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INFORMATION

Hokkaido University conferred the degree of Bachelor of Veterinary Medicine to the following 41 graduates of the School of Veterinary Medicine on March 25, 2014. The authors summaries of their theses are as follows:

Pathogenetic roles originating from the telomeric region of MRL/MpJ-type chromosome 1 in the case of murine inflammatory exocrinopathy

Yuki Okada

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

The MRL/MpJ (MRL) mouse is a model of autoimmune exocrinopathy. The telomeric region of MRL chromosome 1 contains *Mag* as the susceptibility locus for autoimmunity. This study examined the role of *Mag* in the development of autoimmune exocrinopathy. The histology of exocrine glands and the serum level, protein localization, and mRNA expression of pancreatitis markers were examined in females of C57BL/6 (B6), MRL, and B6.MRLc1 congenic strain carrying B6 background and *Mag* at 9–12 months of age. The pancreas was histologically compared between sexes in the above 3 strains as well as 9 inbred strains over 6 months of age.

No histopathological difference was observed in salivary glands among the 3 examined strains. Periductal/perivascular cell infiltration of the pancreas (PCIP) was observed frequently in A/J, AKR/N, B6.MRLc1, C57BL/6, MRL/MpJ, moderately in DBA/1 and DBA/2, and rarely in BALB/c and C3H/He, and females tended to have more PCIPs than males. The PCIPs contained B-cells and T-cells and were larger in MRL and B6.MRLc1 than in B6. The borders between PCIPs and acini were clear in B6 but unclear in MRL and B6.MRLc1 because of invading inflammatory cells between each acinus, and some acinar cells died around PCIPs in the latter strains. No strain difference was observed in the appearance of fibrotic lesions and high endothelial venules in PCIPs. The serum anti-dsDNA antibody and amylase levels, markers of autoimmune-mediated pancreatitis, and mRNA expression of tumor necrosis factor-α and Fc gamma receptor III (FcγRIII, coded on *Mag*) in the pancreas increased in MRL and B6.MRLc1 compared to B6. FcγRIII-positive cells in pancreatic acini were observed in MRL and B6.MRLc1 but not in B6.

In conclusion, B6.MRLc1 developed chronic pancreatitis with an autoimmune phenotype. The data indicated that B6.MRLc1 could be a model of autoimmune-mediated pancreatitis and emphasized the crucial roles of *Mag* in the molecular and genetic pathogenesis of this disease.

Genetic factors characterizing perinatal oocyte developments in MRL/MpJ mice

Yuma Yamashita

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

In perinatal mice, the number of oocytes decreases during nest breakdown, in which nests containing several oocytes break into smaller cysts, and then separated oocytes start to form primordial follicles. Several previous studies have reported unique phenotypes in the gonads of MRL/MpJ mice with their susceptibility loci. The present study focused on the oocyte development of MRL/MpJ during perinatal stages and morphologically compared them with those in C57BL/6 and B6.MRLc1(68–81) congenic strain, carrying one of the MRL/MpJ-derived loci regulating the abnormalities of male germ cell development.

At embryonic day 18.5, although no significant strain difference was observed in the ovarian morphology, oocytes starting folliculogenesis, indicated by the NOBOX oogenesis homeobox (Nobox)-positive reaction, were more frequently observed in MRL/MpJ and B6.MRLc1(68–81) compared to C57BL/6. At postnatal day 0, primordial follicles appeared at the inner ovarian cortices of MRL/MpJ and B6.MRLc1(68–81) but not in C57BL/6, and the total number of oocytes and the Nobox-positive rate in total oocytes were significantly lower and higher in MRL/MpJ and B6.MRLc1(68–81) than in C57BL/6, respectively. The mRNA level of DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*Ddx4*), a marker of germ cells at all developmental stages, and the *Nobox*/*Ddx4* expression ratio tended to be lower and higher in the neonatal ovary of MRL/MpJ than C57BL/6, respectively. Furthermore, one of the responsible loci regulating these ovarian phenotypes of perinatal MRL/MpJ was specialized to chromosome 1 (68–76 cM) by analyzing MRL/MpJ-derived congenic strains.

In conclusion, the morphological characteristics of the perinatal MRL/MpJ ovary were clarified, such as accelerated nest breakdown, folliculogenesis, oocyte reduction, and oocyte development compared to C57BL/6. Furthermore, it was suggested that the genetic locus underlining these phenotypes was located at chromosome 1 (68–76 cM), and murine chromosome 1 (68–76 cM) is thought to be a crucial locus regulating the progress of oocyte development during nest breakdown and early folliculogenesis.

Studies on the plumage reflection spectra of jungle crow (*Corvus macrorhynchos*) and carrion crow (*Corvus corone*)

Atsushi Ichikawa

Laboratory of Physiology, Department of Biomedical Sciences, School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

1. Tetrachromatic avians can perceive a greater range of colors than trichromatic humans.

Some avian species utilize colors, including ultraviolet, as cues, which are invisible and indistinguishable to humans, for intra- and/or inter-species communication. The current study aimed at determining whether any optical differences exist in plumage between various body regions, sexes and species by recording whole, UV and visible refection spectra of the plumage, and discriminative ability was assessed in visual mode.

2. Intensity and chroma were calculated from spectra recorded at the crown, wing coverts, primary remiges, secondary remiges, dorsal tail feathers, ventral tail feathers, back, throat, and breast and analyzed for regional, sex and inter-species differences. Furthermore, brightness under natural sunlight was calculated and the perceptual distance (just noticeable difference: jnd) of plumage colors was assessed.
3. Sex and inter-species differences existed in intensity and chroma, especially at wing coverts of male jungle crow, with higher intensity than male carrion crow, irrespective of whole, UV and visible spectral ranges and with higher chroma than female jungle crow in UV range. The differences were reproducible when brightness was assessed under natural sunlight. In addition, the trends in chroma were consistent with the results obtained with the visual model, indicating that these differences contributed to species' identification.
4. The visual model suggested that plumage color differences are distinguishable by avian eyes; e.g. between secondary remiges and back, and between that and the throat. With human eyes, crow plumage appears mostly and uniformly black but it can be more colorful through crows' eyes, helping to discriminate sex and species.
5. The present results would be helpful for the future investigation of plumage color of various species. Comprehensive analyses of reflective spectra of avian plumage in combination with visual models are of great benefit for better understanding of the visual world of avians.

Low UV reflection found in some plumage regions of avians detected with an ultraviolet-photographable digital camera

Yasuyuki Nomura

Laboratory of Physiology, Department of Biomedical Sciences, School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

1. In addition to three types of retinal cones, tetrachromatic avians possess ultraviolet (UV)-sensitive opsin containing cones, with which they can receive a variety of UV-related information on their surroundings as visual cues. Plumage UV reflectance is one such cue that is indicated to be utilized for the discrimination of sex and/or species. In the current study, we used a UV-photographable digital camera in an attempt to detect possible regional differences in UV reflectance of plumage in various avians.
2. Visual images were also photographed for the comparison of regions of interest between UV and visual images of avians reared in zoos and aquariums in Hokkaido prefecture. The images were gray-scaled and brightness was calculated as indices of the degree of reflectance in the regions of interest when some differences were assessed by visual assessment of the photographs.
3. Clear differences in the brightness were found

in some regions of both mature white pelicans (*Pelecanus onocrotalus*) and king penguins (*Aptenodytes patagonicus*). The UV brightness from the frontal inferior part of the cervical region to the thoracic region was significantly lower than that of visible light or in other body areas. These areas can function as additional cues or signs for various vital activities in UV-recognizable tetrachromatic avians, which are invisible to humans.

4. In immature white pelicans, such regions were not detected. This indicates that the UV reflective pattern of plumage may be a cue to assess the level of maturity in conspecific avians.
5. A UV-photographable camera is a useful tool by which specific regions of low or high UV reflection can be readily assessed. In combination with conventional spectrophotometry, the UV-inclusive multihued pattern of plumage can be analyzed in more detail.

Dynamics of mammary stem cells in the process of ductal development in the mouse mammary gland

Mai Tokai

Laboratory of Biochemistry, Department of Biomedical Sciences, School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

The mammary gland is a unique organ that primarily develops after birth. Mammary ductal formation in the mammary fat pad starts immediately after birth and around 4 weeks of age it increases significantly, with the ducts having terminal end buds (TEBs), highly proliferative structures found at the tips of the invading ducts. This developmental stage lasts until the age of about 7–8 weeks. When the mammary ducts have reached the end of the mammary fat pad, TEBs become less proliferative and decrease in size. Mammary stem cells (MaSCs) play a key role in ductal development and are believed to be located in these developing mammary gland structures. In addition, it is suggested that MaSCs are distributed widely in mammary ducts without TEBs. However, due to the lack of suitable biochemical and histological markers for MaSCs, their precise location and their changes during ductal development have not been determined. Recently, it has been demonstrated that co-expression of two transcription factors, Slug and Sox9, is a pivotal characteristic of MaSCs. Therefore, the present study examined the location of MaSCs expressing both Slug and Sox9 in the process of mouse mammary ductal development and the possible involvement of reproductive hormones in their distribution.

Morphological analyses of mammary glands from female C57BL/6J mice (1 day to 12 weeks old) showed similar developmental phenotypes of ducts as described above, and confirmed the marked increase in ductal elongation and branching and in the number of TEBs from 4 to 8 weeks of age, whereas no TEBs were observed at 12 weeks of age. On the other hand, immunohistochemical analyses revealed that cells co-expressing Slug and Sox9 were present sparsely in the lateral layer of the mammary ducts in the age range tested. Further, it was found that double-positive cells were richly present in TEBs at puberty, especially in the cap cell region located at the tip of TEBs. These results suggest that MaSCs are present in previously supposed regions in the mammary ducts and that the proliferation and self-renewal of MaSCs in TEBs might be

regulated by reproductive hormones.

To test the latter assumption, the effects of estrogen and progesterone on the distribution and proliferation of MaSCs were examined in C57BL/6J mice ovariectomized at 5 weeks of age. Four groups of mice of 6 weeks of age were given a daily single subcutaneous injection of corn oil as a control solvent, estrogen, progesterone or a mixture of estrogen and progesterone for 8 consecutive days. Histological examination revealed that the ducts of control mice consisted of luminal epithelia lined with myoepithelium and a surrounding collagen layer, and there was no difference in the cellular composition among the groups. In addition, the distribution of the cells co-expressing Slug and Sox9 was unchanged from that of the aforementioned non-ovariectomized mice at puberty, in all ovariectomized mouse groups. However, whole-mount analyses of the mammary gland showed that ductal elongation and the number of side branches and TEBs were increased significantly in response to the hormone mixture, compared to the control. Since the number of MaSCs in each TEB seemed to be substantially identical, it is postulated that the proliferation and self-renewal of MaSCs in TEBs may be enhanced in mice given reproductive hormones. Indeed, it was found that cells expressing Slug in the cap cell region at the tip of TEBs strongly expressed PCNA, an indicator of cell proliferation, in response to the hormones. In contrast, those existing in the lateral layer of ducts hardly expressed PCNA. Moreover, the cells expressing PCNA in the branching region of ducts did not express Slug protein.

Real-time PCR analysis showed no difference in Slug expression among the groups but showed an increase in Sox9 expression in mice given both estrogen and progesterone, compared to the control group.

In summary, it has been demonstrated that MaSCs expressing both Slug and Sox9 transcription factors are localized sparsely in the lateral layer of the mammary ducts irrespective of age, and densely in the cap cell region at the tip of TEBs, if present. Furthermore, MaSCs localized in TEBs had a sensitive proliferative response to reproductive hormones, but those in the lateral layer of mammary ducts did not, suggesting that there are at least two distinct populations of MaSC in terms of the hormonal response, and the former MaSC population possibly contributes to the elongation of mammary ducts.

Effect of adenosine metabolic enzyme inhibitors on reflex potentials in rat spinal cord

Yuki Tomonari

Laboratory of Pharmacology, Department of Biochemical Sciences, School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Adenosine is known as a neurotransmitter and modulator, and is reported to have neuroprotective and analgesic effects in the central nervous system. Adenosine inhibits neurotransmission by activating adenosine A_1 receptor. The adenosine level is regulated by adenosine metabolic enzymes, such as adenosine kinase (AK) and adenosine deaminase (ADA), and equilibrative nucleoside transporters (ENTs). In this study, we examined the effects of adenosine metabolic enzyme and/or adenosine transporter inhibitors on reflex potentials and the extracellular adenosine level in the isolated spinal cord of neonatal rats.

An AK inhibitor ABT-702 inhibited both slow ventral root potential (sVRP), which is thought to reflect the nociceptive pathway, and monosynaptic reflex potential (MSR). sVRP was more sensitive to the AK inhibitor than MSR. The ABT-702-evoked inhibition was abolished by an A_1 receptor antagonist CPT. An ADA inhibitor EHNA had little effect on the reflex potentials, but potentiated the inhibition by ABT-702. ENT inhibitors NBTI and dipyridamole (DIP) inhibited the reflex potentials, while coapplication of NBTI and DIP (NBTI/DIP) attenuated the inhibitory effect of ABT-702/EHNA. ABT-702 increased the extracellular adenosine level, which was potentiated by EHNA. NBTI/DIP abolished and attenuated the adenosine increase by ABT-702 and ABT-702/EHNA, respectively.

These results suggest that intracellular adenosine increased by adenosine metabolic enzyme inhibitors is transported to the extracellular space by ENT and inhibits reflex potentials by activating A_1 receptors. As ABT-702 is more effective against sVRP than MSR, AK inhibitors may be used as new analgesic drugs in the spinal cord.

Molecular mechanism of the suppression of interferon alpha and beta secretion in host cells infected with bovine viral diarrhea virus

Yuri Abe

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

Bovine viral diarrhea virus (BVDV) suppresses the secretion of interferon alpha and beta (IFN-α/β) in host cells. Little is understood about the molecular mechanism of the suppression of IFN-α/β secretion by BVDV infection. In the present study, this was analyzed using GBK_E^+ and GBK_E^- strains. The GBK_E^+ strain suppressed IFN-α/β secretion in infected cells and the GBK_E^- strain did not. These viruses had 4 amino acid differences in N^{pro}, NS4B, NS5A, and NS5B. A full-length cDNA clone of the GBK_E^+ strain (pGBK_E^+) was constructed for reverse genetics to identify viral proteins responsible for the suppression of IFN-α/β secretion. Infectious virus generated by pGBK_E^+ (vGBK_E^+) was confirmed to suppress IFN-α/β secretion in infected cells. The viruses with N^{pro} carrying with aspartic acid at position 136 (136D) suppressed IFN-α/β secretion in infected cells, while the viruses with N^{pro} carrying with glycine at position 136 did not. In addition, the viruses with N^{pro} carrying 136D degraded interferon regulatory factor-3 (IRF-3) in infected cells. These results indicate that N^{pro} carrying 136D degrades IRF-3 in infected cells, resulting in the suppression of IFN-α/β secretion in host cells infected with BVDV.

Studies on the role of structural proteins for the pathogenicity of H7N7 influenza virus in chickens

Takaya Ichikawa

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

Highly pathogenic avian influenza viruses, which have multiple basic amino acid sequences at the cleavage site in their hemagglutinin (HA), cause fatal systematic infection in chickens. However, a previous study reported that Vac2sub-P0, which has basic amino acid residues KRRRRR at the HA cleavage site of a low pathogenic avian influenza virus A/duck/Hokkaido/Vac-2/2004 (H7N7), was not pathogenic in chickens (Soda, 2010). In this study, we obtained Vac2sub-P3L4 after seven consecutive passages of Vac2sub-P0 in chickens and investigated the intranasal pathogenicity and biochemical characteristics of the passaged viruses and recombinant viruses.

In PB2, PB1, PA, HA, NA, M1, M2 and NS1, 12 amino acid substitutions and a deletion were identified by comparison of Vac2sub-P0 and Vac2sub-P3L4 sequences. The results of intranasal inoculations in chickens with recombinant viruses indicated that viruses which have HA and NA genes derived from Vac2sub-P3L4 were highly pathogenic in chickens. Accordingly, the biochemical functions of HA and NA were compared between rgP0 and rgP3L4. The HA of Vac2sub-P3L4 showed lower receptor avidity and lower optimal pH than that of Vac2sub-P0. Vac2sub-P3L4 has lower ability of the virus to elute from erythrocytes than Vac2sub-P0. Moreover, it was found that the polymerase activity and M2 ion channel activity were not different between rgP0 and rgP3L4. These results indicate that the functional changes of HA and NA affect the infectivity and growth efficiency of the virus in chickens.

Potency of an inactivated influenza vaccine prepared from A/duck/Hong Kong/960/1980 (H6N2) against a challenge with A/duck/Vietnam/OIE-0033/2012 (H6N2) in mice

Tatsuya Nishi

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

H6 influenza viruses are prevailing in domestic birds and wild birds in Eurasian countries, and have been isolated from pigs and humans. Since H6 viruses have the potential to cause pandemic influenza in humans, a vaccine against infection with H6 viruses should be prepared. In the present study, H6 viruses in the influenza virus library were analyzed genetically and antigenically to select a vaccine strain. The potency of an inactivated whole virus vaccine prepared from the vaccine strain was evaluated in mice.

The HA genes of H6 viruses in the library were phylogenetically classified into Early, Group II, Group III, W312 sublineages, and North America lineage. Antigenic analysis of H6 influenza viruses was performed in hemagglutination-inhibition tests. The antiserum to H6 virus of the early sublineage reacts with viruses of different sublineages. Virus growth was assessed in embryonated chicken eggs of H6 viruses of the early sublineage in the library. A/duck/Hong Kong/960/1980 (H6N2) [Hong Kong/960] replicated the most efficiently and was selected as the vaccine strain. Mice injected with an inactivated whole virus vaccine intraperitoneally were challenged with A/duck/Vietnam/OIE-0033/2012 (H6N2) [Vietnam/2012] of Group II, which showed low cross-reactivity with H6 viruses of other sublineages. To assess protective immunity induced by the vaccine, the serum antibody titers of mice before challenge, virus titers in the lungs, and the body weight of mice after the challenge were measured. Neutralizing antibody titers to the challenge strain were detected in the sera of the mice vaccinated with Hong Kong/960 vaccine. Virus titers in the lungs and the weight loss of the vaccinated mice were significantly suppressed compared with those of the control group. These results indicated that Hong Kong/960 vaccine induced protective immunity in mice and reduced the impact of disease caused by the challenge strain. Furthermore, the potency of the vaccine against the challenge strain was enhanced by two intraperitoneal injections in mice.

The present results indicate that the inactivated whole virus vaccine prepared from a virus strain in the influenza virus library is useful as a vaccine against pandemic influenza.

Functional analysis of bovine programmed death-ligand 1 and 2 IgG1 Fc fusion protein

Asami Nishimori

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

An immunoinhibitory receptor, programmed death-1 (PD-1), and its ligand, programmed death-ligand 1 (PD-L1) and 2 (PD-L2), are involved in immune evasion mechanisms for several pathogens causing chronic infections. Previous studies showed that blockade of the PD-1/PD-L pathway by antibodies specific to either PD-1 or PD-L resulted in the re-activation of immune reactions and is expected to be applied to new therapies for chronic infectious diseases. In the veterinary research field, some reports showed that the PD-1/PD-L1 pathway is involved in disease progression in cattle chronically infected with bovine leukemia virus (BLV). Moreover, these reports showed that blockade of the pathway by anti-PD-1 or anti-PD-L1 antibody augmented antiviral effects *in vitro*. However, no functional analysis of PD-1 and PD-L1 molecules has been reported in cattle. Thus, in this study, recombinant bovine PD-L1 and PD-L2 proteins fused with rabbit IgG1 Fc region (PD-L1-Ig and PD-L2-Ig) were prepared, and then their molecular functions in cattle were analyzed *in vitro*.

After peripheral blood mononuclear cells (PBMCs) from cattle were cultured with either PD-L1-Ig or PD-L2-Ig, IFN-γ production and cell proliferation were analyzed. IFN-γ production and cell proliferation were reduced in the presence of PD-L1-Ig, whereas they were promoted in the presence of PD-L2-Ig. Flow cytometry analysis

confirmed the binding of PD-L2-Ig with bovine PD-1. In order to determine whether PD-L2-Ig could enhance the antiviral effect of anti-PD-1 antibody, the effect of the combination of PD-L2-Ig and anti-PD-1 antibody on IFN-γ production was examined. PBMCs cultured with both PD-L2-Ig and anti-PD-1 produced higher IFN-γ production than those with anti-PD-1 antibody alone.

To examine the antiviral effect of PD-L2-Ig against BLV, PBMCs or B cells from BLV-infected cattle were cultured with PD-L2-Ig, and then the expression of BLV protein, gp51, was analyzed by flow cytometry. The expression of gp51 was increased in the presence of PD-L2-Ig, showing that treatment with PD-L2-Ig alone did not have an antiviral effect against BLV.

This study revealed one of the molecular functions of bovine PD-L1 and PD-L2. Furthermore, PD-L2-Ig could stimulate the immune reaction in both BLV-infected and -uninfected cattle. These results demonstrated that PD-L2-Ig can be potentially applied as a new therapeutic strategy against chronic infections. Further investigations are required to develop a novel vaccine and control method against bovine chronic infections by the use of PD-L2-Ig.

Characterization of an immunosuppressant molecule, Ipis, in *Ixodes persulcatus* ticks

Kochi Toyomane

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

Ixodid ticks not only feed on blood but are also harmful vectors of several pathogens causing infectious diseases, afflicting both human public health and the livestock industry. Recent studies focused on the development of an anti-tick vaccine that inhibits blood feeding and the oviposition of tick. There have been several reports on the identification and characterization of tick molecules as effective targets for anti-tick vaccines.

Ixodes ricinus immunosuppressor (Iris) is a tick salivary gland protein derived from *I. ricinus*, which is the major vector of the Lyme disease agent in Western Europe. Iris has been shown to inhibit the proliferation of mouse splenocytes and the production of several cytokines, IFN-γ, IL-6, TNF-α, and IL-8, from human PBMCs. Iris is also known to bind to monocytes/macrophages and inhibit their ability to secrete TNF-α. In addition, Iris is a member of the serine protease inhibitor superfamily and interferes with coagulation. Thus, Iris is expected to be a new candidate antigen for anti-tick vaccines because of its multi-functional properties. In Japan, *I. persulcatus* is the major vector of the Lyme disease agent. Previously, two Iris homologues were identified from the salivary glands of *I. persulcatus* and were named Ipis-1 and Ipis-2, respectively. However, the function of these Ipis molecules remains unknown. Thus, in this research, the functions of Ipis-1 in blood feeding were studied.

To investigate the function of Ipis, recombinant Ipis expressed as a rabbit IgG Fc-fused protein (Ipis-1-Fc) in COS-7 cells was prepared. The cell proliferation assay and IFN-γ ELISA showed that Ipis-1-Fc inhibits the proliferation and IFN-γ production of bovine peripheral blood mononuclear cells (PBMCs). To identify the target cells for Ipis, a binding assay was performed. Ipis-1-Fc

protein was shown to bind $CD4^+$ cells, $CD8^+$ cells and $CD14^+$ cells, whereas the binding of Ipis-1-Fc with IgM^+ cells and $CD335^+$ cells was not observed. These results suggest that Ipis binds T cells and monocytes/macrophages. In addition, Ipis-1-Fc inhibited the cell proliferation and production of IFN-γ in bovine PBMCs even when $CD14^+$ cells were depleted, suggesting that Ipis could directly interact with T cells and inhibit their functions.

In conclusion, Ipis could contribute to the establishment of environments suitable for tick feeding by suppressing the functions of immune cells, such as T cells and monocytes. Further analysis of the immunosuppressive functions and anti-hemostatic effects of Ipis-1, as well as Ipis-2, will be required to develop an effective vaccine against *I. persulcatus*.

Characterization of canine immunoinhibitory factors PD-1 and PD-L1 and their potential to be a therapeutic target for the treatment of canine tumors

Naoya Maekawa

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

Programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) are an immunoinhibitory receptor and its ligand which induce the 'exhausted' status in antigen-specific lymphocytes, and thus are involved in the immune evasion of tumor cells. In human and mice, it has been reported that some kinds of tumor cells express PD-L1, and that tumor infiltrating lymphocytes, which are specific to tumor antigens, express PD-1. When PD-1 binds to PD-L1, negative signals are transmitted to the lymphocytes, resulting in the suppression of antigen-specific immune responses. Importantly, this suppressive status is reported to be reversible, and blockade of the PD-1/PD-L1 pathway, using anti-PD-L1 antibody or other molecules, can restore the function of exhausted lymphocytes. It is also reported that blockade of the PD-1/PD-L1 pathway results in re-activation of anti-tumor immunity and the subsequent regression of some tumors in human clinical trials. Therefore, this therapeutic strategy is now considered to be promising for the effective treatment of tumors. In domestic animals, however, there are few reports on the PD-1/PD-L1 pathway and its relevance to diseases is almost unknown. In this study, canine tumors were chosen as models, and canine PD-1 and PD-L1 were characterized molecularly. Then, PD-L1 expression on canine tumors and the potential of the PD-1/PD-L1 pathway as a therapeutic target for the treatment of canine tumors were assessed.

Full-length canine *PD-1* and *PD-L1* cDNA sequences were determined, and the deduced amino acid sequences of these molecules showed high homology with those of human and murine PD-1 and PD-L1. The intracellular region of canine PD-1 contained predicted functional motifs, which could transmit immunosuppressive signals to cells, and the motifs were well conserved among species. Recombinant canine PD-1 and PD-L1 proteins were constructed and found to bind to each other, determined by flow cytometric analysis. Anti-bovine PD-L1 monoclonal antibodies were found to recognize and detect canine PD-L1, and thus, expression analysis of canine PD-L1 on tumor cells was conducted using flow cytometry

and immunohistochemistry methods. Canine melanoma, mast cell tumor, renal cell carcinoma, and other kinds of tumors examined in this study expressed PD-L1, whereas some tumors did not express PD-L1. These results suggest that canine tumors could evade immune responses through the PD-1/PD-L1 pathway. In addition, anti-PD-L1 antibody effectively blocked the binding of recombinant PD-1 with PD-L1-expressing cells, and cytokine production of lymphocytes derived either from healthy canine peripheral mononuclear cells or from canine tumor tissues were increased when treated with this antibody. These results showed that the canine PD-1/PD-L1 pathway also suppresses immune responses in dogs, and that this antibody could be used as a new therapeutic strategy for the treatment of canine tumors.

In this study, canine PD-1 and PD-L1 were characterized molecularly, and their potential as therapeutic targets for canine tumors was demonstrated. Further investigations are needed to confirm the ability of anti-PD-L1 antibody to re-activate canine anti-tumor immunity, and its therapeutic potential has to be further discussed.

Multilocus Sequence Analysis of *Leishmania* Species in Ecuador

Hayato Furumoto

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

Leishmaniasis is a protozoan disease caused by the genus *Leishmania*, transmitted by female sand flies. More than 20 species of *Leishmania* have been described as causative agents of human leishmaniasis and the species is the major determinant of clinical outcome (cutaneous, mucocutaneous and visceral forms). In addition, response to the treatment is different among the species infected. Therefore, identification of the infecting parasite species is important for appropriate treatment and prediction of the patient's prognosis. In the present study, cytochrome *b* (*cyt* b), heat shock protein 70 (*hsp70*), mannose phosphate isomerase (*mpi*) and 6-phosphogluconate dehydrogenase (*6pgd*) genes of *Leishmania* species endemic in Ecuador were analyzed, and their utility for the identification of *Leishmania* at the species level was assessed. Furthermore, development of a simple genotyping method of *Leishmania* species circulating in Ecuador was attempted on the basis of a PCR-restriction fragment polymorphism (RFLP) method. Of the four leishmanial genes analyzed, *cyt* b gene analysis discriminated Ecuadorian *Leishmania* species including closely related species, strongly suggesting that the *cyt* b gene is a reliable marker for identification of the parasites. Based on sequence analyses, inter- and intra-species genetic variation was assessed, and species-specific cleavage sites for restriction enzymes were determined on each gene. As a result, PCR-RFLP analysis of the *mpi* gene with *Hae*III and *Hpa*I or that of the *6pgd* gene with *Bsp*1286I and *Hin*fI successfully differentiated *Leishmania* species in Ecuador. The present PCR-RFLP method will be a useful tool for the surveillance of *Leishmania* species in Ecuador. Further study is needed to verify its utility in other endemic areas and other *Leishmania* species.

Epidemiology and Genetic Diversity of *Babesia gibsoni* in Dogs in Bangladesh

Masashi Terao

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

Babesia gibsoni is a tick-borne canine hemoprotozoan parasite known to be distributed in Asian, including Japan, South Korea, Taiwan and Malaysia. In the present study, an epidemiological survey of *Babesia* infections in dogs was conducted in Bangladesh. Blood samples were collected onto FTA® Elute cards from 50 stray dogs in Mymensingh District in Bangladesh. DNAs eluted from the cards were subjected to nested PCR, targeting the *18S rRNA* gene of *Babesia* spp. Approximately 800 bp PCR products were detected in 15 of 50 dogs (30%). Based on RFLP (restriction fragment length polymorphism) and direct sequencing analysis of PCR products, all parasites were identified as *B. gibsoni*. In addition, the *BgTRAP* (*Babesia gibsoni* thrombospondin-related adhesive protein) gene was detected in 13 canine blood samples. Sequence analysis of the *BgTRAP* genes revealed that functional domains of the protein were well-conserved but the repetition structure of some amino acid residues near the C-terminus varied among *B. gibsoni* parasites in Bangladesh. Phylogenetic analysis of the *BgTRAP* genes showed that *B. gibsoni* parasites in Bangladesh formed a cluster, which was genetically different from other Asian *B. gibsoni* parasites. Although all 15 ticks collected from these dogs were morphologically identified as *Rhipicephalus sanguineus*, *B. gibsoni* DNA was not detected in the ticks. These results suggest that *B. gibsoni* parasites are endemic in dogs in Bangladesh. Further studies are required to elucidate the origin, distribution, vector and pathogenesis of *B. gibsoni* parasites circulating in dogs in Bangladesh.

Study on the malformation of the spleen and thymus in *Ednrb*-null rats

Miyu Lee

Laboratory of Laboratory Animal Science and Medicine, Department of Disease Control, School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Contrast-enhanced ultrasonography of hepatic vein for the early diagnosis of canine diffuse hepatic diseases

Akira Hiramoto

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

Contrast-enhanced ultrasonography (CEUS) of the hepatic vein can assess intrahepatic hemodynamic changes, and it has been studied as a noninvasive method to assess the severity of portal hypertension. However, there is no study about its usefulness in veterinary medicine. The purpose of this study was to clarify the utility of CEUS to evaluate hemodynamic changes in canine diffuse hepatic disease.

Firstly, the repeatability of CEUS of hepatic vein variables was investigated in six normal dogs. The time-intensity curve was analyzed using four parameters: hepatic vein arrival time (HVAT), time to peak (TTP), time to peak phase (TTPP), and wash-out ratio (WR). The coefficients of variation (CV) of these parameters were similar to those of human studies.

Secondly, we established a canine portal hypertension model and investigated the correlation between portal vein pressure and CEUS parameters. Catheterization of the portal vein was performed by laparotomy and the outer end of the catheter was fixed subcutaneously in the abdominal wall. Intra-portal injections of 15 mg/kg microspheres at five-day intervals induced stable portal hypertension models within two months. Only TTPP showed a time-dependent reduction that was significantly correlated with the increase of portal vein pressure ($P < 0.05$).

Finally, CEUS parameters were analyzed in 22 clinical cases of hepatic disease that could induce arterialization of hepatic blood flow. Sixteen dogs were categorized as Group I (diffuse hepatic disease) and 6 dogs as Group II (congenital portosystemic shunts). TTPP was significantly reduced in both groups and WR significantly reduced in group I when compared to normal dogs.

In conclusion, these results showed that CEUS of the hepatic vein in dogs has similar repeatability to CEUS studies in people. Furthermore, the measurement of TTPP may be valuable to evaluate the arterialization of hepatic blood flow.

Analysis of *Babesia gibsoni* heat shock protein 90 expression level under stress condition

Natsuki Idaka

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

Babesia gibsoni is exposed to oxidative stress by reactive oxygen species in canine erythrocytes. Diminazene aceturate (DA)-resistant *B. gibsoni* isolates, which had been maintained in our laboratory, were also under continuous stress due to exposure to DA. The present study investigated

the stress response of molecular chaperone heat shock protein 90 in *B. gibsoni* (BgHsp90). Firstly, the expression of BgHsp90 was compared between wild-type and DA-resistant isolates. Secondly, the change in the expression of BgHsp90 in wild-type *B. gibsoni* under oxidative stress was investigated.

The mRNA and protein expression level in BgHsp90 of wild-type and DA-resistant isolates, and its subsequent changes in wild-type *B. gibsoni* under oxidative stress were examined using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and immunoblotting. A template complementary DNA for qRT-PCR was synthesized from total RNA extracted from *B. gibsoni* in cultures. For immunoblotting, rabbit anti-recombinant BgHsp90 polyclonal antibody was developed by immunization of a rabbit with recombinant BgHsp90.

The mRNA and protein expression level of DA-resistant isolates were significantly lower than in the wild type. This indicates that the expression of BgHsp90 was reduced due to persistent stress by DA. BgHsp90 requires energy to maintain its function; however, a paradoxical increase in glucose consumption of DA-resistant isolates in spite of the decreasing expression of BgHsp90 was observed. This suggests that an increase in BgHsp90 was not necessary to maintain the DA-resistant capacity in *B. gibsoni.*

The expression of BgHsp90 was not altered in wild-type *B. gibsoni* exposed to oxidative stress, indicating that BgHsp90 was not required to protect the parasites from oxidative stress.

In conclusion, real-time qRT-PCR and immunoblotting methods for the measurement of BgHsp90 expression were successfully established. In the present study, the BgHsp90 expression of DA-resistant isolates was decreased compared to the wild type. Moreover, the BgHsp90 expression of the wild type exposed to oxidative stress was not altered. The mechanism and meaning of these results are still unclear at the present, and further studies are necessary to clarify the functions and roles of BgHsp90.

Expression of Toll-like receptors in the colorectal mucosa of inflammatory bowel disease in miniature dachshunds

Miho Tanaka

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

Inflammatory colorectal polyps (ICRPs) in miniature dachshunds were recently recognized as a major cause of large bowel diarrhea in this dog breed in Japan. ICRPs are characterized by the formation of multiple small polyps (small polyps) and/or a space-occupying large polyp (large polyp) in the colorectal area, and are thought to be a novel form of inflammatory bowel disease (IBD). In humans, increased expressions of Toll-like receptor (TLR) 2 and TLR4 are thought to play important roles in the pathogenesis of IBD. Thus, in this study, the TLR gene and protein expressions were examined in the colorectal mucosa of dogs with ICRPs.

Colorectal mucosal biopsy samples were obtained from healthy control dogs (n = 12), normal miniature dachshunds (n = 3), and areas of non-inflamed colonic mucosa (n = 4), small polyps (n = 9) and large polyps (n = 15) of ICRPs by endoscopy or surgical excision. The expression levels of *TLR2*, *TLR3* and *TLR4* mRNA were analyzed by quantitative real-time PCR. The

TLR2 and *TLR4* gene expression levels were significantly increased in large polyps and small polyps compared to in controls. In contrast, *TLR2* and *TLR4* gene expression levels in non-inflamed colonic mucosa of ICRPs were similar to those in controls. There was no significant difference in *TLR3* gene expression levels among the groups.

Next, immunoblot analysis was performed to examine TLR2 and TLR4 protein expressions in the colonic mucosa of healthy dogs and large polyps of ICRP dogs. Both anti-TLR2 and anti-TLR4 antibodies detected proteins with approximate molecular sizes of 70 and 50 kDa in the colorectal mucosa. The 70 and 50 kDa proteins were clearly detected in large polyps of ICRP dogs.

Lastly, the locations of TLR2 and TLR4 proteins were examined by immunohistochemistry. In the small polyp area, increased expression of TLR4 was observed at the luminal surface of the crypt epithelium, and mildly increased expression of TLR4 was observed in the cytoplasm of goblet cells. In the large polyp area, increased expression of TLR4 was clearly observed at the luminal surface of the crypt epithelium and in the cytoplasm of goblet cells. Specific staining for TLR2 was not observed in control or ICRP dogs.

This study demonstrated that TLR2 and TLR4 expressions were increased in the colorectal polyps of ICRP dogs. Up-regulation of TLR2 and TLR4 could lead to the activation of innate immunity, which, in turn, may play an important role in the pathogenesis of ICRPs in miniature dachshunds.

Isolation and characterization of canine tumor endothelial cells

Yusuke Izumi

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

Effects of iron-metabolism-related protein expression in canine tumor cell lines on sensitivity to dihydroartemisinin

Takafumi Anekawa

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

The antitumor effect of artemisinin has been reported *in vitro* and *in vivo* in many human tumor cell lines. In canine tumor cells, anti-tumor effects have been reported in osteosarcoma, histiocytic sarcoma and mammary carcinoma cell lines. The purpose of this study was to evaluate the cytotoxicity of dihydroartemisinin (DHA) on canine tumor cells and to clarify factors affecting the antitumor effect of artemisinin.

Since artemisinin showed cell cytotoxicity in the presence of iron, artemisinin sensitivity of tumor cells was expected to increase *in vivo*. Sensitivity to DHA, an artemisinin derivative, was evaluated by MTT assay for cell lines of

canine osteosarcoma, canine histocytic sarcoma, canine transitional cell carcinoma, canine hemangiosarcoma, canine malignant melanoma and murine squamous cell carcinoma and fibroblasts derived from a dog and a mouse. Cell activity decreased in a DHA concentration-dependent manner in all tumor cells. Antitumor effects of DHA were examined in BALB/cAJcl-nu/nu mice, into which canine histocytic sarcoma cells were transplanted, and DBA/2J Jcl mice, into which murine squamous cell carcinoma cells were transplanted. The increase of the tumor volume rate was reduced in murine squamous cell carcinoma, which showed low sensitivity of DHA under culture conditions in a dose-dependent manner. From this result, certain factors, such as the participation of iron in cell cytotoxicity that was not evaluated under standard culture conditions, are important in the cell toxicity of DHA.

Sensitivity of DHA decreased in all tumor cells markedly when iron was chelated by deferoxamine supplementation to culture media. Sensitivity of DHA was then reinforced in tumor cells except canine malignant melanoma cells by ferric ammonium citrate supplementation to culture media. From these results, it was shown that the existence of the ferric ion participated in the cell cytotoxicity of DHA. The IC_{50} of DHA decreased in cell lines of canine osteosarcoma, canine histocytic sarcoma, canine transitional cell carcinoma and murine squamous carcinoma cells by supplementation of holo-transferrin derived from humans. Sensitivity of DHA may be enhanced in these cells by taking in iron through transferrin receptor (TfR). On evaluation of the expression of TfR genes in the tested cell lines, there was no relation between the expression of TfR and *in vitro* DHA sensitivity. Highly enhanced expression of TfR in murine squamous carcinoma cells might be somehow related to the ability of the high uptake of iron *in vivo* rather than the sensitivity of DHA. On quantification of ferritin (Ft) gene expression, the level of Ft genes was high in canine cell lines of histocytic sarcoma and canine hemangiosarcoma cells. Sensitivity of DHA was increased in the presence of iron in these cells, so Ft may therefore participate in DHA sensitization with iron. Regardless of the lower expression of TfR and Ft genes, the examined cells, in which sensitivity to DHA could be disturbed by chelating iron ions, could be reinforced by iron supplementation. This suggested that factors apart from TfR and Ft could be candidates for enhancers of DHA sensitivity. From this study, the different sensitivity of canine tumor cells to DHA between *in vitro* and *in vivo* was revealed, and the iron metabolism factors of the cell were more likely to participate in the extent of cytotoxicity of DHA.

Clinical evaluation of disease process of experimentally induced chondral and osteochondral defects on distal femoral surfaces in dogs

Akio Odani

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

Articular cartilage is a tissue that never fully heals after injury. Repaired cartilage defects are mostly filled with fibrocartilage, not with functional hyaline cartilage. Despite many research projects

on the reconstruction and regeneration of hyaline cartilage, none has been effectively established for clinical use. Recently, several studies have reported that hyaline cartilage could be induced by prostaglandin (PG) E_2 signal via EP_2 receptor, leading to the clinical examination of a new therapeutic approach using EP_2 selective agonists. In the clinical field of veterinary medicine, computed tomography (CT) and magnetic resonance imaging (MRI) have not yet been widely used for imaging the pathology of joint disorders, while tomographic images of the intra-articular structure can be analyzed by CT and MRI in humans. The objective of this study was to evaluate the clinical effects of EP_2 selective agonists on injured articular hyaline cartilage in dogs and to analyze the time-course changes of the joint structure using CT and MRI.

In experiment 1 (E1), five one-year-old female beagles were assigned to full-thickness cartilage defects on the distal articular surface of the lateral femoral condyle. After clinical observation for 12 weeks, necropsy and histological analysis were performed. In experiment 2 (E2), five one-year-old male beagle dogs were subjected to osteochondral defects on the distal articular surface of the medial femoral condyle and femoral trochlea. Clinical observation was performed for 12 weeks. Necropsy and morphological analysis were performed 20 weeks after injury to evaluate the repair of the cartilage structure. In the E1 and E2 experiments, three dogs were randomly assigned to the treatment group and two dogs to the placebo group. In treatment groups, sequential injections of EP_2 selective agonists (0.050 ml/kg) were given into the affected articular space every two weeks from two weeks after injury to the end of the experiment. In placebo groups, a placebo drug injected as in treatment groups. Clinical observations included the condition of dogs and X-ray, CT and MRI of both sides of knee joints.

In E1, histological changes were observed in the subchondral structure and synovial tissues in both groups at 12 weeks. Cellular infiltration into subchondral bone was observed and mild to moderate synovitis was induced by intra-articular injection of EP_2 selective agonists. Formation of enlarged trabecular bone and bone cyst-like structures in subchondral bone could indicate changes encouraging bone metabolism in the structure. While these findings in subchondral bone were impossible to observe by conventional X-ray, CT could reveal osteogenesis as a high signal intensity and bone remodeling as a low signal intensity. MRI also clearly showed changes of signal intensity indicating structural changes, as on CT. In the E2 treatment group, regeneration of cartilage tissue was visualized on the surface of osteochondral defects 20 weeks after injury. Both CT and MRI, rather than X-ray, showed signal changes indicating not only reactive changes in subchondral bone, but also chondrogenesis on the surface of osteochondral defects. These findings occurred markedly in treatment groups, suggesting the reconstructive abilities of the substances.

In conclusion, EP_2 selective agonists could accelerate metabolism within osteochondral structures that might promote the regeneration of a hyaline cartilage-like structure when chondrogenic cells were supplied into the sites. Furthermore, compared to X-ray, CT and MRI were more useful in veterinary use for detailed diagnosis of structural changes in small animals.

Trial for development of an animal model of influenza virus-associated encephalopathy —Effect of intracerebral administration of inactivated viral antigens to influenza virus-infected mice—

Marino Oichi

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

Influenza virus-associated encephalopathy (influenza encephalopathy) is a severe central nervous system (CNS) disorder in humans, especially in infants. The pathogenesis remains unclear and effective treatments have not been established. Because not all patients with influenza develop the CNS disorder, infection with influenza virus is necessary but not sufficient for the development of influenza encephalopathy. As raised concentrations of pro-inflammatory cytokines such as TNF-α and IL-6 are frequently detected in plasma and cerebrospinal fluid in the affected children, hypercytokinemia is suspected to be involved in the pathogenesis. In the present study, in order to verify the hypothesis that the entry of infinitesimal influenza virus antigens into CNS could induce encephalopathy, BALB/c mice were intranasally inoculated with a strain (A/Aichi/2/68) of H3N2 influenza A virus and survivors (at day 13 post-infection) were intracerebrally inoculated with inactivated influenza viral antigens. The brains of mice inoculated with the antigens showed significantly increased expressions of IL-10 and IL-6 compared with those of mice without administration of the inactivated antigens, but there was no significant difference in the expression of TNF-α and IL-1β in both plasma and brains between the antigen-inoculated and mock-inoculated groups. Additionally, there was no pathological finding suggesting encephalopathy, including neural symptoms and cerebral edema around vessels, in any mice. These results suggest that a pathological condition similar to human influenza encephalopathy could not be experimentally reproduced just by intracerebral inoculation of infinitesimal inactivated influenza virus antigens into mice.

The intracellular localization of rabies virus phosphoprotein and its significance in rabies virus-infected cells

Naoki Tsunekawa

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

Rabies is a lethal zoonotic disease that is caused by rabies virus infection. Rabies virus belongs to the genus *Lyssavirus* in the family *Rhabdoviridae*. Rabies virus phosphoprotein (P) is a multifunctional protein involved in rabies virus pathogenicity. However, its detailed localization

and functions in infected cells are unknown. Furthermore, the relationship between rabies virus infection and cellular autophagy remains unclear. In this study, the intracellular localization of P and its significance in rabies virus-infected cells were investigated. Immunohistochemical staining for P in the brains of mice infected with rabies virus (challenge virus standard strain, CVS strain) revealed positive signals at the edge of the intracytoplasmic vacuoles in the cytoplasm of neurons and some of them colocalized with microtubule-associated protein light chain 3 (LC3) as an autophagosome marker and lysosomal-associated membrane protein 1 (LAMP1) as a lysosome marker, suggesting the localization of P in the autophagy pathway. Additionally, mice challenged with rabies virus after the administration of rapamycin to induce autophagy showed a tendency toward delayed onset compared to control mice. In addition, ATG5 knock-out mouse embryonic fibroblasts (MEF) showed a higher rabies virus infection rate than wild-type MEF. These results suggest that rabies P is partially digested by autophagolysosomes in the infected neurons, which leads to temporary inhibition of virus replication.

Experimental study on retrovirus-inducing myocardial abnormalities

Sayaka Kishi

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

Subgroup A of avian leukosis viruses (ALVs-A) belong to *alpharetrovirus* in the family *Retroviridae* and generally induce hematopoietic neoplasms. Some strains of ALV-A, including fowl glioma-inducing virus prototype (FGV-p) and Km_5666, have been suggested to rarely induce myocardial abnormalities, but the details remain unclear. In this study, chick embryos were inoculated with Km_5666 by the yolk sac route at 4 days of incubation, which is earlier than the routine protocol (at 6 days of incubation), in order to clarify the causal relationship. The inoculated chicks showed matrix inclusions in cardiomyocytes and Purkinje cells (10/12; 83%) and disarray of myocardial fibers (9/12; 75%) at 10 days of age after hatching. The frequency of these lesions and intracardial viral RNA levels were significantly higher in chicks inoculated at 4 days of incubation than in those at 5 days of incubation. Also, they had matrix inclusions (18/18 chickens; 100%), non-suppurative myocarditis (17/18; 94%), myocardial fibrosis (8/18; 44%) and focal to multifocal cardiomyocyte hypertrophy with large atypical nuclei (17/18; 94%) at 35 days of age. Immunohistochemistry revealed that these hypertrophied cardiomyocytes were positive for vimentin and desmin, whereas they were negative for α-sarcomeric actin and myosin. Additionally, the severity of these cardiac changes was associated with the intracardial viral RNA level. These results suggest that ALVs cause cardiomyocyte abnormalities, including cardiomyocyte hypertrophy, and these abnormalities are disorders of myocardial differentiation induced by ALVs, implying a pathogenic mechanism different from insertional mutagenesis.

Effect of the exposure of bovine frozen semen to ambient air on characteristics of thawed spermatozoa

Yasuhiro Imada

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

It is well known that the handling of bovine frozen semen affects the characteristics of spermatozoa. However, no report has investigated the relationship between the handling of frozen semen and the capacitation-like changes of bovine spermatozoa. In the present study, firstly, the development of a procedure for capacitation-like changes by double staining with propidium iodide and Fluo-3 AM staining with flow cytometry analysis was attempted. Calcium ionophore A23187 was added to thawed semen and the change in the intracellular calcium concentration of spermatozoa was examined. As a result, in accordance with the extension of the reaction time, the percentage of spermatozoa showing high intracellular calcium concentration and an intact cell membrane was increased. Then the effects of repeated exposure of frozen semen to ambient air on the characteristics of spermatozoa were evaluated by flow cytometry and a sperm motility analyzer. Frozen semen in straws was subjected to static air or a light breeze at 30°C for 2–30 sec and placed back into liquid nitrogen, and this exposure was repeated five times. As a result, the percentage of spermatozoa showing capacitation-like changes was low at 0 or 3 hr after thawing, although the percentage of dead spermatozoa was significantly higher at 3 hr than at 0 hr. Detailed analysis of damage to spermatozoa by repeated exposure to ambient air suggested that mitochondria in sperm seemed to be damaged before acrosome damaged. Also, it was clarified that long-term exposure (30 sec) to ambient air as static air or as a light breeze (10 sec) caused severe damage to mitochondria and acrosomes of spermatozoa. In conclusion, a procedure for detection of the survival and high intracellular calcium concentration of sperm was established in the present study, but further research on the detection of capacitation-like changes after thawing is necessary. In addition, it was indicated for the first time that mitochondrion injury in sperm seems to occur before acrosome injury.

Evaluation of stress before parturition by changes in rumination behavior and plasma cortisol concentration in dairy cows and heifers

Hiyori Kinoshita

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

The objective of this study was to quantify stress before parturition using the time spent in

rumination and other behaviors as stress indicators and to investigate the relationship between these behaviors and the plasma cortisol concentration in dairy cows and heifers. The times of rumination, feeding and lying were monitored in 4 cows and 2 heifers for 96 h until rupture of the chorioallantois. Blood samples were collected from 5 cows and 3 heifers from six days before to one day after calving.

Cows and heifers spent more time in rumination (466 ± 25.3 min/day, mean ± SE) during the 24 h before rupture of the chorioallantois (calving period) than during the 1 to 3 days before (411 ± 13.6 min/day, $p < 0.05$). Also lying time during the calving period (585 ± 99.4 min/day) tended to be shorter than that 3 days before the calving period (668 ± 91.2 min/day, $p = 0.07$). The increase in total rumination time mostly depended on the increase in rumination time in a standing position and the time spent lying without rumination seemed to decrease during the calving period. Rumination time started to decline 2 h before rupture of the chorioallantois. Plasma cortisol concentration started to increase one day before calving in heifers and on the calving day in cows. No difference was observed between cows and heifers in the mean value of the plasma concentration throughout the experiment.

In conclusion, total rumination time and the plasma cortisol concentration increased on the day of parturition in the present study, even though a decrease in rumination time before parturition was prospected. A decrease in rumination time immediately before the onset of calving was observed, suggesting that cows and heifers were feeling severe stress at that time.

Folliculogenesis and steroidogenesis of follicles during development and maturation in Hokkaido sika deer (*Cervus nippon yesoensis*)

Sumiyo Kobayashi

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

Follicle formation and the ability of steroid synthesis in sika deer ovary were considered as physiological factors that control breeding activities such as estrus, pregnancy and parturition. Folliculogenesis and the expression of steroidogenic enzymes (P450scc, 3βHSD, P450c17 and P450arom) were examined by hematoxylin-eosin staining and immunohistochemical staining, using ovaries collected from fetus, fawn and adult female sika deer. Also, in order to investigate the relationship between the intensity of enzyme expression and steroidogenesis, peripheral estradiol-17β concentrations were measured. Primordial, primary, and antral follicles were observed in the fetus on fetal day 50 to 80, day 120 and day 150 to 200, respectively. Postnatal sika deer had many types of follicles, i.e. primordial to antral. Because 3βHSD and P450scc were expressed in granulosa and luteal cells of the fetus in the late pregnancy period and in postnatal deer, progesterone was expected to be synthesized in these cells. The expression of P450c17 was detected in theca interna cells of the fetus at about 200 days of pregnancy and postnatally. Moreover, P450arom was expressed in granulosa cells of the fetus at about 225 days of pregnancy and in postnatal deer. These data indicate that these two cells were likely to synthesize estradiol-17β cooperatively

from 225 days of gestation. P450arom and P450c17 were also detected in luteal cells; therefore, estradiol-17β was probably synthesized in luteal cells. The intensity of P450arom staining in luteal cells was relatively low in early pregnancy compared to in the mid- and late period, but there was no difference in peripheral estradiol-17β concentration between these periods. The present results suggested that steroid hormone synthesis in sika deer ovaries is carried out in the granulosa, theca interna and luteal cells of the fetus after the late pregnancy period, and that the expression site of these steroid synthesis enzymes is unique to sika deer.

Distorted *α-β* spectrin interaction and tetramer formation caused by focal structural alteration of *α*-spectrin due to E91K substitution

Eri Kitaguchi

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

Endoplasmic reticulum-associated degradation of R664X mutant of bovine AE1 anion exchanger: selective interaction of derlin-2 with the mutant

Roka Shimada

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

Study on replication mechanism of encephalitic flaviviruses in primary mouse neuronal culture

Minato Hirano

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

Neurological diseases caused by encephalitic flaviviruses are severe and are associated with a high level of mortality. However, the detailed mechanism of viral replication in the brain and the features of viral pathogenesis remain poorly understood. We carried out comparative analysis

of the replication of neurotropic flaviviruses, West Nile virus, Japanese encephalitis virus, and tick-borne encephalitis virus (TBEV) in primary cultures of mouse brain neurons.

All flaviviruses multiplied well in primary neuronal cultures from the hippocampus, cerebral cortex, or cerebellum. Distribution of viral-specific antigen in the neurons varied; TBEV infection induced the accumulation of viral antigen in neuronal dendrites to a greater extent than infection with other viruses. An experiment using a chemical inhibitor showed that microtubules affect accumulation. Viral structural proteins, non-structural proteins, and double-stranded RNA were detected in dendritic regions in which viral antigens accumulated after TBEV infection. Infection of virus-like particles of TBEV also induced antigen accumulation. Further, electron microscopic observation confirmed that TBEV replication induced characteristic ultrastructural membrane alterations in the neurites; a newly formed laminal membrane structure containing virions.

This is the first report describing viral replication in and ultrastructural alterations of neuronal dendrites, possibly causing neuronal dysfunction. These findings encourage further study to understand the molecular mechanisms of viral replication in the brain and the pathogenicity of neurotropic flaviviruses.

Adaptation of Hantaan virus strain AA57 to Vero E6 cells affects pathogenicity in a mouse model.

Masahiro Maki

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

Hantaviruses belong to the genus *Hantavirus* within the family *Bunyaviridae*. Rodents and Soricomorpha species are known to be natural reservoirs for these viruses. Several rodent-borne hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans. HFRS and HPS are epidemiologically important due to their high pathogenicity in humans. However, since there are few suitable animal models for hantavirus infection, little is known about the mechanisms of the pathogenicity of the disease. Recently, it was found that infection with a newly isolated strain of a Hantaan virus (HTNV), AA57, caused severe acute pulmonary symptoms in 2-week-old ICR mice, indicating that it could be a good disease model for hantavirus infection. It has been shown that the passage of viruses in cultured cells causes adaptation of the virus to cell culture and reduces the pathogenicity of the viruses. In this paper, AA57 was passaged in Vero E6 cells 30 times, and the viruses were characterized in cultured cells and the mouse model.

The viruses passaged 20 times (P20) and 30 times (P30) grew significantly more than the virus passaged 3 times (P3) in primate Vero E6 (African green monkey) and A549 (human) cells.

Morbidity and mortality in mice infected with P20 or P30 were significantly lower than in those infected with P3. While a peak of the virus titer (8.8×10^4 ffu/gram) was observed 5 days post-inoculation in the lungs of P3-infected mice, only a few virions (lower than the detection limit) were detected in mice infected with P30 throughout the experiments.

The expressions of inflammatory cytokines, IL-1β and TNF-α, were induced in the lungs of

P3-infected mice, but no expression was observed in mice infected with P30.

One amino acid substitution was found in the S segment of P20 and P30 at nucleotide position 163, resulting in a substitution of amino acid position 43 in nucleocapsid protein from Ala to Thr. Comparing P3 with P20 and P30, there were four amino acid substitutions at positions 200 (Lys → Thr), 772 (Glu → Asn), 1,662 (Gly → Glu) and 2,096 (Asp → Glu) in viral polymerase encoded by the L segment. Moreover, there were several substitutions in the 5' non-coding region of each segment (S, M, and L), and one substitution in the 3' non-coding region of the S segment.

These results indicate that mutations by the passage of AA57 in Vero E6 cells induced high virus multiplicity in primate cells and reduced multiplicity in mice, resulting in lower virulence. This paper provides novel information about hantavirus pathogenicity and host response in hantavirus infection.

Local structural alteration of bovine *α*-spectrin leads to instability of red cell membrane

Momoko Fujimoto

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

Recent studies reported that some Japanese black cattle homozygous for R664X mutation of band 3 (anion exchanger 1, AE1) possessed red cells with extremely fragile membranes and reduced spectrin contents. Moreover, in this band 3 deficiency, substitutions of Glu^{91} to Lys^{91} have been also reported to occur in bovine α-spectrin allele B-K91 (SpαB/K91). These findings suggest that the E91K mutation modulates red cell phenotypes in band 3 deficiency. The purpose of the present study was to elucidate the local structural alteration of α-spectrin by E91K. In this study, recombinant proteins of α-spectrin with E91 and K91 (SpαB[0-1]E91, SpαB[0-1]K91) including N-terminus to segment 1 were created, respectively. This region has only two Trp residues (Trp^{59} and Trp^{131}). Additionally, recombinant proteins substituting Trp^{59} with Phe^{59} (SpαB[0-1]E91_W59F, SpαB[0-1]K91_W59F) and substituted Trp^{131} with Phe^{131} (SpαB[0-1]E91_W131F, SpαB[0-1]K91_W131F) were created. To obtain local structural information around two tryptophan residues of α-spectrin, the fluorescence spectra (Ex; 295 nm, Em; 310–350 nm) derived from tryptophan residues in these recombinant α-spectrins were measured and the susceptibility (Stern-Volmer constant; K_{SV}) of Trp-derived fluorescence against a hydrophilic collisional quencher (acrylamide) was also estimated. K_{SV} of SpαB[0-1]K91 was significantly higher than that of SpαB[0-1]E91. In the replacement of a fluorescent Trp with a non-fluorescent Phe in recombinant α-spectrin, K_{SV} of SpαB[0-1]K91_W59F was significantly higher than that of SpαB[0-1]E91_W59F, although K_{SV} of SpαB[0-1]E91_W131F and SpαB[0-1]K91_W131F were almost equal. These observations indicate that the substitution of Glu^{91} with Lys^{91} leads to a more hydrophilic environment around Trp^{131}, suggesting that this local structural alteration to a more hydrophilic environment around Trp^{131} induces instability of the red cell membrane in band 3 completely deficient cattle.

The effect of downregulation of DNA repair enzyme apurinic/apyrimidinic endonuclease 1 on extracellular matrix-related gene expression and cellular physiology

Yuri Sakai

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

Apurinic/apyrimidinic endonuclease 1 (APE1) is known to have two major functions, DNA repair and redox regulation of transcriptional factors. However, recent discoveries of new functions of APE1 have suggested that APE1 has more as yet unknown functions. Furthermore, it has not been well understood how these functions of APE1 affect cellular physiology as a whole. Recent evidence has indicated that APE1 influences the expression levels of extracellular matrix (ECM)-related genes. Intrigued by this finding, the effect of APE1 downregulation by small interfering RNA (siRNA) on the ECM-related gene expression and cellular physiology was evaluated to reveal a new function and the biological role of APE1.

The endogenous APE1 level was downregulated by siRNA in HeLa cells and A549 cells, and the mRNA levels of ECM-related genes were analyzed. TGF-β1 gene expression was significantly upregulated in HeLa cells and A549 cells. While obvious accumulation of F-actin was observed in HeLa cells by APE1 siRNA, disappearance of actin stress fibers was found in A549 cells by the same treatment. Accumulation of F-actin in HeLa cells after the suppression of APE1 expression decreased when the TGF-β signaling pathway was inhibited.

In summary, siRNA-induced APE1 downregulation promoted TGF-β1 gene expression, followed by reorganization of the F-actin structure. Since the actin cytoskeleton plays important roles in various cellular functions such as cell mitosis, motility and intracellular trafficking, it is suggested that APE1 could regulate these functions via TGF-β1 signaling. The results in this study imply that gene expression control by APE1 could affect various new cellular functions, providing novel insights to help understand the wide-ranging biological roles of APE1.

Influence of Drp1-mediated mitochondrial morphological change after X-irradiation on cellular radioresponses

Tomoya Sasagawa

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

Ionizing radiation induces DNA damage and triggers various responses in cells. Mitochondria have been demonstrated to play important roles in cellular radioresponses through various mechanisms including the production of reactive oxygen species (ROS) and the activation of

apoptosis signaling. It has been shown that mitochondria are dynamic organelles that constantly fuse and divide, and the control of fusion and fission is important for the maintenance of cellular homeostasis. Recent evidence suggests that dynamin-related protein 1 (Drp1), which is a mitochondrial fission protein, is involved in multiple cellular functions such as ROS production and the execution of apoptosis. Although there are several reports showing mitochondrial morphological changes after exposure to ionizing radiation, it has not been fully elucidated whether mitochondrial dynamics plays a role in cellular radioresponses such as alterations in mitochondrial function, cell cycle arrest and radiation-induced cell death. Therefore, this study aimed to address this point.

When the effect of X-irradiation on mitochondrial morphology was evaluated in NIH3T3 cells and in mouse embryonic fibroblasts (MEFs) derived from either wild-type or Drp1-deficient mice, mitochondrial fragmentation was induced in NIH3T3 cells and in Drp1 wild-type MEFs after X-irradiation. This radiation-induced mitochondrial fragmentation was suppressed by the Drp1 inhibitor mdivi-1 and the genetic knockout of Drp1. Furthermore, mitochondrial fragmentation was accompanied by the concomitant increase of Drp1 phosphorylation at Ser616, which is associated with its activation. Mdivi-1 treatment attenuated the increase of mitochondrial mass after irradiation, but mitochondria-derived ROS levels and mitochondria membrane potential were not affected. On the other hand, none was altered by the deletion of Drp1. While radiation-induced G2/M arrest was upregulated by mdivi-1 treatment, genetic inhibition had no effect. Radiation-induced cell death by mitotic catastrophe was suppressed by both mdivi-1 and Drp1 gene deletion. These results suggest that X-irradiation induces mitochondrial fragmentation through Drp1 activation, and Drp1 is involved in radiation-induced mitotic catastrophe.

In conclusion, the present study suggests that mitochondrial morphological change after ionizing radiation influences the determination of the cell fate after irradiation, providing new insights into the regulatory mechanism of cellular radioresponses.

The effect of environmental pollution on terrestrial animals caused by extensive mining activity in Obuasi Municipality, Ghana.

Go Otani

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

Obuasi Municipality in the Republic of Ghana has one of the biggest gold mines in the world. Therefore, there has been concern about the effect of environmental pollution caused by mining activity on the residents, especially arsenic pollution. In chronic arsenic intoxication, cancer and nervous symptoms have been seen as typical diseases. There have been various studies on human health around Obuasi, and the cancer risk is a concern. However, there are limited studies about the effect of metal pollution on terrestrial animals in Obuasi, such as domestic ruminants, chickens and wild rodents. This study aimed to reveal the current situation of environmental metal pollution in Obuasi and the effect of pollution caused by mining activity on

terrestrial animals.

Soil, ground water and food plants were collected in Obuasi as environmental samples, and metal and metalloid concentrations (As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn) were measured. In 67% of soil samples (n = 116), arsenic concentration were much higher than the benchmark value (170 mg/kg, USEPA, ECO-SSL). In 32% of ground water samples (n = 25), including samples from the mining area, the concentrations were higher than the benchmark value (10 μg/l, WHO, Guidelines for drinking water). These results suggest that terrestrial animals in the mining area are at a higher risk of arsenic exposure than those in non-mining areas.

Since edible animal parts are considered an arsenic exposure route in humans, organ and muscle samples were collected from domestic animals (cattle, goats, sheep and chickens). Arsenic concentration in these samples was not higher than that in arsenic-polluted areas in other countries or in non-polluted areas. However, in rats (*Rattus rattus*), which are a sentinel animal for environmental pollution, the arsenic concentration in the mining area in Obuasi was significantly higher than that in non-mining areas in Obuasi. Moreover, the nitrogen stable isotope ratio ($\delta^{15}N$) in the rat liver showed positive correlations with arsenic concentrations in organs. These results suggest that the food habitat of rats in the mining area is different from that of rats in non-mining areas, and the amount of arsenic exposure by rats resulted in a higher concentration in organs.

In summary, 1) in a mining area of Obuasi, the level of arsenic exposure from soil and groundwater was higher than in non-mining areas, and 2) rats in the mining area had a higher risk of suffering from chronic arsenic intoxication than other rodents in Obuasi. Further studies are needed, such as the mobility of arsenic from soil and ground water to animals, species differences in arsenic metabolism and the effect on the expression of tumor suppressor gene.

Enzymological characteristics of cytochrome P450 responsible for diazepam metabolism in horses

Aki Hayami

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

It is necessary to elucidate the drug metabolic system for the safe use for drugs. However, in equine medicine, there are only limited data about drug metabolism. The metabolic reaction catalyzed by cytochrome P450 (CYP) is an important factor in drug pharmacokinetics. This study aimed to elucidate the characteristics of CYP in horses using diazepam as a model compound. Diazepam is in widespread clinical use in horses as an anesthetic and sedative, and is mainly metabolized in the liver by CYPs.

Diazepam metabolic activities were measured using horse and rat liver microsomes to obtain the enzymatic kinetics parameter of each metabolite (tamezepam (TMZ), nordiazepam (NDZ), *p*-hydroxydiazepam (*p*-OH-DZP), oxazepam (OXZ)) and to compare the metabolic capacity of horses with rats. In horse microsomes, the metabolic activity of *p*-OH-DZP was significantly lower than in rats. The metabolic activity of TMZ made up the majority of diazepam metabolism in horse microsomes. From inhibition assays with a

CYP-specific inhibitor and antibodies, it was suggested that CYP3A was the main enzyme responsible for diazepam metabolism in horses. Four major horse CYP3A isoforms were expressed in Cos-7 cells and the diazepam metabolic activity was measured. The major metabolite was TMZ for all expressed CYP3A isoforms. However, there was a significant difference in the ratio of the production of TMZ and NDZ among CYP3As, suggesting differences in the metabolic capability of each isoform. Further study is needed to elucidate the metabolic characteristic of each CYP3A isoform.

Contamination status and accumulation characteristics of metals and a metalloid in wild birds

Chihiro Ishii

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

Metal and metalloid pollution is a serious issue for bird species. It has been reported that marine animals accumulate high concentrations of metals and metalloids, such as mercury (Hg) and cadmium (Cd). Seabirds also accumulated high levels of Hg, Cd and As and this type of poisoning is a concern. At present, concentrations of Hg in arctic marine animals are about 10–12 times higher than those in pre-industrial times, indicating that the Hg accumulated is derived from anthropogenic sources. Lead (Pb) poisoning caused by accidental ingestion of Pb pellets or bullets is also a serious problem for raptors and waterfowl. Thus, many birds are affected by metal pollution caused by human activities.

However, there are few reports on metal pollution and accumulation characteristics among species. The contamination of birds due to metal pollution and their associated accumulation characteristics were therefore investigated. The present study examined the accumulation patterns of eight heavy metals (Hg, Cd, Cr, Co, Ni, Cu, Zn, and Pb) and a metalloid (As) in the liver and kidney of various bird species.

This study showed differences in the accumulation characteristics of metals and a metalloid among seabird species. For instance, Hg was easily accumulated in the northern fulmar (*Fulmarus glacialis*), whereas Cd was accumulated in the tufted puffin (*Fratercula cirrhata*) and As was high in the spectacled guillemot (*Cepphus carbo*). Therefore, exposure to these metals is a great risk to wild birds. It was also found that Hg pollution in sea birds was increased in 2010 compared with 1990. Further studies are needed to clarify species-specific differences in sensitivity to metals and metalloids and to assess toxic effects in seabird species.

Moreover, the results presented in this study clearly show that Pb poisoning in bird species is still a serious problem in Hokkaido. Pb poisoning in birds has also been reported in Honshu; nevertheless, there is only restricted prohibition of using Pb shot in small areas. To clarify the present status of Pb pollution in birds, studies not only in Hokkaido but also in main island in Japan are important. Furthermore, the origin of Pb should be identified by measuring the isotope ratio, which indicates the source of Pb, such as the shot type or sinker, to discuss the prevention of Pb poisoning.

Evaluation of nutritional condition of brown bears (*Ursus arctos*) using kidney fat and femur marrow fat indices

Mariko Mori

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

For the conservation of bears, it is important to elucidate the factors involved in bears' intrusion into human residential areas. Generally, the main factor is thought to be the availability of their main food in autumn, nuts and fruits, and therefore their nutritional condition. However, the nutritional condition index of brown bears (*Ursus arctos*) has not been established. The purpose of this study was to examine the efficiency of kidney fat and femur marrow fat as nutritional indices and to evaluate the annual changes and regional differences of Hokkaido brown bears using these indices.

The kidney fat index (KFI), modified kidney fat index (mKFI) and femur marrow fat (FMF) were measured in the kidneys and femurs of brown bears killed in 2006 to 2012.

KFI and mKFI had a positive correlation. This means that mKFI is a more useful index than KFI. FMF decreased following the decrease of mKFI. This result indicates that kidney fat is consumed earlier than femur marrow fat. mKFI and FMF were low in summer and increased in autumn/winter.

mKFI in 2011 was higher than in any other years, although the number of captured bears was also higher in 2011 than in any other years. This suggests that a poorer nutritional condition does not necessarily lead to more bear intrusions into human residential areas. mKFI in the northeastern region was higher than that in the southern region in summer. This might have been caused by the regional differences of food habits, such as predation of deer in the northeastern region. Unusually, mKFI and FMF in Shiretoko peninsula were lower than in other regions in summer 2012. They were also lower than in any other years. This result suggests that an unusual decrease of the nutritional condition occurred in Shiretoko peninsula in summer 2012. This might have been caused by the ascent delay of pink salmon and the low amount of creeping pine seeds.

Study on gene flow of brown bears (*Ursus arctos*) in Shibetsu town, Eastern Hokkaido

Natsuo Nagano

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

Conflicts between bears (*Ursus arctos*) and people are a concern in Shibetsu town, Eastern Hokkaido. For the appropriate conservation of brown bears, ecological information is necessary. The objective of this study was to reveal the gene flow of brown bears in Shibetsu town by behavioral

tracking, mitochondrial DNA (mtDNA) analysis, and microsatellite DNA (msDNA) analysis.

It was confirmed that males, in which the mtDNA cluster was different from that of females in Shibetsu, were present, and that these individuals (migrants) were from other regions. Behavioral tracking revealed that some migrants moved to Nemruo district, and suggests that migrants came from Nemuro. To confirm this expectation, we compared genetic differentiation between migrants and the Nemuro group, north Akan group, Shiranuka group and residents in Shibetsu town by msDNA analysis. From this result, the degree of genetic differentiation between migrants and residents in Shibetsu town was the smallest, and the degree between migrants and the Nemuro group was the second smallest. By analysis of the genetic structure, it was clarified that one migrant was from north Akan and two migrants were from Nemuro. In addition, kin analysis revealed mating with residents in Shibetsu town and two migrant males. These results indicated that most of the migrants were from Nemuro regions and that there is enough gene flow between migrants and residents in Shibetsu town. Furthermore, since one migrant moved to Shiretoko Peninsula for breeding, Shibetsu town is important not only as a place but also as a pathway for the gene flow of brown bears from different places of origin.

Gene flow is an essential element for the maintenance of genetic diversity. Thus, Shibetsu town is an important region for the conservation and management of brown bears in eastern Hokkaido. Further studies are needed to reveal the detailed gene flow in eastern Hokkaido, including Shibetsu town.

Analysis of prion infection in primary cortical neurons

Ai Fujiwara

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

Prion diseases are fatal neurodegenerative disorders that are characterized by the accumulation of an abnormal isoform of prion protein (PrP^{Sc}), reactive astrogliosis, microglial activation and the vacuolation of neurons and neuropils in the central nervous system. Although multiple events, including ER stress caused by the accumulation of PrP^{Sc} aggregates, activated astrocytes and/or microglia or synaptic and dendritic alterations, have been suggested to be involved in neurodegeneration, its molecular mechanism is not fully understood yet. Neuronal cell lines used for analyses of the cellular mechanism of prion propagation have so far shown little cytopathic effect. Thus, a novel ex vivo experiment system, in which the generation of PrP^{Sc} in neurons and neurodegeneration can be reproduced, is required to elucidate the mechanism of neurodegeneration in prion diseases. This study therefore analyzed prion infection in primary cortical neurons to establish an experimental system for the analysis of neurodegeneration caused by prion infection. Four different prion strains could effectively produce PrP^{Sc} in primary cortical neurons, confirming the prion infection. A slight decrease in cell viability and in the expression of synaptic proteins such as PSD95 and N-cadherin was observed; however, neither obvious neurodegeneration nor apoptosis was observed in primary cortical neurons infected

with prions. Efficient PrP^{Sc} generation in neurons without neuronal cell death suggests that certain causes other than neurons, such as factors produced from activated astrocytes and/or microglia play a critical role in the neurodegeneration caused by prion infection. Analyses of neuron-glia interaction using prion-infected primary cortical neurons co-cultured with astrocytes or microglia may provide a clue to elucidate the neurodegenerative mechanisms of prion diseases.

Immunohistochemical studies for the identification of organelles involved in prion propagation in the brain

Mao Saito

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

Prion diseases are fatal neurodegenerative disorders that are characterized by the vacuolation of neurons and neuropils and the accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system. Propagation and accumulation of PrP^{Sc} play a central event in the pathophysiology of prion diseases; therefore, identification of the intracellular site where conversion of PrP^{C} into PrP^{Sc} occurs is important for clarification of the pathogenesis. However, intracellular localization of PrP^{Sc} in the central nervous system, the target tissue of prions, remains unclear due to the difficulty of detecting PrP^{Sc} specifically. Recently, our laboratory established a method for PrP^{Sc}-specific detection in an immunofluorescence assay using anti-PrP monoclonal antibody 132, which recognizes amino acid 119-127 of mouse PrP. Using the PrP^{Sc}-specific detection method, this study analyzed the localization of PrP^{Sc} in the brains of mice infected with prions, particularly in the early and middle stage of prion infection, in order to identify the intracellular organelle where PrP^{Sc} is generated. Double immunofluorescence staining with mAb132 and intracellular organelle markers emphasized the presence of PrP^{Sc} co-localized with lysosomal-associated membrane protein 1 (LAMP1) in the early stage of infection (45 days post-inoculation [dpi]) at which PrP^{Sc} signals became detectable. Nearly half of PrP^{Sc} co-localized with LAMP1 was co-localized with Rab9a or cation-independent mannose-6-phosphate receptor (M6PR), suggesting the presence of PrP^{Sc} co-localized with LAMP1 in late endosomes. Multiple immunofluorescence staining with mAb132, LAMP1 and cell marker molecules revealed the presence of PrP^{Sc} both in microtubule-associated protein 2 (MAP2)-positive neurons and glial fibrillary acidic protein (GFAP)-positive astrocytes, and furthermore, the co-localization of PrP^{Sc} with LAMP1 in those cells. These results suggest that PrP^{Sc} is generated in LAMP1-positive late endosomes of neurons and astrocytes in the early stage of prion infection and therefore imply that LAMP1-positive late endosomes could be one of the sites where the conversion of PrP^{C} to PrP^{Sc} takes place.