hepatic microsomes from Sprague-Dawley (SD) and Brown Norway (BN) rats had 300-fold higher diazepam *p*-hydroxylation activity than Dark Agouti (DA) and W rats at a low diazepam concentration. Kinetic

studies indicated that diazepam *p*-hydroxylation in SD and BN rats proceeded with lower *K_m* and higher *V_{max}* than it did in DA and W rats. However, the expression levels of CYP2D1 did not co-segregate with the activity. DA rats showed 3- and 2-fold higher diazepam 3-hydroxylation and N-desmethylation activities, respectively, than the other rat strains. In agreement with this, DA rat liver microsomes had higher expression of CYP3A2, which is responsible for diazepam 3-hydroxylation and partly responsible for N-desmethylation. Values of *CL_{int}* indicated that *p*-hydroxy-diazepam is the major metabolite in SD and BN rats, while 3-hydroxy-diazepam is the major metabolite in DA and W rats.

I studied to identify the unknown diaze-

pam *p*-hydroxylase in SD, BN and EM W rats. Until recently, diazepam *p*-hydroxylation was thought to be catalyzed by CYP2D1. However I found that diazepam *p*-hydroxylation was not catalyzed by CYP2D1 or CYP2D2. I managed to separate the specific protein expressed in liver microsomes of SD, BN and EM W rats, but not expressed in DA and W rats. Since the specific protein (CYP2DX) was shown only in EM of diazepam *p*-hydroxylation, there was a strong possibility that the protein was diazepam *p*-hydroxylase. N-terminal amino acid sequence of CYP2DX exactly corresponded to those of CYP2D3. In conclusion, CYP2D3 is the most likely enzyme involved in diazepam *p*-hydroxylation in EM rats.

The original papers of this thesis appeared in *J. Pharm. Sci.*, 93 : 1271-1278 (2004) and *Drug Metab. Dispos.*, 32 : 959-965 (2004).

Immune responses to *Echinococcus multilocularis* infection and immune modulation by the parasite in the definitive hosts

Naoko KATO

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

Echinococcus multilocularis distributes widely in the Northern Hemisphere and is of public health importance. To reduce the infection pressure to human echinococcosis, novel immune prophylaxis to the definitive host would be effective in addition to deworming of the definitive hosts. Despite the need, the information about immunity to *Echinococcus* infection in the definitive hosts is limited.

The humoral and cellular immune responses to the intestinal infection of *E. multilocularis* in dogs (genuine definitive hosts)

and prednisolone-untreated Mongolian gerbils (rodent definitive hosts) were investigated. In dogs orally infected with *E. multilocularis* metacestodes (containing ca. 230,000 proto-scolecocytes), serum antibody responses against the parasite antigens were induced from 10 days post-infection (DPI) for IgG1 and from 7 DPI for IgG2, with the highest responses against protoscolex excretory-secretory antigen (PES). Concanavalin A (Con A)-induced proliferative responses of peripheral blood mononuclear cell (PBMC) at 7 DPI decreased

remarkably and were restored thereafter. During the 21-day infection period, the lymphocyte proliferative responses to the parasite antigens were not induced in PBMC or the splenocytes at 21 DPI. However, Peyer's patch cells from one of two dogs showed proliferative responses to PES. Con A-induced lymphocyte proliferative responses were suppressed by the addition of parasite antigens. The suppressive effect was remarkable in PES.

In gerbils orally inoculated with protozoetes (ca. 20,000), significant antibody responses (both serum IgG and intestinal IgA) were induced. However, significant lymphocyte proliferative responses to the parasite antigen at 14 DPI were observed only in Peyer's patch cells.

The immune suppressive effect of the parasite was assessed by co-cultivation of splenocytes from naive gerbils with mitogens (Con A and LPS) and parasite antigens. Almost all parasite antigens reduced Con A-induced lymphocyte responses in dose-dependent manner, but not LPS-induced responses. The maximum suppressive effect on

Con A-induced lymphocyte responses was obtained by adding PES.

The characteristics of *E. multilocularis* antigens were examined. The smeared patterns of immunoblotting with serum IgG1 and IgG2 and fecal IgA from the infected dogs indicate that antibodies are produced against carbohydrate moieties of the parasite antigens. High carbohydrate/protein ratio was recognized in PES. Lectin blotting revealed that the parasite antigens consist of α -D-mannose, *N*-acetyl-D-glucosamine, sialic acid and sialyl-galactose (β 1-3) *N*-acetyl-D-galactosamine, but little of α -fucose. The present study first identified that adult *E. multilocularis* possess and secrete mucin-type *O*-linked glycans.

The results indicate that humoral and cellular immune responses are induced by the intestinal infection with the parasites. It is also suggested that the parasites suppress host immune responses by secreting carbohydrate-rich antigen(s) for their establishment and survival in the intestine of the definitive host.

The original papers of this thesis appeared in *Parasitology Research*, 95 : 339-345 (2005), and *Parasitology Research*, 96 : 12-17 (2005)