Japanese Journal of Veterinary Research 61(1&2): 25–38, 2013

INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on December 25, 2012 to 1 recipient.
The title of thesis and other information are as follows:

Evaluation in tumor microenvironment after multi-kinase inhibitor sorafenib in human renal cell carcinoma xenograft by positron emission tomography probes

Masahiro Murakami

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

Recently, many kinds of molecular targeted therapy agents are clinically available. However, responses to molecular targeted therapy vary with characters of the tumor and the patient. Thus, evaluations of not only therapeutic effect but also responses in tumor microenvironment after molecular targeted therapy enable a patient-specific therapy. However, it has not been established yet. Positron emission tomography (PET) is a non-invasive functional imaging method using radiolabeled tracers. By selecting a specific radiolabeled tracer such as a hypoxia tracer ^{18}F-fluoromisonidazole (FMISO), a tumor proliferation tracer ^{18}F-fluorothymidine (FLT), and others, PET imaging can evaluate various specific functions. Thus, the principal aim of this study was to clarify whether FMISO and FLT imaging can detect tumor responses after the therapy with multi-kinase inhibitor sorafenib, by evaluating early changes of FMISO and ^{3}H-fluorothymidine (^{3}H-FLT) uptake in the tumor microenvironment after sorafenib treatment in a renal cell carcinoma (RCC) xenograft.

In chapter 1, the author clarified the changes in the antiangiogenic tumor microenvironment such as a reduction in blood flow and "tumor starvation" at early time-points and determined whether FMISO hypoxia imaging can detect the changes in the tumor microenvironment after sorafenib treatment in an RCC xenograft. A human RCC xenograft (A498) was established in nude mice. The mice were randomly assigned to the control and sorafenib-treated groups (80 mg/kg, per os). Mice were sacrificed on Days 1, 2, 3 and 7 in the histological study, and on Days 3 and 7 in autoradiography (ARG) after sorafenib treatment. Tumor volume was measured every day. ^{18}F-FMISO and pimonidazole were injected intravenously 4 and 2 hours before sacrifice, respectively. Tumor sections were stained with hematoxylin and eosin and immunohistochemically with pimonidazole and CD31. Intratumoral ^{18}F-FMISO distribution was quantified in ARG. Tumor volume did not significantly change on Day 7 after sorafenib treatment. In the histological study, hypoxic fraction significantly increased on Day 2, mean vessel density significantly decreased on Day 1 and necrosis area significantly increased on Day 2 after sorafenib treatment. Intratumoral ^{18}F-FMISO distribution significantly increased on Days 3 (10.2-fold) and 7 (4.1-fold) after sorafenib treatment. The sequential histological evaluation of the tumor microenvironment clarified tumor starvation in A498 xenografts treated with sorafenib. ^{18}F-FMISO hypoxia imaging confirmed the tumor starvation.

In chapter 2, the author clarified the changes in the tumor proliferative microenvironment at early time-points and determined whether FLT can reflect tumor response after sorafenib treatment in an RCC xenograft in comparison with a tumor proliferation marker Ki-67. Mice bearing A498 tumor were assigned to the control and sorafenib-treated groups. The tumor volume was measured every day. ^{3}H-FLT was injected 2 hours before sacrifice on Days 3 and 7 after the treatment. ^{3}H-FLT ARG and Ki-67 immunohistochemistry (IHC) were performed using adjacent tumor sections. In the visual assessment, intratumoral ^{3}H-FLT uptake level diffusely increased on Days 3 and 7 after sorafenib treatment, while no apparent changes were observed in Ki-67 IHC. The intratumoral ^{3}H-FLT uptake level significantly increased by 2.7- and 2.6-fold on Days 3 and 7 after the treatment, while tumor volume and Ki-67 index did not significantly change. Thus, an increased FLT uptake level was demonstrated after the treatment, which may indicate the suppression of thymidylate synthase and compensatory upregulation of TK-1 activity by sorafenib.

In conclusion, the result of the present study indicated that the both of FMISO and FLT PET could reflect the response in tumor microenvironment after sorafenib treatment in an RCC xenograft. To evaluate hypoxia status after antiangiogenic therapy by FMISO PET may differentiate tumor responses between tumor starvation and vascular normalization to select optimum patient specific therapy. To evaluate FLT uptake after sorafenib treatment by FLT PET may reflect the activity of salvage pathway in DNA synthesis and may be able to monitor the treatment effects. Thus, this study could be a huge first step towards making definitive diagnosis and patient-specific therapy with this noninvasive diagnostic method, FMISO PET imaging of tumor starvation and FLT PET imaging of salvage pathway activity. In the future, highly efficient and minimally invasive monitoring method of cancer molecular targeted therapy using FMISO and FLT PET may be applied not only in human medicine but also in veterinary medicine.

The original paper of this thesis appeared in *Int. J. Oncol.*, **41**: 1593–1600 (2012).

Hokkaido University conferred the degree of Doctor of Philosophy on March 25, 2013 to 10 recipients.
The titles of theses and other information are as follows:

Development of the animal model resembling hantavirus infection in natural rodent hosts and isolation of hantavirus using a newly established cell line derived from a wild rodent species

Takahiro Sanada

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

Hantaviruses belong to the *Bunyaviridae* family and cause two severe human illnesses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Hantaviruses are maintained in a variety of Rodentia and Soricomorpha species in the nature. More than 40 viruses have been reported in the genus *Hantavirus* and each hantavirus is carried by the specific host.

In contrast to human infection, hantavirus does not cause any symptoms in the host rodent and persist for a long period. However, the mechanisms mediating persistent infection in natural hosts remain unknown because of the lack of suitable animal model mimicking hantavirus infection in the natural host. The author reports that Syrian hamsters infected at subadult with Puumala virus (PUUV), which is the etiologic agent of HFRS, showed persistent infection. Persistent infection despite the presence of neutralizing antibodies, without any clinical symptoms, is the characteristic feature of PUUV infection in subadult hamster, which is quite similar to the infection in natural host rodents. These data indicated that hamsters could be a suitable animal model for studying hantavirus infection in the reservoir host.

Although VeroE6 cells are widely used for hantavirus research, growth and isolation of hantaviruses in this cell line is very slow and difficult. To develop efficient method for the propagation and isolation of hantaviruses, a new cell line, MRK101 derived from a kidney of gray red-backed vole (*Myodes rufocanus*), the natural host of Hokkaido virus (HOKV) was established. The MRK101 cells showed a significantly higher susceptibility to PUUV hosted by *Myodes glareolus* than VeroE6 cells did. The propagation of PUUV in MRK101 cells was also higher than in VeroE6 cells during the early phase of infection. In contrast, MRK101 cells showed low or no susceptibility to Amur or Hantaan virus, *Apodemus*-borne hantaviruses. Using MRK101 cells, HOKV, which has not been isolated to date, was successfully isolated. The newly isolated HOKV propagated in MRK101, but not in VeroE6, cells. These data suggest that the MRK101 cell line is a useful tool for the isolation and propagation of *Myodes*-borne hantaviruses. This is the first report on hantavirus isolation in a cell line that originated from the natural host. MRK101 cells and HOKV will likely contribute to clarification of the mechanisms of hantavirus replication, host-specificity, pathogenesis, and

persistent infection in wild rodents.

In vivo and *in vitro* tools developed in this research, which are the hamster model resembling hantavirus persistent infection in the natural host and the MRK101 cell line derived from *Myodes rufocanus bedfordiae*, would promote the studies in unsolved questions remained in the hantavirus research field.

The original papers of this thesis appeared in *Virus Res.* **160**: 108–119 (2011) and *J. Gen. Virol.*, **93**: 2237–2246 (2012).

Studies on the epidemiology of hantavirus infection in Mexico and the role of nucleocapsid protein N-terminus domain in serological cross-reactivity

Ngonda Saasa

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

Hantaviruses are increasingly becoming important pathogens all over the world. A deeper understanding of the hantavirus infection in rodents is critical for a better understanding of the virus-rodent interaction. The existence of a vast number of rodent species, all with the potential to harbor hantaviruses imply an possibilities of the hantaviruses yet to be recognized. Mexico presents an interesting geographical area for studying the epidemiology of hantaviruses because there are yet no HPS reports but the surrounding countries have recorded cases of HPS in humans. The identification of infected rodents as well as the possibility of several distinct hantaviruses circulating in the Guerrero and Morelos States presents an opportunity to further investigate the diversity of hantaviruses in the region.

The role of serological diagnosis in the detection and diagnosis of hantavirus infections can not be over emphasized. The strong Ab response generated towards, in addition to the broad cross-reactivity renders the N protein a flexible tool in the detection of hantaviruses. Nevertheless, despite the realization that the N-terminus of the protein as the source of cross-reactivity, details on the why and how the protein sustains these characteristics are yet to be fully investigated.

The ecological study of hantaviruses revealed that the Mexican MTNV is predominantly carried by a *Peromyscus. beatae* species, different from other previously identified hosts for hantaviruses in North and South America. In addition, CARV was found to be carried predominately by *Reithrodontomys. sumichrasti.* The phylogenetic analysis helped elucidate some of the existing taxonomic relationships between the closely related Mexican *Peromyscus* and *Reithrodontomys* rodent species. The rodent reservoir information obtained and the range of the pathogen within the rodent hosts provides insights into the virus-host interactions in these areas that could ultimately facilitate prediction of where human disease could occur.

More work however is still required in order to fully assess the on-going hantavirus-host interactions. There is still need to extend the geographical coverage within Mexico in order to assess the regional distribution of the rodent

hosts and hantaviruses in the various habitat types of Mexico. The relative prevalence of infection among demographic subpopulations of the *Peromyscus* and *Reithrodontomys* rodents in Mexico is still unclear. Temporal and spatial patterns of host-pathogen dynamics as well still needs to be clarified through systematic and regular longitudinal studies in the area.

The mAbs generated to the N protein of Mexican MTNV were extensively cross-reactive. In the search for the source of this cross-reactivity, we probed the N-terminus with these highly cross-reactive mAbs. The analysis of the reactivity of the mAbs revealed that the N-terminus is the source of the strong cross-reactivity associated with hantaviruses infections.

The findings suggest that despite the strong reactivity of anti-hantavirus antibodies in WB that would suggest that the epitopes were linear in nature. To the contrary evidence obtained strongly point to the N-terminus possessing strongly immunogenic conformation-dependent epitopes. It is possible that the N-terminus retains its structure and is refractory to the denaturing effects of SDS treatment in WB. Overall, the analysis revealed that the cross-reactivity arising from the N-terminus is heavily reliant on conformational integrity of the viral protein.

The original papers of this thesis appeared in *Virus Res.*, **168**: 88–96 (2012) and *Virology*, **428**: 48–57 (2012).

Effects of anti-cancer drugs and radiation on invasive ability of tumor cells

Masato Eitaki

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

There are various strategies in anti-cancer drug treatment, and a variety of medicines are used according to situations. Similarly, radiotherapy is also performed widely, and many cancer kinds including breast and lung cancer are therapeutic objectives. However, some say that anti-cancer drugs and radiation have undesirable side effects for cancer therapy. For example, doxorubicin and X-ray irradition are reported to enhance invasion and metastasis of breast and pancreatic cancer cells. Because the mechanisms are not clear well, basic research results associated with the side effects have not been able to be reflected in clinical yet. Hence, it is meaningful to report more and more basic data in this field.

In the first experiment, I assessed effects of four anti-cancer drugs, which have different functional mechanisms, on invasive ability in human gastric adenoma-derived MKN45 cells. At first, I showed that microtubule polymerization inhibitor vincristine enhanced invasive ability of MKN45 cells, and this enhancement was mediated by guanine nucleotide exchange factor-H1 (GEF-H1)/RhoA/Rho-associated protein kinase (ROCK)/myosin light chain (MLC) signaling pathway. RhoA/ROCK/MLC signaling pathway has been reported to be involved in amoeboid-like motility, a novel type of invasive cancer movement whose existence has become clear in recent years. As the features, the cells move without extracellular matrix degradation, which is common in cellular invasion, and show round shape with membrane

blebs. In this study, formation of membrane blebs and amoeboid-like motility were observed in vincristine-treated cells, and the formation was inhibited by GEF-H1 siRNA and ROCK inhibitor. Therefore, it was strongly indicated that amoeboid-like motility was involved in the vincristine-induced enhancement of cellular invasive ability in MKN45 cells. These results suggest that vincristine induces amoeboid-like motility via GEF-H1/RhoA/ROCK/MLC signaling pathway, leading to high cellular invasiveness. This study shows that an anti-cancer drug vincristine can enhance invasive ability of tumor cells, and GEF-H1 is related with amoeboid-like motility for the first time.

In the second experiment, I assessed effects of indirect function of X-irradiation on invasive ability of human breast adenoma-derived MDA-MB-231 cells and human lung adenoma-derived A549 cells. At first, it was revealed that irradiated cell CM enhanced invasive ability of MDA-MB-231 cells and A549 cells. This result suggested the existence of some factors enhancing cellular invasive ability in the irradiated cell conditioned medium (CM). Next, I showed that epidermal growth factor (EFG) mRNA was increased in X-irradiated MDA-MB-231 cells, and EGF concentrations were increased in irradiated cell CM of MDA-MB-231 cells and A549 cells. These results suggested that EGF was involved in the irradiated cell CM-induced enhancement of cellular invasive ability. Then, I also showed that irradiated cell CM activated epidermal growth factor receptor (EGFR), and it enhanced invasive ability and extracellular matrix (ECM) degradation activity via EGFR in MDA-MB-231 cells. These results suggest that X-ray irradiation induces EGF expression of tumor cells, and increased-EGF in the irradiated cell CM enhances ECM degradation activity of the surrounding cells, resulting to higher invasive ability of them. In this study, it was shown that X-ray irradiation indirectly enhanced invasive ability via EGFR in tumor cells for the first time.

As described above, I presented the two effects of vincristine treatment and X-ray irradiation which enhance invasive ability of tumor cells. From this study, it is expected that medical treatment efficiency is reinforced by inhibition of GEF-H1/RhoA/ROCK/MLC and EGFR signaling pathway in vincristine treatment and X-ray irradiation against tumor, respectively. The new information about undesirable activation of tumor invasion after vincristine and radiation treatments might be important in the clinical cancer therapy.

The original paper of this thesis appeared in *BMC Cancer*, **12**: 469 (2012).

Molecular evolution of xenobiotic metabolizing enzymes

Yusuke Kawai

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

For protecting against various chemicals, animals have evolved xenobiotic metabolizing system (phase I and phase II reactions). Compounds are hydroxylated by phase I enzymes and then are conjugated by phase II enzymes. In this study, we focused on the evolution of xenobiotic metabolizing enzymes, especially cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), and glutathione S-transferase (GST). In vertebrates CYP 1 to 3

families are involved in xenobiotic metabolism phase I reaction and UGT, SULT, and GST are involved in phase II reaction.

In chapter 1, I analyzed vertebrate CYP1D1 genes and elucidated the evolutionary history of CYP1D1 gene in vertebrate lineage. Members of the cytochrome P450 family 1 (CYP1s) play a role in the detoxification and bioactivation of numerous environmental pollutants and phytochemicals, such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines, and flavonoids. The vertebrate CYP1 gene consists of four subfamilies, CYP1A, CYP1B, CYP1C, and CYP1D. Firstly, the CYP1D1 gene was identified in fish, and subsequently in the platypus. These findings indicated that common ancestor of mammal and fish possessed functional CYP1D1 gene, and suggested that mammals possessed a functional CYP1D subfamily. However, there is little information on the mammalian CYP1D gene. In this study, I investigated genomic location of CYP1D genes in mammals and other vertebrates, performed phylogenetic analysis, and calculated the identities and similarities of CYP1D sequences. The data from synteny, phylogenetic analyses, and calculating similarities of CYP1D genes demonstrated the evolutionary history of the CYP1 gene family. Although in some mammalian species, CYP1D1 became pseudogene, in other mammalian species CYP1D1 is intact and conserved. The pseudogenization event of CYP1D1 occurred in several lineages independently. I also confirmed expression of CYP1D1 mRNA in eastern grey kangaroo (*Macropus gigantues*) and slow loris (*Nycticebus coucang*). These result suggested that in many mammals CYP1D1 possess xenobiotic metabolizing function. I also estimated the selection pressure on substrate recognition sites (SRSs) of CYP1D1. The result suggested that positive selection occurred three times in early CYP1D1 lineage. First positive selection occurred in divergence from CYP1A1. Second selection happened in ancestor of tetrapod. Third event occurred in ancestor of terrestrial tetrapod. These findings suggested there are species differences in substrate affinity of CYP1D1 among vertebrates.

In chapter 2, I focused on xenobiotic metabolizing enzymes expressed in ostrich liver and identified 10 CYP isoforms, 2 UGT genes, 6 SULT genes, and 10 GST genes using next generation sequencer. Ostrich is a member of Palaeognathae with the earliest divergence from other bird lineages at least 66 million years ago. Comparing ostrich result and other bird's genomic information, it was indicated that in chicken CYP2AF1 became pseudogene and in zebra finch CYP2G19 became pseudogene. This result also suggested that SULT2B1, SULT3A1, GSTM2 became pseudogene in zebra finch and GSTM3 became pseudogene in chicken and zebra finch. Moreover, I compared expression levels among CYP1A2, CYP2C23, CYP2C45, CYP2D49, CYP2G19, CYP2AF1, and CYP3A37 in liver. CYP2G19 showed highest expression in the ostrich liver. It was suggested that CYP2G19 which has not been focused in previous bird studies is also important in ostrich xenobiotic metabolism. Investigating synteny and phylogenetic analysis results indicated that bird CYP2G19 and mammal CYP2G1 is not orthologous. Moreover there are two amino acid differences in SRS region between ostrich CYP2G19 and mammalian CYP2G1. These result suggested that bird CYP2G19 possess different function from mammal CYP2G1.

In this study, I investigated the molecular evolution of xenobiotic metabolizing enzymes. In chapter 1, I showed the possibility that xenobiotic metabolizing enzyme changed the substrate affinity along with adaptation of vertebrates to terrestrial environment. In chapter 2, I elucidated that xenobiotic metabolizing enzyme genes were duplicated in bird lineage. Moreover even in bird species there is species difference in expression of xenobiotic metabolizing enzymes between Palaeognathae and other birds. These findings contribute to understanding of species difference in sensitivity to environmental pollution.

The original paper of this thesis appeared in *Mamm. Genome*, **21**: 320–329 (2010).

The distribution and mechanisms of Super rats — warfarin resistant black rats (*Rattus rattus*) — in Japan

Kazuyuki Tanaka

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

Warfarin is commonly used worldwide as a rodenticide. It inhibits blood coagulation by inhibiting vitamin K 2,3-epoxide reductase (VKOR) activity leading to hemorrhage. However, it has been reported that repeated or long-term treatment with warfarin results in resistance emerging in wild rodents called super-rats. Such resistance may explain why it is difficult to control rodents in many regions in Japan. In this report, I studied the distribution of super-rats and their mutations in the *VKOR* gene (including the VKOR complex subunit 1 (*VKORC1*)), while also analyzing VKOR and clotting factor activity in black rats (*Rattus rattus*) in order to understand better the mechanism of warfarin resistance in this species.

I sequenced the *VKORC1* gene from 275 rats living in the wild in Japan. I found several types of novel base substitutions, some of which conferred warfarin resistance. These substitutions found in super-rats were first confirmed in this research, and there was no report also in a brown rat. When I made phylogeographic analysis using AFLP fragment data, it suggested that there were multi-origin of super rats with Ala41Val.

About functional difference of VKOR enzyme, there was no difference in coagulation times between warfarin-sensitive and resistant rats measured under physiological conditions. However, after warfarin administration, no effect was noted in warfarin-resistant rats, although a prolonged coagulation time was noted in warfarin-sensitive rats. This means warfarin resistant rats can produce vitamin K-dependent blood clotting factor regardless of warfarin intake.

I also determined the kinetic differences in hepatic microsomal VKOR-dependent activity between warfarin-resistant and sensitive rats. Warfarin-resistant rats showed 2-3-fold lower V_{max}/K_m values than did sensitive rats. In addition, I report that resistant rats found in the Tokyo area had a VKOR activity which was poorly inhibited by warfarin.

About structural difference of VKOR enzyme, I performed docking simulation between VKOR enzyme and warfarin. The distance between VKOR enzyme and warfarin in warfarin resistant rats is longer than that in sensitive rats. It suggested that electron transfer rate between VKOR enzyme and warfarin of warfarin resistant rats was below one-quarter of that in sensitive rats.

The functional and structural difference occurred in VKOR substitution found in super-rats made advance for warfarin resistance. So I conclude that warfarin resistance in the Japanese black rat might be due to mutations in the *VKORC1* gene. However, further study is needed to clarify how such rats can maintain adequate vitamin K-dependent clotting factor levels, while simultaneously exhibiting low VKOR activity and warfarin resistance.

The original paper of this thesis appeared in *Pestic. Biochem. Physiol.*, **103**: 144–151 (2012).

The ecologies of *Borrelia* spp. in Hokkaido, Japan

Kyle Rueben Taylor

Laboratory of Wildlife Biology and Medicine, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

The ecologies of *Borrelia* spp. are very specific to location, since they are dependent upon the spirochete species, the vectors, and the host vertebrates present. The intent of this research was to describe the poorly studied ecological interactions of each of the most common *Borrelia* spp. found in Hokkaido, Japan, with their respective vectors and suspected host species. To this end, this research was endeavored in order to identify factors involved in *Borrelia* spp. maintenance by cross-comparison between sampling areas within Hokkaido (eastern and central), across time (May through September), among host species (deer, five rodent species and three shrew species), and between important vector ticks (*Ixodes persulcatus* and *Ixodes ovatus*).

First, a study was performed to examine questing tick infection rates with various *Borrelia* spp., and to contrast those rates between the two sampling areas in Hokkaido. Furthermore, comparison was made for tick abundance between the two areas by species and stage. Notably, tick abundance was significantly greater in eastern Hokkaido, yet infection rates of *I. persulcatus* with each *Borrelia* spp. were similar between areas. *Borrelia garinii*, a Lyme disease *Borrelia* sp., was the most common species found carried by questing *I. persulcatus*, and *Borrelia miyamotoi*, a relapsing fever *Borrelia* sp., was the least common.

Second, a study was performed on whether deer act as important reservoir hosts for Lyme disease *Borrelia* spp. Although it is difficult to determine with conviction that deer do not act as hosts, the results of this study suggest that, if deer are involved, they play a minor role. However, a novel *Borrelia* sp. similar to *Borrelia lonestari*, a relapsing fever *Borrelia* sp. also found among deer in the United States, was discovered during this study, and is herein reported for the first time. The absence of this *Borrelia* sp. among rodents or shrews, and the prevalence among deer suggest that deer may be an important host for this species.

Third, a study was performed on wild rodents to determine the infection rates with various *Borrelia* spp., the level of exposure to feeding nymphs and larvae of both *I. persulcatus* and *I. ovatus*, and the level of exposure to Lyme disease *Borrelia* spp. Furthermore, comparisons were made for rodent abundance, infection rates, and tick exposure between the two sampling areas by species. Notably, rodent abundance was greater in eastern Hokkaido, but infection rates between respective rodent species were generally similar between areas. Furthermore, differential infection rates among rodent species were correlated with tick burdens of the respective vectors of each Lyme disease *Borrelia* sp. Also, this study showed for the first time, in Hokkaido, that infections with Lyme disease *Borrelia* spp. are age-dependent. For *B. miyamotoi*, however, this is not true, and this species likely does not cause persistent infections in rodents the way that the Lyme disease *Borrelia* spp. do. Moreover,

B. miyamotoi infections in blood were highly correlated with larval *I. persulcatus* attachment, suggesting that larvae may be important vectors of this *Borrelia* sp.

Finally, a study determined the infection rates of shrew species with various *Borrelia* spp. This study agreed with previous research suggesting that these species are only moderately involved in the maintenance of pathogenic Lyme disease *Borrelia* spp. More importantly, this is the first report of *B. miyamotoi* in shrew species, and the herein recorded data indicates that shrews may be at least as important for the maintenance of this *Borrelia* sp. as rodents.

The original papers of this thesis appeared in *Vector Borne Zoonotic Dis.*, **13**: 92–97 (2013) and *J. Vet. Med. Sci.*, in press.

Studies on cellular mechanism of prion propagation using abnormal isoform prion protein specific detection method

Takeshi Yamasaki

Laboratory of Veterinary Hygiene, Department of Applied Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders pathologically characterized by neuronal vacuolation, astrocytosis and accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in central nervous systems (CNSs). PrP^{Sc} is an only known proteinaceous component of prions, the causative agents of TSEs, therefore, generation of PrP^{Sc} is a key event on prion propagation. Clarification of the intracellular dynamics of PrP^{Sc} facilitates understanding of the mechanism of prion propagation in cells. Therefore, I established a reliable PrP^{Sc}-specific detection method in immunofluorescence assay (IFA) and analyzed the intracellular events that are involved in prion propagation.

I analyzed intracellular localization of PrP^{Sc} in cells persistently infected with prions with the PrP^{Sc}-specific staining. The extensive analyses revealed the presence of PrP^{Sc} throughout endocytic compartments. In particular, some of the granular PrP^{Sc} signals appeared to be localized in endocytic recycling compartments (ERCs) at a peri-nuclear region of the cell. Interestingly, the peri-nuclear distribution of PrP^{Sc} was dispersed to the peripheral regions during the incubation of the cells at 20°C, but the dispersed PrP^{Sc} appeared to return to a peri-nuclear region within 30 min during subsequent incubation at 37°C. These results suggest that PrP^{Sc} in cells persistently infected with prions dynamically cycles between a peri-nuclear region and peripheral regions including the plasma membrane through ERCs, via the endocytic-recycling pathway.

The association of PrP^{Sc} with membrane trafficking in cells is thought to be important for the continuous generation of prions. However, intracellular events required for the establishment of prion infection, especially in the early stage after introduction of prions still remain to be elucidated. Therefore, I analyzed the intracellular dynamics of PrP^{Sc}, both inoculum-derived and newly generated PrP^{Sc} during the early stage after the PrP^{Sc} inoculation. Within 24 hours after inoculation, the newly generated PrP^{Sc} was evident at the plasma membrane, in early endosomes, and in late endosomes, but this PrP^{Sc}

was barely evident in lysosomes; in contrast, the majority of the inoculated PrP^{Sc} was transported to late endosomes and lysosomes. However, during the subsequent 48 hours, the newly generated PrP^{Sc} increased remarkably in early endosomes and ERCs, suggesting that compartments on the endocytic-recycling pathway such as early endosomes and ERCs are major site for the PrP^{Sc} generation. *De novo* generation of PrP^{Sc} after the inoculation of PrP^{Sc} was partly inhibited by overexpression of wild-type of Rab22a and a dominant-negative mutant of Rab11a, both of which impair membrane trafficking along the endocytic-recycling pathway. New PrP^{Sc} formation was also partly inhibited by overexpression of a dominant-negative Rab7 mutant that affects membrane trafficking along the endo-lysosomal pathway. Trafficking of inoculated PrP^{Sc} to late endosomes and lysosomes but the appearance of newly generated PrP^{Sc} in early endosomes and ERCs suggest that the transfer of exogenous PrP^{Sc} and/or newly generated PrP^{Sc} from the endo-lysosomal pathway to the endocytic-recycling pathway is important for the initiation of prion propagation that leads to the establishment of prion infection.

In this thesis, using reliable PrP^{Sc}-specific staining by IFA, I found intracellular dynamics of PrP^{Sc} that had not been identified previously. Although multiple trafficking pathways may be involved in the intracellular transport of PrP^{Sc}, the trafficking of PrP^{Sc} along membrane trafficking via the endocytic-recycling pathway may provide a significant advantage for continuous prion propagation. The generation of PrP^{Sc} is tightly associated with neuronal degeneration in CNSs in prion diseases. Although further studies are required for a thorough understanding of the mechanisms of neurodegeneration caused by prion infection, the information provided in this thesis greatly contributes to the elucidation of the mechanism of neurodegeneration caused by prion propagation.

The original paper of this thesis appeared in *J. Gen. Virol.*, **93**: 668–680 (2012).

Antibody-mediated inhibition of Marburg virus budding

Masahiro Kajihara

Division of Global Epidemiology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Host-induced antibodies play a pivotal role in protective immunity against filovirus infection. The only target of protective antibodies is envelope glycoprotein (GP), because GP is the sole surface protein on virions and the principal mediator of virus entry into host cells. However, the knowledge about the mechanisms underlying antibody-mediated inhibition of Marburg virus (MARV) infection and MARV escape from antibody pressure is quiet few.

In the chapter I, I demonstrate a novel function of MARV GP-specific antibodies, namely inhibition of MARV budding. In the presence of MARV GP-specific monoclonal antibodies (MAbs) AGP127-8 and MGP72-17, MARV particles were not released from infected cells efficiently and virus-like particles (VLPs) were densely tangled and accumulated on the cell surface. The most plausible mechanism of this phenomenon is that the MAbs form highly intricate cross-linkage *via* interaction between antibodies and GPs and mechanically interfere with the driving force for

MARV budding.

In the chapter II, I discuss novel mutations in MARV GP to escape from immunological pressure of the budding inhibition MAbs. A couple of escape variants had deletion of mucin-like region (MLR) in GP1 subunit including the epitope of MGP72-17. Some mutant GPs showed decreased cleavability due to a single amino acid substitution in the furin-recognition motif, indicating that the MAb-specific epitopes are unexposed on the uncleaved GP. These extraordinary structural flexibility and variability of MARV GP may contribute to evasion from antibody-mediated pressure.

The present thesis provides the idea that the "classical" neutralizing activity may not be the only indicator of a protective antibody that may be available for prophylactic and therapeutic use. Recent studies using nonhuman primate models highlighted the passive immunization with GP-specific antibodies as a promising prophylactic and/or therapeutics intervention for filovirus infection. On the other hand, possible risks that the escape mutants selected under antibody pressure interfere with this intervention should be also studied in parallel. The present thesis provides new insights into the development of prophylactic and therapeutic measures utilizing antibodies.

The original papers of this thesis appeared in *J. Virol.*, **86**: 13467–13474 (2012) and *J. Gen. Virol.*, **94**: 876–883 (2013).

Correlation between drug resistance and amino acid substitutions in DNA gyrase of *Mycobacterium leprae*

Kazumasa Yokoyama

Division of Global Epidemiology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Leprosy is a chronic human infectious disease caused by *Mycobacterium leprae* which may cause severe disabilities due to damage to the peripheral nerves. The World Health Organization (WHO) reported the global number of registered new cases in 2010 to be 228,474, while during 2009 it was 244,796. Although the number of new cases detected globally fell by 16,322 (6.7%) during this period, new leprosy cases are still detected every year, mainly in Asia, Latin America, and Africa.

In the 1980s, the WHO introduced multidrug therapy (MDT), composed of dapsone (DDS), rifampin (RIF), and clofazimine (CLF). Multibacillary leprosy is usually treated by administering DDS, RIF, and CLF in the combination, where single skin lesion paucibacillary leprosy is recommended to be treated by administering RIF, ofloxacin (OFX), and minocycline (MIN).

The emergence of multidrug-resistant (MDR) leprosy, resistant to both DDS and RIF owing to therapeutic failure or low compliance, has been reported, and fluoroquinolones (FQs) are thought to be important.

FQs inhibit type II DNA topoisomerases, DNA gyrase, and topoisomerase IV, which play crucial roles in DNA replication during cell division. As *M. leprae* has only DNA gyrase, this is the sole target of FQs. DNA gyrase, consisting of two GyrA and two GyrB, catalyzes the negative supercoiling of the circular bacterial

chromosome by cleaving double strands and passing the enwrapped DNA, followed by resealing the double strands. FQs prevent DNA replication and transcription by inhibiting DNA strand resealing step.

The present thesis consists of two chapters; in chapter I, I have elucidated the influence of an amino acid substitution at position 95 in GyrA to FQ resistance by *in vitro* DNA supercoiling and cleavage assays in the presence or absence of FQs.

Amino acid substitutions at position 89 or 91 in GyrA of FQ-resistant *M. leprae* clinical isolates have been reported. In contrast, those at position 94 in *M. tuberculosis*, equivalent to position 95 in *M. leprae*, have been identified most frequently. To verify the possible contribution of amino acid substitutions at position 95 in *M. leprae* to FQ resistance, I conducted an *in vitro* assay using wild-type and mutant recombinant DNA gyrases. FQ-inhibited supercoiling assay and FQ-induced cleavage assay revealed the potent contribution of an amino acid substitution of Asp to Gly or Asn at position 95 to FQ resistance. These results suggested that the possible future emergence of FQ-resistant *M. leprae* isolates with these amino acid substitutions and the usefulness of detecting these mutations for the rapid identification of FQ resistance in leprosy.

In chapter II, I conducted assays same as chapter I and revealed the contribution of amino acid substitutions at positions 464, 502, and 504 in GyrB to FQ resistance.

OFX is a FQ used for the treatment of leprosy. Mutations conferring FQ resistance have been reported to be found only in the gene encoding A subunit of this enzyme (*gyrA*) of *M. leprae*, although there are many reports on the FQ resistance-associated mutation in *gyrB* in other bacteria, including *M. tuberculosis*, a bacterial species in the same genus as *M. leprae*.

To reveal the possible contribution of mutations in *gyrB* to FQ resistance in *M. leprae*, we examined the inhibitory activity of FQs against recombinant DNA gyrases with amino acid substitutions at position 464, 502 and 504, equivalent to position 461, 499 and 501 in *M. tuberculosis*, which are reported to contribute to reduced sensitivity to FQ. The FQ-inhibited supercoiling assay and FQ-induced cleavage assay demonstrated the important roles of these amino acid substitutions in reduced sensitivity to FQ with marked influence by amino acid substitution, especially at position 502. Additionally, effectiveness of SIT, a FQ, to mutant DNA gyrases was revealed by low inhibitory concentration of this FQ.

Data obtained in this study suggested the possible emergence of FQ-resistant *M. leprae* with mutations in *gyrB* and the necessity of analyzing both *gyrA* and *gyrB* for an FQ susceptibility test. In addition, potential use of SIT for the treatment of problematic cases of leprosy by FQ-resistant *M. leprae* was suggested.

The original papers of this thesis appeared in *Antimicrob. Agents chemother.*, **56**: 697–702 (2012) and *PLoS Negl. Trop. Dis.*, **6**: e1838 (2012).

Development of cytotoxic T lymphocyte-inducing peptide vaccine

Toru Ichihashi

Division of Collaboration and Education, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

The current vaccination strategy based on inactivated vaccine or component vaccine is to produce antibodies which recognize surface proteins of pathogens. On the other hand, live attenuated vaccine is able to induce both antibody and cytotoxic T lymphocyte (CTL), so that live attenuated vaccine is considered to be more effective than other vaccines. However, live attenuated vaccine is not applicable to all pathogens because of the possibility of harmful side effects and the difficulty of selecting of an appropriate strain.

In this study, the author employed the strategy of peptide immunization to develop CTL-inducing vaccine without using live attenuated vaccine.

In chapter I, the possibility of developing CTL-based influenza vaccine which is broadly protective was explored. HLA-A*2402-restricted immunogenic epitope peptides from influenza A virus internal proteins were identified using web-based CTL epitope prediction programs and *in vivo* cytotoxicity assay. The protective effect of identified immunogenic peptides against lethal influenza A virus infection was examined in regard to the number of peptide required for the sufficient protection, effective administration route, and the difference in effector and memory CTL phase.

As the results, three epitope peptides as potential vaccine components for HLA-A*2402-positive personnel were selected. Although vaccination with any of the single epitope peptide showed weak protection, intranasal vaccination with mixture of the three peptides protected mice against influenza A virus challenge regardless of virus subtypes tested almost perfectly. Furthermore, this study revealed that T cell recruitment in lung was important for highly efficient protection against influenza virus infection. Unfortunately, protective efficacy of memory CTL phase was less than that of effector CTL phase.

In chapter II, a simple and effective novel peptide carrier used as an alternative to liposome was explored for developing a practical peptide vaccine. Micelles consisted of phosphatidylserine (PS) were selected among the candidate materials as a peptide carrier. Furthermore, immune-enhancing effect of PS micelles as a carrier was examined. This analysis demonstrated that PS conjugation enhanced delivery effect of antigens to professional antigen-presenting cells (APCs), especially conventional dendritic cells that were main APCs to stimulate epitope-specific CTL. This adjuvant effect of PS was also applicable to the *in vivo* induction of peptide-specific T helper cells.

In conclusion, the evaluation system of immunogenic CTL peptides used in chapter I contributes to the development of broadly protective influenza vaccine. PS micelles selected in chapter II are very practical carrier for a peptide vaccine. Furthermore, this study may contribute to the development of peptide-based vaccine against any intracellular pathogens.

The original papers of this thesis appeared in *PLoS ONE*, **6**: e24626 (2011) and *PLoS ONE*, **8**: e60068 (2013).