Japanese Journal of Veterinary Research 57(1):37–69, 2009

INFORMATION

Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 40 graduates of the School of Veterinary Medicine on March 25, 2009. The authors summaries of their theses are as follows:

Analysis of caspases associated with skeletal myogenesis in mouse embryo

Teppei Ikeda

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

Caspases are a family of cysteine proteases that are known to regulate apoptotic signaling. Apoptosis by activation of caspases is strongly associated with embryonal development and regeneration in many organs, therefore indicating that a disorder caused by homozygous mutation in caspase genes gives rise to embryonic lethality. Recently, it has been reported that the members of caspase family are activated during skeletal myogenesis, one of their functions in developmental biology. In the present study, the author investigated the relationship between skeletal myogenesis and caspase activation by analyzing their dynamics in the expression of caspases during mouse embryogenesis.

Individual fresh myogenetic tissues and their total RNAs were obtained from C57BL/6 mouse embryos aged 12.5–17.5 prenatal days, and the expression of caspases was analyzed by histochemical and molecular biological methods. Immunoreaction for caspase-3, -9 and -12 was first detected on myoblasts increasing according to embryonal development, in which myoblasts differentiate into myotube cells. Western blot analysis of caspase-3 confirmed that the active form was expressed in embryonal tongue tissues. On the other hand, the immunoreaction for ssDNA, well-known as an apoptosis marker, was little detected throughout skeletal myogenesis. Quantification analysis for caspase mRNAs expression by RT-PCR as well as by *in situ* hybridization showed the highest peak at 15.5 but not 17.5 prenatal days. Similar dynamics was detected on *Myod1* mRNA, a muscle regulatory factor, but not on *Fasl*, *Bax* and *Rock1*, apoptosis-associated factors, during skeletal myogenesis.

These results suggest that the activation of caspases in skeletal myogenesis is closely associated with myoblast differentiation, but is not directly related to apoptosis.

MRL/MpJ mice, a model for hydrocephalus — Analysis of strain differences to establish their suitability —

Keisuke Hino

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

Hydrocephalus is an intractable disease characterized by the excessive accumulation of cerebrospinal fluid (CSF) in the cerebral ventricle, mainly caused by stenosis of the cerebral aqueduct; however, in many cases the causes can not be determined clearly, so the mechanism of hydrocephalus is not yet well understood. In this study, differences in the cerebral ventricles in 5 inbred mice (MRL/MpJ, C57BL/6, C3H/He, DBA/2 and BALB/c) were analyzed by histological techniques to investigate their dilation mechanism and the potential for establishing a new mammalian model of hydrocephalus.

There were significant differences in the volume and surface area of lateral ventricles in 5 inbred mice, showing that MRL/MpJ mice had the largest lateral ventricles. Moreover, it was clear that MRL/MpJ mice had the largest third, cerebral aqueduct and fourth ventricles among the 5 inbred mice. In addition, comparing MRL/MpJ mice to BALB/c mice 0 day after birth, MRL/MpJ mice had larger lateral ventricles than BALB/c mice. Ependymal cells, constructing the lateral wall of lateral ventricles, were ultrastructurally classified into 6 types. Although there was not significant difference in cell ratio between MRL/MpJ mice and BALB/c mice, the number and diameter of lipid droplets in MRL/MpJ mice were interestingly lower than in BALB/c mice. It is well known that ependymal cells absorb nutritional substances in the cerebrospinal fluid by endocytosis, suggesting that their depression is the cause of dilation in MRL/MpJ cerebral ventricles.

In conclusion, it is suggested that MRL/MpJ mice had the most dilated cerebral ventricles of the examined strains, indicating their suitability as a potential model of mild hydrocephalus, but without severe clinical signs. Further investigation would be expected to provide a new model of hydrocephalus by examining its mechanism and candidate factors.

The Role of G protein-coupled estrogen receptor GPR30 in the regulation of breast cancer cell proliferation

Kenji Suzuki

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

Estrogen plays pivotal roles in the development and progression of breast cancer. Until recently, estrogen was thought to transmit its signal through intracellular estrogen receptors (ERs), nuclear receptors which function as a transcription factor for target genes. However, it has been revealed that estrogen also transduces its signal via GPR30, a cAMP-producing G protein-

coupled receptor.

In this study, to clarify the role of GPR30 signaling in the regulation of breast cancer cell growth, I examined the effects of the selective GPR30 agonist G-1 and membrane-impermeable 17β-estradiol (E_2)-conjugated BSA (E_2 BSA) on the cell proliferation in two human breast cancer cells, MCF-7 and MDA-MB-231, and in four primary cells derived from canine mammary tumors, all of which express GPR30 mRNA.

Treatment of MDA-MB-231 cells with G-1 dose-dependently inhibited the cell proliferation, and G-1 at 1 μM decreased the number of cells to 30～40% of the control. Treatment with E_2, E_2 BSA, or Forskolin that activates cAMP production and mimics GPR30 signaling, also induced the inhibition only by 20%. Treatment with siRNA for GPR30 abolished its expression and reduced significantly G-1-induced inhibition by 60% to 50%. These results suggest that GPR30 signaling is negatively involved in MDA-MB-231 cell proliferation, but G-1 also induces growth retardation through GPR30-independent pathway.

Treatment of MCF-7 cells with G-1 dose-dependently inhibited the cell proliferation by 20%. In contrast, treatment with E_2, E_2 BSA, or Forskolin enhanced the cell proliferation. The results indicate that GPR30 signaling is positively involved in MCF-7 cell proliferation, and confirm that G-1 induces growth inhibition in a GPR30-independent manner.

Next, I revealed partial nucleotide sequence of canine GPR30 cDNA, indicating that the primary structures of GPR30 genes are highly conserved among human, mouse, rat and dog. Treatment of cells derived from four canine mammary tumors with G-1 inhibited the cell proliferation.

These results, together with the above results, suggest that GPR30 transmit estrogen signal in a cell-type dependent manner, and that G-1 or its derivatives could be used for breast cancer therapeutics in future, as G-1 has GPR30-independent pharmacological action.

Neurotrophin-3 and-4 induce proliferation of the stromal vascular cells from rat white adipose tissue in an autocrine/paracrine manner

Yoshifumi Murakita

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

Neurotrophins are defined as survival factors for neurons, and control neuronal development and function in sensory and sympathetic nervous systems. In addition, neurotrophins are shown to be present in non-neuronal tissues and play roles in a variety of cell functions. However, their roles in the adipose tissue remain obscure. Therefore, in the present study, I first examined mRNA expression of four neurotrophin family proteins and their corresponding receptors in the brown adipose tissue and four different depots of white adipose tissues isolated from Wistar rats. The mRNA for nerve growth factor (NGF), neurotrophin-3 (NT-3), and NT-4 were richly found in all the adipose tissues tested, although mRNA for brain-derived neurotrophic factor (BDNF) was not. Four gene transcripts for high and low affinity neurotrophin receptors were also detected in both the brown and white adipose tissue. In addition, transcripts of three neurotro-

phins except BDNF and four neurotrophin receptors were present in both mature adipocytes and stromal vascular cells (SV cells) separated from inguinal white adipose tissue. These results suggest that neurotrophins produced by adipocytes and SV cells act on themselves and/or adjacent cells.

To test this hypothesis, I next examined the effect of neutralizing antibodies for neurotrophins on the proliferation of SV cells in culture, which were isolated from inguinal subcutaneous and retroperitoneal intra-peritoneal adipose tissues. Addition of either anti-NT-3 antibody or anti-NT-4 antibody significantly decreased the proliferation of SV cells from two depots, while addition of anti-NGF antibody did not. Simultaneous addition of anti-NT-3 and NT-4 antibodies additively decreased the proliferation, and inclusion of recombinant NT-3 or NT-4 with the respective antibodies abolished the decrease. These results strongly indicate that NT-3 and NT-4 are produced by SV cells and act independently as the autocrine/paracrine factors to induce cell proliferation.

Matricellular protein SPARC interacts with neurotrophins, and potentiates the bioactivity of nerve growth factor

Takeshi Imao

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

Secreted protein, acidic and rich in cysteine (SPARC) is a Ca^{2+}-binding glycoprotein that is secreted and deposited mainly into matricellular region. SPARC is known to modulate cell-cell and cell-matrix interactions, possibly by binding a variety of cell adhesion molecules and growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). As a consequence, SPARC is suggested to regulate formation, remodeling, and regeneration of various tissues spatiotemporally. In the present study, to elucidate novel pathophysiological roles of SPARC, I examined the possible interaction between SPARC and neurotrophins (NTs), because NTs belong to β-cysteine knot cytokine family that also includes PDGF or VEGF.

First, to clarify whether SPARC was co-localized with NTs such as nerve growth factor (NGF), NT-3, and NT-4 in neuronal and non-neuronal tissues, these mRNA expressions were investigated in various rat tissues. SPARC transcript was ubiquitously detected in all tissues examined. In contrast, mRNA expression of each NT was tissue-dependent: NGF mRNA was highly expressed in the heart and kidney, while NT-3 and NT-4 transcripts were richly detected in the heart, kidney, and stomach, and in the lung, kidney, stomach, and testis, respectively.

Next, possible interactions between SPARC and NGF, NT-3, or NT-4 were determined by surface plasmon resonance method. NGF interacted with immobilized SPARC whose the dissociation constant (K_D) was ~100 nM, irrespective of the presence or the absence of Ca^{2+} in the running buffer. NT-3 interacted with SPARC whose K_D either in the presence or absence of Ca^{2+}, were 2.12 nM and 46.7 nM respectively. Similarly, NT-4 bound to SPARC with KD of 52.9 nM and 2.97 μM either in the presence or absence of Ca^{2+}. These results indicate that SPARC directly interacts with NGF, and NT-3 and NT-4 in a Ca^{2+}-independent and -dependent manners.

Finally, I examined whether SPARC could

influence NT functions. Treatment of PC12 cells with NGF, but not with SPARC induced phosphorylation of p44/p42 mitogen-activated protein kinase (ERK 1/2), an indicator of NT receptor signaling, and neurite outgrowth, a marker for NGF function. However, simultaneous treatment with NGF and SPARC enhanced both the phosphorylation and the neurite outgrowth, indicating that SPARC interacts with NGF and potentiates its bioactivity.

Although the role of SPARC and NT-3 or NT-4 interaction has not determined, present results suggest that SPARC interacts with NTs, thereby modulating pathophysiological function of NTs.

Effects of endogenous 5-hydroxytryptamine on monosynaptic reflex and nociceptive reflex in isolated spinal cord of neonatal rat *in vitro*

Takeyuki Iwasaki

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

In order to investigate the mechanisms of modulation of synaptic transmission by endogenous 5-hydroxytryptamine (5-HT), I studied the effect of DL-p-chloroamphetamine (pCA) on monosynaptic reflex potentials (MSR) and slow ventral root potentials (sVRP) in isolated spinal cord of the neonatal rat *in vitro*. I also examined the effect of pCA on the release of 5-HT and amino acids from the spinal cord with high performance liquid chromatography (HPLC).

pCA (3～30 μM) increased 5-HT release from the spinal cord in a concentration-dependent manner. pCA (1～10 μM) also caused a concentration-dependent depression of MSR and sVRP, which was mimicked by exogenously applied 5-HT (3 nM～10 μM). The pCA-induced release of 5-HT was reduced by a 5-HT_3 receptor antagonist, tropisetron, and was prolonged by a 5-$HT_{1B/1D}$ receptor antagonist, GR 127935. On the other hand, a 5-HT_3 receptor agonist, SR 57227 (30～100 μM), increased 5-HT release from the spinal cord in a concentration-dependent manner. The pCA-induced depression of MSR was attenuated by tropisetron and was abolished by a 5-HT_{2A} receptor antagonist, ketanserin, but was prolonged by GR 127935. The pCA-induced depression of sVRP was also attenuated by tropisetron and ketanserin but not affected by GR 127935. Ketanserin attenuated the 5-HT-induced depression of MSR and sVRP. A 5-HT_{2A} receptor agonist, α-methylserotonin (0.1～10 μM), depressed MSR and sVRP in a concentration-dependent manner.

Respective $GABA_A$ and glycine receptor antagonists, bicucullin and strychinine, significantly potentiated sVRP without any effects on MSR. Futhermore bicucullin or strychinine markedly attenuated the pCA-induced depression of sVRP, but did not affect that of MSR. pCA (30 μM) significantly increased GABA release and also tended to increase glycine release from the spinal cord, which was blocked by ketanserin.

These results indicate that endogenously released 5-HT depresses MSR and sVRP via 5-HT_{2A} receptors in isolated spinal cord of neonatal rat. It is suggested that the depression of sVRP but not MSR is mediated by GABA and glycine, which are released by the activation of 5-HT_{2A} receptors. It is likely that endogenous

5-HT increases 5-HT release via 5-HT_3 receptor activations, resulting in the potentiation of the 5-HT-induced depression of MSR and sVRP. Moreover, the increase of extracellular 5-HT concentration activates 5-$HT_{1B/1D}$ receptors, which reduces the release of 5-HT.

Potentiation of transient receptor potential V1 (TRPV1)-mediated responses to protons by histamine in mouse primary sensory neurons

Yuusuke Kajihara

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

In inflamed tissues, extracellular pH decreases to around 5 and acidosis is an important source of pain. Histamine is released from mast cells under inflammatory conditions. It is known that histamine evokes not only itch but also pain sensation, but the cellular mechanism of histamine-induced pain has not been well understood. In the present study, I examined the effects of histamine on responses to protons through TRPV1 in mouse dorsal root ganglion (DRG) neurons.

In many of capsaicin-sensitive DRG neurons, protons evoked $[Ca^{2+}]_i$ increases in a dose-dependent manner. $[Ca^{2+}]_i$ responses to histamine are observed in a subset of neurons, most of which are sensitive to capsaicin. Histamine potentiated $[Ca^{2+}]_i$ responses to protons ($<$ pH 6.0) which were mediated through TRPV1 activation. The $[Ca^{2+}]_i$ responses in DRG neurons were potentiated regardless of their size or IB4-staining. Proton-induced membrane depolarization was also enhanced by histamine. RT-PCR indicated the expression of histamine H1, 2, 3 and 4 receptor subtypes in mouse DRG. H1 but not H2-4 receptor agonists mimicked the potentiating effects of histamine and only H1 receptor antagonist inhibited the potentiating effects of histamine. The potentiating effects of histamine were not affected by the inhibition of lypoxgenase. Histamine failed to potentiate $[Ca^{2+}]_i$ responses to protons in the presence of inhibitors for PLC and PKC. The inflammation evoked by the injection of carrageenan or complete Freund's adjuvant into the hemirateral hind paws decreased the paw withdrawal latency to thermal stimuli. But it didn't affect the potentiating effects of histamine on $[Ca^{2+}]_i$ responses to protons in the DRG neurons of L4-L6 segment.

These results suggest that histamine sensitizes TRPV1 via H1 receptor-PKC pathway, the action of which may be involved in the generation of inflammatory pain.

Mechanism of hypoxia-induced purine release from isolated spinal cord of neonatal rat

Takumi Takahashi

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

In order to reveal the mechanism of extracellular purine accumulation during hypoxia in the CNS, I examined the effect of hypoxia on purine release from the isolated spinal cord of neonatal rats *in vitro*.

The spinal cord preparations were incubated at 35°C in artificial cerebrospinal fluid (ACSF) gassed with 5% CO_2+95% O_2 (normal ACSF), which was followed to ACSF gassed with 5% CO_2+95% N_2 (hypoxic ACSF). The amount of purine released in ACSF was measured by high performance liquid chromatography or ELISA assay. The hypoxic ACSF increased the release of adenosine and inosine, but not AMP, ADP, ATP and cAMP. At 25°C, the adenosine level was lower than that at 35°C, and hypoxia failed to induce purine release. Removal of extracellular Ca^{2+} did not affect the release of adenosine and inosine induced by hypoxia. On the other hand, incleasing concentrations from1.25 mM to 2.5 mM inhibited hypoxia-induced purine release.

Pretreatment of EHNA, an adenosine deaminase inhibitor, increased adenosine level but decreased that of inosine. Hypoxia-induced adenosine release was potentiated by EHNA, while hypoxic inosine release was disappeared. An ecto-ATPase inhibitor, ARL67156, a phosphodiesterase (PDE) 4 inhibitor, rolipram, ecto-PDE inhibitors, DPSPX and cGMP, did not affect purine release induced by hypoxia. The mixture of equilibrative nucleoside transporter (ENT) inhibitors, NBTI and dipyridamole, decreased hypoxia-induced inosine release, but not adenosine release. A multidrug resistance protein 4 inhibitor, sulfinpyrazone, a gap junction hemichannel inhibitor, carbenoxolone, a $P2X_7$ receptor inhibitor, brilliant blue G and a maxi-anion channel inhibitor, arachidonic acid, did not affect purine release induced by hypoxia. A glia-specific metabolic inhibitor, fluoroacetic acid, abolished the purine release by hypoxia.

These results indicate that hypoxia releases adenosine *per se* from the isolated spinal cord of neonatal rats. Inosine is produced from intracellular adenosine by adenosine deaminase and released to extracellular space via ENT during hypoxia. Glial cells are suggested to play a key role in hypoxia-induced purine release.

Antigenic and genetic analyses of H3N8 influenza viruses isolated from horses in Japan and Mongolia

Shingo Asakura

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

In August 2007, outbreaks of influenza occurred among horses in Japan, and also in Mongolia

in October 2007. The present study analyzed the causative strains of these outbreaks of equine influenza genetically and antigenetically.

Nasal swabs were collected from horses showing clinical signs in Ishikawa and Hokkaido Prefecture in Japan and in Tuv and Arkhangai Prefecture in Mongolia. The HA genes of influenza viruses detected from nasal swabs were genetically analyzed. Phylogenetic analysis of the H3 HA genes showed that epidemic strains in Japan and Mongolia were classified into the sublineage Florida of American lineage, but were of a different group, although they belonged to the same sublineage, and the homology of the HA genes of the viruses isolated in each country was high.

Cross-hemagglutinin inhibition tests of H3 equine influenza viruses with chicken antisera revealed that the isolates from Japan and Mongolia, and vaccine strains were antigenically closely related. A panel of monoclonal antibodies recognizing 4 different epitopes on the hemagglutinin molecule of A/equine/Kanazawa/1/2007 (H3N8) was established and used for antigenic analysis. Vaccine strains reacted with 3 of 4 monoclonal antibodies. Reactivity of the HA of the isolate in Japan with the panel of monoclonal antibodies was similar to that of the isolate in Mongolia.

The present results indicate an antigenically close relationship between isolates in Japan and Mongolia, although slight antigenic drift was found between the vaccine strains and isolates in Japan and Mongolia.

Molecular analyses of pathogenicity of influenza A virus to ducks

Masahiro Kajihara

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

Ducks are a natural host of the influenza A virus, and basically do not show any clinical signs when infected with avian influenza viruses (AIVs); however, some H5N1 highly pathogenic AIVs isolated after 2002 are lethal to ducks. To reveal the molecular basis of the pathogenicity of AIVs to ducks, A/Hong Kong/483/1997 (H5N1) [HK] low pathogenic and A/whooper swan/Mongolia/3/2005 (H5N1) [Mon] highly pathogenic to ducks, and genetic reassortant viruses generated between HK and Mon by reverse genetics (RG) were inoculated intranasally into ducks.

In experimental infection studies of RG HK and RG Mon to 1-day, 2-week and 4-week old ducks, their clinical signs and virus titers in the brain indicated that 2-week-old ducks are appropriate for this analysis. By inoculation of reassortant viruses into 2-week-old ducks, it was revealed that 5 gene segments, PB2, PA, HA, NP and NS, are responsible for the pathogenicity to ducks. The present results showed that the pathogenicity of influenza A virus to ducks correlated with extensive replication in multiple organs, including the brain. The mechanism of how the proteins translated from these 5 genes contribute to the pathogenicity to ducks is currently being studied.

Expression kinetics of interferon promoter stimulator-1 (IPS-1) inducing innate immune response in cells infected with classical swine fever virus

Takushi Nomura

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

The expression of interferon regulatory factor-3 is suppressed by the non-structural protein Npro expressed in cloned porcine kidney (CPK) cells infected with virulent classical swine fever virus (CSFV) strain ALD/A76 via the proteasome pathway, thereby controlling the production of interferon (IFN)-β that stimulates innate immunity. On the other hand, it has been reported that non-structural viral proteins NS3/4A produced in cells infected with hepatitis C virus of Flaviviridae, cleave and inactivate interferon promoter stimulator-1 (IPS-1), a mitochondria-associated protein, thereby down-regulating type-1 IFN production. The aim of the present study was to determine the effect of CSFV infection on the expression kinetics of swine IPS-1 (swIPS-1), and to assess the possibility that CSFV controls type-1 IFN-mediated innate immunity via swIPS-1.

Two monoclonal antibodies (Mabs) to swIPS-1 were produced from mice immunized with purified recombinant swIPS-1 expressed in E. coli. The sub-cellular localization and expression level of swIPS-1 in CPK cells infected with CSFVs were determined by the immunofluorescence method and Western blotting analysis using MAbs 47/A3 and 00/1. The swIPS-1 was detected on the mitochondria of both cells infected or not infected with CSFVs and no significant difference was found in the sub-cellular localization of swIPS-1. No significant change was detected in the expression level and molecular weight of swIPS-1 in cells infected with CSFV/ALD/A76, CSFV/GPE-or not infected. From these findings, it is concluded that CSFV infection does not directly suppress the type-1 IFN production pathway via swIPS-1.

Although the expression level of the full length of swIPS-1 was low in cells infected with Newcastle disease virus (NDV) used as a control, the expression of low molecular size swIPS-1 was found. The present findings suggest that NDV may control type-1 IFN-mediated innate immunity via swIPS-1.

Studies on the molecular mechanisms for Marek's disease virus to increase its virulence in the fields

Tomoyuki Hashiguchi

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

Marek's disease virus (MDV) causes T cell lymphoma in chicken. Marek's disease (MD) has

been controlled by the administration of live vaccines of nonpathogenic strains of MDV. Currently, MDV strains are classified into four groups, mild, virulent, very virulent and very virulent plus (vv+) MDV, based on their virulence. Recently, increase in the virulence of several MDV field isolates and several MD cases even in vaccinated chickens have been reported, suggesting the risk of future outbreak in chicken and wild bird. For these reasons, it will be necessary to understand the mechanism of lymphomagenesis by MDV, and subsequently to develop new vaccines to control virulent MDV in the field. In this study, viral genes of MDV isolated from domestic chickens in several areas of Japan were sequenced, and their nucleotide sequences were compared with those of several MDV strains isolated in the United States. Moreover, the effect of the point mutations in the MDV *Eco*RI Q (Meq) protein, a candidate oncoprotein of MDV, on its functions were analyzed.

Though no notable nucleotide polymorphisms were identified in the *UL49* and *MDV074* genes, amino acid substitutions were found in Meq of the MDV strains isolated in Japan, and these substitutions were identical to those reported in highly virulent MDV strains isolated in the U.S. Thus, the polymorphisms found in Meq coincided with the differences in virulence among MDV strains, showing that Meq is the most important determinant of the MDV virulence.

Since Meq shares significant homology with the Jun/Fos family, and is known as a transcriptional factor, the effects of amino acid substitutions in Meq on its transactivation potentials were analyzed. Various degrees of transactivation were observed depending on the amino acid substitutions introduced into Meq, and especially, the substitutions at positions 77 and 80 in the Basic Region of Meq markedly influenced its transactivation potential. Then, in the next experiment, transforming potentials of the Meq mutants in which these amino acid substitutions were introduced were evaluated based on their anchorage-independent cell growth on the transformation system using chicken fibroblast cell line, DF-1. Significant differences were observed in the numbers and sizes of the formed colonies among various Meq mutants depending on the amino acid substitutions introduced into these mutants, showing that polymorphisms in amino acid substitutions in Meq could alter its transforming potential as well as transactivation potentials.

This study showed that polymophisms in Meq could be one of the determinants for the virulence of MDV. In addition, deduced amino acid sequences of Meq detected from some MDV field isolates in Japan were similar to those of highly virulent MDV strains previously reported, indicating that highly virulent MDV strains spread to poultry in Japan. This study also raise the possibility that highly virulent MDV strains could emerge in the future, such as MDV carrying Meq with the combination of lysine and tyrosine residues at positions 77 and 80, respectively, which will be more virulent than vv+MDV. Thus, not only exact roles of Meq and other viral factors such as viral telomerase RNA on the transformation process by MDV must be clarified, but also further surveillance and characterization of MDV field isolates is required in chickens and wild birds.

Identification and functional analysis of tick immunosuppressant, Salp15, in *Ixodes persulcatus*

Akina Mori

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

Ixodid ticks transmit several pathogens that cause diseases important for both livestock industry and human public health. Recent studies are focused on the development of anti-tick vaccine that inhibits blood feeding of tick. There have been several reports on the identification and characterization of tick molecules as effective targets for anti-tick vaccine.

Salp15, a 15-kDa tick salivary gland protein, has been known for its several suppressive activities against host immunity, and critical functions for the transmission of Lyme borrelia in *Ixodes scapularis* and *I. ricinus*, the major vectors for Lyme borreliosis in North America and Western Europe. Salp15 inhibits the activation of $CD4^+$ T cells through the repression of both TCR triggered calcium fluxes and IL-2 production. Furthermore, Salp15 adheres the spirochaeta and specifically interacts with its outer surface protein C. The binding of Salp15 with *Borrelia burgdorferi* protect it from antibody-mediated killing *in vitro*. Thus, the aim of this study is to identify the Salp15 genes in *I. persulcatus*, which is the specific vector for human Lyme borreliosis in Japan.

Two cDNA clones encoding the Salp15-like sequence were obtained from salivary glands of fed female ticks, and termed as *Salp15 Iper-1* and *-2*. These genes encoded 135-and 132-amino acid proteins, designated Salp15 Iper-1 and Salp15 Iper-2, which have signal peptide sequences, and were predicted as secretory proteins. Salp15 Iper-1 and-2 showed 51.8% to 68.2% homology to *I. scapularis* Salp15, respectively. The C-terminal part, which is known for the binding domain to the CD4 molecule in *I. scapularis* Salp15, was well conserved in both *Salp15 Iper-1* and *-2*. RT-PCR analysis showed that *Salp15 Iper-1 and-2* were expressed specifically in the salivary glands through life cycle stages of the ticks, and its expression was up-regulated by blood feeding. However, recombinant Salp15 Iper-2 produced in *Escherichia coli* did not inhibit mouse splenocytes proliferation stimulated with anti-CD3/CD28 antibodies.

In the future, it will be necessary to analyze immunosuppressive functions of Salp15 Iper-1 and-2 and their interaction with *Borrelia* sp.

Identification of blood meal sources of Phlebotomine sandflies in Pakistan

Keisuke Aoshima

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

Cutaneous leishmaniasis caused by *Leishmania major* is now endemic in the southern dry

areas of Pakistan. To elucidate the mechanisms of disease transmission, identification of the blood-meals and host preference of sandflies is useful to determine the reservoir animals in these areas. Phlebotomine sandfly in the Old World includes two major genera: the genus *Phlebotomus*, which is known to take blood from warm-blooded animals and act as a vector of leishmaniasis, and the genus *Sergentomyia*, which is believed to take blood from cold-blooded animals and to not transmit leishmanial parasites. In this study, the sources of blood-meals of both sandfly genera in endemic areas were examined.

From May 28th to June 2nd, 2007, sandflies were collected using CDC or Shannon light traps in four villages of Sindh province in southern Pakistan. DNA was individually extracted from 42 blood-sucked female sandflies and subjected to whole genome amplification using a multiple displacement amplification system. PCR amplification and sequencing of the 18S ribosomal RNA genes were used for identification of sandfly species. To determine the blood sources of sandflies, PCR amplification, subcloning and sequencing of the vertebrate mitochondrial cytochrome b (cyt b) genes were performed. The sequence data of cyt b genes were analyzed by BLAST program and validated by comparison with those from domesticated animals, wild rodents and lizards collected in the study areas.

Among 42 samples, both sandfly species and blood-source animals were determined for 28. The sandflies included two *Phlebotomus* species, *P. alexandri* (n = 1) and *P. papatasi* (n = 1), and four *Sergentomyia* species, *S. clydei* (n = 4), *S. dentate* (n = 1), *S. dubia* (n = 10) and *S. ghesquierei* (n = 11). From *P. alexandri*, cyt b sequences of *Homo*, *Bos*, *Capra* and *Canis* species were detected. From *P. papatasi*, DNA sequences of *Bos* and *Rattus* species were found. Interestingly, *S. clydei*, *S. dubia* and *S. ghesquierei* were also demonstrated to take blood from humans and animals such as *Bos*, *Bubalus*, *Capra*, *Ovis*, *Equus*, *Canis*, *Rattus* or *Tatera* species residing in the study areas. Most female sandflies of these species were shown to take blood-meals from more than one animal species, indicating multiple feeding characteristics. *Sergentomyia dubia* contained the blood of *Hemidactylus* species of Reptilia. *Sergentomyia dentata* contained only the blood of an unknown reptilian species. These results suggest that dogs and wild rodents can be reservoir animals for leishmaniasis transmitted by *Phlebotomus* sandflies in southern Pakistan. Further investigations are needed to elucidate the role of *Sergentomyia* sandflies as vectors for not only leishmaniasis but also other blood-transmitted diseases, including sandfly fever.

Genotype of *Echinococcus granulosus* in cattle and prevalence of intestinal parasites in dogs in Zambia

Seita Nakamura

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

Echinococcus granulosus is a major public health concern, since its larval stage causes the life-threatening zoonotic disease cyctic hydatidosis in many parts of the world. The parasite comprises a series of genotypes or strains which differ in biological characters, including intermediate host range. Though it is commonly believed that genotype G1 (sheep strain) exists in Zambia, there has been no study on the genotypes of *E. granulosus* in Zambia. The aim of the

present study was to identify the genotypes of *E. granulosus* in cattle, known as the most important intermediate host in Zambia, and to survey the prevalence of intestinal parasites in dogs, known as the definitive host.

In 2006, a hydatid cyst was collected from the lung of a cattle in Kafue, Lusaka, Zambia. In 2007, 15 hydatid cysts were collected from the lungs of 10 cattle in Mongu and Senanga, Western, Zambia. Nucleotide sequencing of the mitochondrial cytochrome *c* oxidase subunit I (COI), NADH dehydrogenase subunit 1 (ND1) and 12S ribosomal RNA (12S rRNA) genes was used to identify their genotypes. DNA sequences of COI and ND1 revealed that all 16 cysts were genotype G5 (*Echinococcus ortleppi*). Genotype G1 was not found. In DNA sequences of 12S rRNA, heteroplasmy of mitochondria was observed. G5 and a 'variation sequence', which is not reported in GenBank, were mixed in a cyst and a protoscolex.

In 2005, 2006 and 2007, rectal feces were collected from 726 domestic dogs in 4 districts, Lusaka or Eastern, Zambia. These samples were examined by several methods. The sugar flotation method showed that the prevalence of taeniid eggs was 11.4%. Other zoonotic helminthes, *Ancylostoma* spp., *Toxocara canis* and *Dipylidium caninum* were found in dogs. Multiplex PCR for taeniid identification revealed that all samples of taeniid eggs were *Taenia* spp., and their COI genes sequences showed that most were *Taenia hydatigena* and others were *Taenia multiceps*. Although sandwich ELISA for the coproantigen was positive for *Echinococcus* in 54 samples (7.4%), there was no concrete evidence that dogs are infected with *E. granulosus* in Zambia. This is the first record of ths presence of *E. granulosus* G5 in Zambia. The parasite was found in cattle but not in dogs.

Antioxidant systems and their related genes of larval *Echinococcus multilocularis*

Mio Hiramatsu

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

It is widely thought that parasites are exposed to reactive oxygen species (ROS) generated by the host immune response. Parasites have therefore developed a set of defense systems to mitigate ROS attack. This study aimed to determine the antioxidant activity of protoscoleces of *Echinococcus multilocularis*, to search for antioxidant-related genes in the cDNA library of larval *E. multilocularis* and to produce a recombinant protein for these genes.

The first experiment was carried out to determine the antioxidant activity of protoscoleces (PSC) of *E. multilocularis*. PSC were cultured with hydrogen peroxide (H_2O_2) of various concentrations at different time-points. The viability of PSC was determined by the trypan blue exclusion test. At the low concentrations (final concentration of $H_2O_2 \leqq 200$ μM), PSC were not killed, whereas high concentrations (final concentration of $H_2O_2 \geqq 500$ μM) were found to be toxic. The second experiment was carried out to determine the H_2O_2 consumption of PSC. The level of H_2O_2 in medium decreased with short incubation, suggesting that PSC metabolized H_2O_2. According to these experiments, it is assumed that PSC of *E. multilocularis* has an antioxidant system.

The antioxidant molecules, including thioredoxin (TRx), thioredoxin peroxidase (TPx) and

glutaredoxin (Grx), were predicted in the larval *E. multilocularis* cDNA library. The full-length cDNA sequences of two types of TRx (*Em*TRx-1 and *Em*TRx-2) and TPx (*Em*TPx-1 and *Em*TPx-2), and one type of *Em*Grx were predicted by *in silico* analysis. *Em*TRx-1 was a 11.6 kDa protein with a Cys-Gly-Pro-Cys active site; however, *Em*TRx-2, a larger protein, 18.2 kDa with a Cys-Asn-Pro-Cys active site, was identified. *Em*TPx-1 was a 21.5 kDa protein and the larger *Em*TPx-2 was 25.1 kDa. Both *Em*TPx-1 and *Em*TPx-2 contained conserved 2-Cys residues that were essential for enzymatic activities. *Em*TRx-1 and *Em*TPx-1 were predicted to be located in cytosol; on the other hand, *Em*TRx-2 and *Em*TPx-2 were assumed to be located in mitochondria. The deduced amino acid sequences of each *Em*TRx and *Em*TPx were highly identical to each orthologue of *E. granulosus*, which were described previously. *Em*Grx was predicted as a mitochondrial monothiol-single domain Grx5, 21.2-kDa protein with a Cys-Gly-Phe-Ser active site. Expression of *Em*TRx-1 and *Em*TPx-1 recombinant proteins was attempted and is an on-going process.

In conclusion, a positive association between the viable PSC of *E. multilocularis* and their antioxidant enzymes was discovered that involved a new type of thioredoxin, thioredoxin peroxidase and glutaredoxin.

Analysis of chromosomal region including genetic locus responsible for X-ray hypersensitivity 1 (*xhs1*) in the LEC rat

Keisuke Shimada

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 canine pancreas with the second generation contrast agent sonazoid

Kazuya Kato

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

The aim of this study was to assess the perfusion pattern and perfusion dynamics in the normal canine pancreas using contrast harmonic imaging, and to evaluate the usefulness of contrast-enhanced ultrasound (CEUS) in the diagnosis of canine pancreatic diseases.

Seven normal dogs without clinical or ultrasonographic evidence of pancreatic disease were injected with an intravenous bolus of Sonazoid. The pancreas was characterized by marked parenchymal enhancement following the pancreatic artery in all dogs. The enhancement of the pancreas was earlier and shorter than that of the liver.

Next, CEUS of experimental models of edematous and necrotizing pancreatitis were investigated. The pancreatic parenchyma was enhanced homogeneously in edematous pan-

creatitis, while a region with no enhancement was seen in necrotizing pancreatitis. In clinical cases, dogs with acute pancreatitis (n = 3) also had a region with no enhancement. In addition, in a patient with insulinoma, the pancreatic mass lesion was enhanced earlier and stronger than the adjacent parenchyma.

These results indicate that contrast-enhanced ultrasound is useful for the detection of parenchymal necrosis in acute pancreatitis and the differentiation of pancreatic mass lesions in dogs.

Finally, peak enhancement from baseline, time to initial upslope and time to peak enhancement from initial upslope were calculated for quantitative analysis. The changes of these parameters in acute pancreatitis were assessed. Time to initial upslope became shorter not only in experimental models of edematous pancreatitis but also in patients with acute pancreatitis. Although no histopathological examination was performed, pancreatic edema might cause this shortening in patients with acute pancreatitis. Quantitative analysis may therefore be suitable for the diagnosis of acute pancreatitis.

In conclusion, it was demonstrated that contrast-enhanced ultrasound with Sonazoid was useful in the diagnosis of canine pancreatic diseases. Further study in patients with pancreatic diseases is necessary to improve the diagnostic accuracy of acute pancreatitis and differential diagnosis of pancreatic mass lesions.

A study on the development and application of a simple and easy method for measuring canine lipoproteins

Yukari Takasaki

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

Canine lipoproteins are analyzed infrequently and their clinical significance is still unclear because simple and easy methods for measuring canine lipoproteins are not available at present; therefore in this study, it was attempted to develop simple and easy methods for measuring canine lipoproteins to investigate canine lipoproteins in many clinical cases.

First, canine lipoproteins were analyzed using the ultracentrifugation method, known as a standard method for analyzing lipoproteins in medicine. Dog sera were overlaid with 154 mM NaCl and centrifuged at 164,000×g, 4°C for 18 hr. Very-low-density lipoproteins (VLDL) were removed from the top fraction. The infranatant, containing low-density lipoproteins (LDL) and high-density lipoproteins (HDL), was added to 1 U/μl heparin and 101 mM $MnCl_2$ to precipitate LDL. Canine lipoproteins were accurately analyzed by this method. Second, canine lipoproteins were analyzed by three homogeneous assays, which are simple and easy methods for measuring the concentrations of HDL-cholesterol (HDL-C) or LDL-cholesterol (LDL-C) in medicine, and compared with the ultracentrifugation method. It was shown that the concentrations in certain homogeneous assays correlated and coincided with those by the ultracentrifugation method, but that others were not appropriate for measuring canine lipoproteins. Accordingly, appropriate homogeneous assays should be employed for measuring canine lipoproteins in veterinary medicine. Additionally, canine lipoproteins were analyzed by the precipitation method with heparin and $MnCl_2$. Though VLDL and LDL were precipitated depending on the concentration of

heparin, the concentration of HDL-C did not coincide with that by the ultracentrifugation method, suggesting that canine lipoproteins could not be analyzed accurately by this method.

Finally, the concentrations of canine HDL-C or LDL-C in many clinical cases were measured by appropriate homogeneous assays. Since homogeneous assays are easy and quick methods, and require a small volume of serum for measurements, canine lipoproteins of many cases could be analyzed in a year. In healthy beagles, the concentrations of HDL-C (126.73 ± 35.11 mg/dl) and LDL-C (25.09 ± 12.89 mg/dl) were measured by appropriate homogeneous assays as reference values. In some healthy miniature Schnauzer and Shetland sheepdogs, the concentrations of HDL-C or LDL-C were higher than in healthy beagles. Moreover, the concentrations of HDL-C or LDL-C were increased in dogs with various diseases, such as hyperadrenocorticism, hypothyroidism, diabetes and hepatic diseases. In the present study, however, the number of clinical cases of each disease was not abundant; therefore, the analysis of more cases is necessary to clarify the characteristics of canine lipoproteins in each disease and their clinical significance in veterinary medicine.

The present study showed that appropriate homogeneous assays could be utilized to measure concentrations of canine HDL-C and LDL-C. It is expected that these concentrations will be measured routinely and that their clinical significance will be clarified in many diseases using homogeneous assays.

Differential expression pattern and subcellular localization of claudin 1-5 in the dog gastrointestinal tract and its evaluation using endoscopic biopsy specimens

Tomoki Yamaguchi

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

Canine inflammatory bowel disease (IBD) is a refractory chronic gastrointestinal tract disorder and its cause and pathophysiology are presently unknown. An altered mucosal barrier contributes to the unsuppressed immune response by increased exposure of immune cells to bacteria and luminal antigens. The barrier function of the epithelial cell monolayer is regulated by a tight junction (TJ), and the major component of TJ is the integral membrane protein, claudin. As the first step in understanding the roles of claudins in the pathogenesis of canine IBD, the expression pattern and subcellular localization of claudins 1～5 in the dog gastrointestinal tract, especially in the duodenum and the colon, were investigated by immunoblotting and immunofluorescence microscopy. Additionally, the usefulness of endoscopic biopsy specimens in the analysis of the expression of claudins was investigated.

Analysis of the claudin expression pattern by immunoblotting with full-length specimens revealed that claudin-1～5 expression varied in each gastrointestinal segment. In the duodenum, high expression of claudins 3 and 5, and low expression of claudin-2 were detected, whereas high expression of claudins 2, 3, 4, and 5, and low expression of claudin-1 were observed in the colon. By immunofluorescence microscopy, constant intense staining of claudins 3 and 5, and weak staining of claudin-2 along the crypt-to-villi

axis in the duodenum were observed. In the colon, claudins 3 and 5 were uniformly expressed along the crypt-to-luminal surface axis, whereas a crypt-to-luminal surface decrease in claudin-2 expression was observed. Using endoscopic biopsy specimens, the expression patterns and subcellular localization of claudins in the duodenum and colon were confirmed in a similar way. The present study demonstrates the differential expression patterns of claudins 1～5 in the dog gastrointestinal tract, which are thought to explain the differences in paracellular permeability properties in each gastrointestinal segment. This study also confirmed that endoscopic biopsy specimens allow the investigation of claudin expression in dogs with IBD.

Gene expression of common melanoma-associated antigens and experimental application of glycoprotein-100 peptide vaccine to canine oral melanoma

Kayoko Yamada

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

Cancer immunotherapy is currently considered to be a novel antitumor intervention with minimum side-effects. Human malignant melanoma expresses a variety of common tumor-associated epitopes, and could be a target tumor, to which therapeutic cancer vaccine is employed. In canine oral melanoma, limited information is available on its immunological property and therapeutic approaches using immunology. The purpose of this study is to provide basic information of clinical application of peptide vaccine to oral malignant melanoma in dogs.

First, mRNA expression of three melanoma-associated antigen including MART-1, gp100 and tyrosinase, recognized in human melanoma patients, was examined in several canine melanoma cells and tissues using reverse transcription-polymerase chain reaction (RT-PCR). Second, clinically healthy dogs were immunized with the gp100 peptide (IMDQVPFSV), which was detected in all examined cells and tissues. This vaccination-induced changes were investigated in general physical condition, hematological and serological status, proportions of peripheral blood lymphocytes and serum IFN-γ concentration. Synthesis of IFN-γ from canine peripheral blood mononuclear cells (PBMC) and tumor cells specific cytotoxic activity related to the vaccination were also evaluated using enzyme-linked immunospot (ELISpot) assay and Rose Bengal Assay, respectively. Delayed-type hypersensitivity (DTH) reactions were then examined clinically and histopathologically at the site of the injection with the antigen.

After the vaccination, the ratio of CD8+ T cell was increased and the cytotoxicity of PBMC to cultured canine malignant melanoma cells (CMeC) was reinforced. Number of IFN-γ secreting cells was significantly expanded by stimuli of whole cells of CMeC or gp100 peptide. DTH test detected clearly positive in one dog in all three animals with intracutaneously injected gp100 peptide. DTH site revealed infiltration of mononuclear cells, which positively stained with CD4 and CD8 antibodies.

In conclusion, melanoma-associated antigens, especially gp100, were highly expressed in canine malignant melanoma. The gp100 peptide vacci-

nation could successfully induce the immunity to canine malignant melanoma cells and the peptide. These results suggested that peptide vaccine would have potential to be as an applicable and effective therapeutic tool for dogs with oral malignant melanoma.

Pathogenicity of the avian leukosis virus isolated from glioma-affected brain of a White Leghorn layer chicken

Tsuyoshi Tadano

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

So-called fowl glioma is caused by fowl glioma-inducing virus (FGV), which belongs to an avian leukosis virus subgroup A (ALV-A), and characterized by multiple nodular growths of astrocytes associated with disseminated non-suppurative encephalitis. FGV infection has been suggested to be prevalent in Japanese fowls in Japan. On the other hands, some outbreaks of an ALV-A infection, which caused mesenchymal neoplasms in cephalocervical cutaneous tissues of commercial layers, have occurred in our country from 2003. Besides, fowl glioma and undifferentiated round cell tumor was also noted in the brains of these affected layers. An ALV strain, TymS_90, was isolated from the affected brains and the isolate had different viral genome from that of FGV. In this study, Specific-pathogen-free chickens (C/O) were inoculated with TymS_90 through yolk sac on the sixth day of incubation in order to clarify the pathogenicity of the isolate. Histologically, brain lesions, including nonsuppurative encephalitis, remnant of external granular layer of crebellum, disorganization of Purkinje cell layer and proliferation of arachnoid cells, were observed in all of 8 inoculated chickens and fowl glioma was noted in three of four birds at the age of 100 days. In addition, undifferentiated round cell proliferation was noted in the cerebrum of two inoculated birds.

The hypervariable region in the SU coding sequence of *env* gene in viral mRNA extracted from the affected brains at the age of 50 days showed 97-99% identity with the corresponding region of inoculated TymS_90. Similarly, the same region of isolates from 100 day-old inoculated chickens had 99% identity to that of TymS_90. By immunohistochemistry and real time RT-PCR, the viral antigens and viral mRNA was detected in various organs, including the brain. These results revealed that TymS_90, like FGV, can cause fowl glioma and cerebellar hypoplasia in chickens but has also ability to induce proliferation of periventricular round cells and arachnoid cells. TymS_90 is considered as the second fowl glioma-inducing ALV.

Study on antibody induction in cerebrospinal fluid by intranasal inoculation of inactivated virus

Aya Morito

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

Intrathecal immunization is a vaccination method, in which the antigens are directly injected into subarachnoid cavity or brain parenchyma, resulting in high antigen-specific antibodies induction not only in the blood but also in the cerebrospinal fluid (CSF). In our previous study, a large amount of antigen-specific immunoglobulins was induced in intrathecally immunized mice and rabbits, and the animals showed resistance against lethal dose of rabies virus inoculation. But intrathecal inoculation is difficult and not safe method. In this study, I tried to detect antigen-specific antibody in the CSF of the rabbit, based on the hypothesis that exposure of the viral antigens to olfactory epithelium in deep nasal cavity of rabbits induces antibody production in CSF following uptake and transport of the antigens into the olfactory bulb by olfactory cells.

The inactivated pseudorabies virus (PrV) suspended in membrane-attachment gels was directly inoculated three times into deep nasal cavity of rabbits by using teflon catheter. The small pieces of PrV genome was detected in olfactory bulb by nested-PCR, suggesting that the inoculated antigens were transported into the brain. By ELISA, it was revealed that anti-PrV antibodies in the serum increased depending on the times of immunization. Because the antibodies contained IgA class of immunoglobulin, I speculated that common mucosal immune system was induced in these rabbits. The antibodies were not detected in the CSF of these rabbits, whereas the rabbits immunized in subarachnoid cavity had high antibody titers in both serum and CSF. The antibodies induced by the intrathecal immunization also contained IgA, and it was assumed that unknown mechanism similar to common mucosal immunization was stimulated by the intrathecal immunization.

From the results of this study, the antibody induction in the CSF was not observed by the immunization in deep nasal cavity of the rabbits. Further modification for intranasal immunization is required to improve the efficiency of uptake and release the antigens into the brain.

Pathological study on atoxoplasmosis in passerine birds

Maiko Mori

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

Atoxoplasmosis is a protozoan disease caused by the isosporoid coccidian parasite, *Atoxoplasma* spp. and the disease has been recognized especially in immature passerine birds. Atoxoplas-

mosis is a main cause of breeding difficulties in Bali mynahs (*Leucopsar rothschildi*), which is one of critically endangered species. The disease has also been reported in gray starlings (*Sturnus cineraceus*) and tree sparrows (*Passer montanus*) in our country. The affected birds reveal lymphocytic and histiocytic infiltration and aggregation with intracytoplasmic protozoa in multiple organs. However, there are limited data on the pathological manifestations of this disease. In this study, 104 passerine birds, including 7 avian species preserved or kept in zoological gardens in Japan, were examined in order to clarify the athological condition of atoxoplasmosis. Of the examined birds, 36 (49%) tree sparrows, 13 (65%) gray starlings and 4 (100%) Bali mynahs had infiltration of mononuclear cells with intracytoplasmic *Atoxoplasma*-like protozoa in the liver. The protozoa were also observed in the spleen, heart, lungs, small intestine, cerebrum and other systemic organs in these birds. The lesions in sparrows were characterized by severe lymphocytic enteritis, whereas the characteristic lesion in gray starlings and Bali mynahs was multifocal hepatocytic necrosis. These protozoa immunohistochemically showed cross-reactivity with anti *Toxoplasma gondii* antibody. Ultrastructurally, the protozoa in mononuclear cells in the liver of a tree sparrow had haracteristic structures, an apical complex with a conoid, rhoptries and micronemes. In addition, the protozoa were categorized in *Atoxoplasma* spp. based on sequence analysis of 18S rRNA gene although the sequences from tree sparrows showed up to 8% differences compared to those of *Atoxoplasma* spp. previously reported. From these results, these birds having protozoa in mononuclear cell cytoplasm were diagnosed as atoxoplasmosis. The results suggest that the pathologic conditions of atoxoplasmosis in passerine birds can be devided into at least 2 types, enteritis type and hepatic necrosis type.

Viability of primordial germ cells in zebrafish (*Danio rerio*) embryo cryopreserved with vitrification solution

Yoshiki Eto

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

The cryopreservation of primordial germ cells (PGCs) has important applications in fish production and conservation. In this study, freezing of dechorionated zebrafish embryos was attempted using vitrification solution for the cryopreservation of PGCs. GFP-*nos1* 3'UTRmRNA was injected into the dechorionated embryos at the 1-to 8-cell stage to visualize their PGCs. GFP-labeled embryos developed to the 10-to 25-somite stage were subjected to freezing with or without yolk. In the first experiment, yolk-intact and yolk-removed embryos were exposed to equilibrium solution and vitrification solution in a step-wise manner before being plunged into liquid nitrogen. After thawing, the number of live PGCs recovered from yolk-removed embryos (7.4 cells/embryo) was higher than that of yolk-intact embryos (2.4 cells/embryo) and was similar to that of fresh (non-frozen) embryos (10.1 cells/embryo). In the next experiment, a single PGC isolated from frozen embryos (yolk-removed embryos at the 10-to 14-somite stage) was transplanted into the marginal region of each blastula-stage embryo (recipient embryo) in which the development of PGCs was blocked by injecting a *dead end (dnd)* antisense morpholino oligonucleotide.

Two days after transplantation, 33% of recipient embryos possessed a single fluorescent PGC (live PGC), and 24% of live PGCs were located in the genital ridge region of recipient embryos. These results indicate that the present embryo freezing procedure can be used as a PGC cryopreservation technique and applied for surrogate production in fish.

Effects of thawing procedures on the survival of *in-vitro*-produced bovine blastocysts frozen with ethylene glycol and sucrose

Yohsuke Hamajima

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 clarify the effects of thawing procedures on the survival of *in-vitro*-produced bovine blastocysts frozen with 1.5 M ethylene glycol and 0.1 M sucrose in a 0.25-ml plastic straw using a conventional slow freezing protocol. Embryos were thawed by holding straws in the air for a short period, prior to being plunged into water at 35°C. The thawed embryos were checked for damage to their zonae pellucidae, and were cultured for 48 hr to evaluate their viability. In experiment 1, the effect of straw exposure time in air (0 to 30 sec) on the survival of thawed embryos (the rate of viable, expanded, hatching and hatched blastocysts) was examined. High survival rates were observed when frozen embryos were thawed by exposing the straws to air for 0 to 15 sec. Embryo survival rates tended to be decreased when embryos were thawed by holding the straw in the air for 20 sec, and was significantly decreased by holding the straws for 30 sec. About 30% of embryos had damaged zonae pellucidae when they were thawed by holding the straws in the air for 0 and 5 sec. Experiment 2 was conducted to determine the effects of holding straws in a light breeze (wind scale: 2) on the viability of thawed embryos. Embryo survival rates were decreased when embryos were thawed by holding the straws for 5 and 10 sec in a light breeze. Experiment 3 was conducted to examine the effects of repeatedly holding straws in no or a light breeze. Embryo viability after 20-sec repeated exposure was lower than after 15-sec repeated exposure. When straws were exposed repeatedly to a light breeze for 5 sec, embryo viability was significantly decreased. These results demonstrate that *in-vitro*-produced bovine embryos frozen with 1.5 M ethylene glycol and 0.1 M sucrose should be thawed by holding the straws in calm air for 10 sec before plunging into water.

The role of the N-terminal region of the cytoplasmic domain containing the ΦXΦXΦ sequence in the trafficking of erythroid anion exchanger 1, AE1 to the plasma membrane

Wataru Otsu

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

Anion exchanger 1 (AE1, band 3) is the most abundant transmembrane protein in red blood cells. Various mutations of the human AE1 gene cause dominant hereditary spherocytosis and distal renal tubular acidosis. Recent findings on affected vesicular transport of AE1 mutants, such as the deletion mutant of the C-terminal 11 amino acid residues (ΔCt11) that exhibited impaired trafficking to the plasma membrane, suggested that some characteristic sequences within the AE1 polypeptide can regulate membrane traffic of AE1. Our previous studies have demonstrated that the N-terminal region containing the sequence of Ser^{25}-Val-Ser-Ile-Pro-Met^{30} in bovine AE1 has a key role in facilitating the traffic of AE1 in non-polarized HEK293 cells. The purpose of the present study was to define and characterize the specific sequence essential for effective trafficking of AE1 to the plasma membrane. We created bovine AE1 and human AE1ΔCt11 mutants with various substitution mutations in the N-terminal region, including the Ser^{25}-Met^{30} sequences and model proteins in which the N-terminal cytoplasmic sequence of Ly49E was replaced by the AE1 N-terminal sequence. Fluorescence microscopy and cell-surface biotinylation studies of these mutants demonstrated that a characteristic sequence of ΦXΦXΦ (Φ is hydrophobic and X is any amino acid residue), corresponding to the Val-Ser-Ile-Pro-Met sequence in bovine AE1, increased the trafficking efficiency of AE1. Disruption of this motif caused endoplasmic reticulum (ER) retardation of AE1 and Ly49E mutants possessing *N*-glycan (s) sensitive to endoglycosidase H and the disappearance of cell-surface expression, as observed for proteins with ΦXΦXΦ motif in the presence of brefeldin A and/or the dominant-negative mutant of a COPII component Sar1. These findings suggest that AE1 is transported to the plasma membrane through the conventional secreting pathway and that the ΦXΦXΦ sequence alone can accelerate ER exit or transport from the ER to the Golgi of AE1.

Polymorphism of spectrin genes in cattle: possible implication in diverse red cell phenotypes in band 3 deficiency

Ayumi Kawano

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

Band 3 deficiency in Japanese black cattle is a novel disorder due to R664X mutation of the

band 3 (anion exchanger 1, AE1) gene, exhibiting hemolytic anemia associated with hereditary spherocytosis. Our recent studies revealed that some animals homozygous for R664X mutation possessed red cells with extremely fragile membranes and reduced spectrin contents, while others did not. Moreover, substitutions of various amino acid residues have been found to occur in bovine α- and β-spectrin due to genetic polymorphisms. These findings suggest that polymorphism of the spectrin genes plays a modulatory role in red cell phenotypes in band 3 deficiency. The purpose of the present study was to define the genotype related to the reduced level of spectrins in red cells. We determined the genotypes for α- and β-spectrin in ～160 animals free from R664X mutation of the band 3 gene, and found several distinct alleles for the α-spectrin gene, including SpαA, SpαB, SpαB-K91, and SpαAB. Among them, allele SpαB-K91 was found in Japanese black but not in Holstein cattle. Allele SpαB-K91 was previously found in a band 3-deficient animal whose red cells also showed marked spectrin deficiency. Animals possessing the SpαB-K91 allele that generates α-spectrin with Lys^{91} in homozygous and heterozygous states showed a reduction in red cell spectrin contents by 20% and 11%, respectively, although no morphological defects and hemolysis were evident in these animals. No other genetic variations for α- and β-spectrin were found to cause reductioin in red cell spectrin contents. These findings suggest that a genotype for α-spectrin SpαB-K91 that is silent in normal animals can exacerbate red cell membrane lesions in band 3 deficiency.

Idetification of unique isoforms of bovine ABCC4, ABCC4.1 and ABCC4.4, generated by gene duplication

Satoko Yamaguchi

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

Ruminant red cells contain a unique transmembrane glycoprotein gp155 with an apparent molecular mass of 155,000. Although we have recently identified that gp155 is an ATP-binding cassette transporter subfamily C type 4 (ABCC4), the function of ABCC4 in ruminant red cells remains to be clarified. In order to define the physiological significance of high ABCC4 expression in ruminant red cells, we analyzed ABCC4 genes and proteins in the bone marrow and red cells in cattle. We found four ABCC4-related genes (ABCC4.1-4.4) in the bovine genome database, probably due to gene duplication in chromosome 12. To explore the multiple ABCC4 isoforms in bovine bone marrow cells, we employed RT-PCR and isolated two isoforms, ABCC4.1 and 4.4. The primary structure of ABCC4.1 is very similar to ABCC4 from other mammals, whereas 4.4 showed atypical features, such that it lacked the C-terminal PDZ domain-binding motif and *N*-glycosylation sites. ABCC4.4 proteins were detected as the non-glycosylated product of 127 kDa in transfected HEK293 cells, although both ABCC4.1 and ABCC4.4 were predominantly localized to the plasma membrane. RT-PCR revealed that ABCC4.1 mRNA was more abundant than that of 4.4 in bone marrow cells, suggesting that the major isoform in bovine red cells is ABCC4.1. Indeed, bovine red cell ABCC4 proteins were detected as the major 155 kDa and a minor 127 kDa species, presumably representing ABCC4.1

and ABCC4.4, respectively, in immunoblotting. Furthermore, the expression of these multiple ABCC4 proteins was more abundant in band 3-deficient red cells than in normal cells, suggesting that ABCC4 is upregulated in band 3-deficient erythroid cells. This implies that ABCC4 compensates for the membrane mechanical stability deficient in red cells lacking band 3. These results demonstrate that multiple ABCC4 isoforms due to gene duplication are actually present and may have some undefined functions in cattle. Such diversity of ABCC4 isoforms might participate in higher expression levels of ABCC4 in ruminant red cells.

Development of diagnostic methods applicable to various serotypes of hantavirus infections

Takahiro Sanada

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

Hantaviruses exist worldwide in association with rodent carriers. Some of them cause severe human illnesses, such as the hemorrhagic fever with renal syndrome (HFRS) and the hantavirus pulmonary syndrome (HPS). Although no human HFRS cases have been reported in Japan since 1984, brown rats (*Rattus norvegicus*) in several port area and field grey red-backed voles (*Myodes rufocanus*) in Hokkaido have been found to be hantavirus-positive. In addition, there is a risk of invasion of infected rodents via ships from endemic countries, and more than 200,000 rodents are imported in Japan annually. Therefore, there is a possibility of re-emergence of human hantavirus infections in Japan.

Diagnostic methods for hantavirus infections require serotype-specific reagents since the antigenic and genetic characteristics of hantavirus varies among the serotypes of the viruses.

To develop the diagnostic method applicable to various hantavirus infections with a single sets of reagents, the antigen detection ELISA was established by using a monoclonal antibody clone E5/G6 which crossreacts with various serotypes of hantavirus nucleocapsid proteins (NPs). By the antigen detection ELISA, the NP of Hantaan, Seoul, and Puumala viruses were detected from hantavirus-infected cells and virus stock. In addition, the NP was detected from lung tissues of Puumala-infected Syrian hamsters (*Mesocricetus auratus*) at 7 to 14 days postinoculation.

The antibody detection ELISA was also developed by using recombinant NPs of three serotypes as antigens and peroxidase-conjugated protein G. By the antibody detection ELISA, the antibodies against Hantaan, Seoul, Amur, Puumala, and Sin Nombre viruses were detected. In the serum samples from Puumala-infected Syrian hamsters, the antibodies were detected at 14 to 70 days postinoculation. In addition, the antibodies in wild rodent sera from brown rats, field grey red-backed voles, striped field mice (*Apodemus agrarius*), and Korean field mice (*Apodemus peninsulae*) were detected with the sensitivity of 90.9%, 100%, 60%, and 60%, respectively, and the specificity of 100%, 100%, 100%, and 94.7%, respectively.

These data suggest that the antigen and antibody detection ELISAs can be useful tools to detect the hantavirus infections in rodents.

The establishment of a neutralization test using the virus-like particles of tick-borne encephalitis virus expressing heterologous gene

Yumiko Chiba

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

Tick-borne encephalitis (TBE) virus is one of the major human pathogenic flavivirus, and causes disease that can impact on public health in the endemic countries of Asia and Europe. In October 1993, a human case of encephalitis was diagnosed as TBE in Kamiiso, Hokkaido. TBE virus strain Oshima 5-10 was isolated from a sentinel dog in the same area, and identified as the Far-Eastern subtype of TBE virus. Since the susceptible vector ticks and reservoir rodents are widely found in Japan, it is possible that TBE virus is endemic in the other parts of Japan. In addition, another flavivirus, Japanese encephalitis virus, is widely distributed in Japan. These flaviviruses show high cross-reactivity in serological diagnosis. To prevent an endemic of TBE virus in Japan, it is necessary to develop new methods that allow differentiation between TBE and JE.

For the diagnosis of flavivirus infection, a neutralization test is generally used due to its high specificity. However, appropriate biocontainment facilities are required to handle live TBE virus, which is highly pathogenic to humans. Therefore in this study, the recombinant particles of TBE virus expressing heterologous gene were constructed and applied to neutralization tests.

In our previous study, TBE virus replicon RNAs were constructed. These replicons contain the genes necessary for genome replication in cells, but lack the genes for viral structural proteins. Therefore no progeny virus is produced in the cells transfected with replicon RNAs. In this study, a luciferase gene was inserted into TBE virus replicon as a reporter gene. The expression of the luciferase gene was observed in the replicon-replicating cells. Furthermore, virus-like particles (VLPs) that packaged replicon RNA containing reporter gene were secreted after the sequential transfection of the replicon RNA and a plasmid expressing viral structural proteins. The single-round infectivity of VLPs enables safer manipulation than live virions. Luciferase activity was confirmed in the cells infected with VLPs containing luciferase gene, and depended on infectious units of VLPs. Moreover, the infection of VLPs was inhibited by neutralizing antibody to TBE virus, and their neutralization titers were determined by using the luciferase activity as an indicator. Furthermore, a positive correlation was demonstrated in the results between neutralization tests using VLPs and live virions, and the sensitivity and specificity were both 100%.

In conclusion, VLPs with luciferase genes are useful tools as a safe and quantitative alternative to live virus in neutralization test. Furthermore, these data also suggest that these replicon RNA and packaging systems have a potential for further applications such as a gene therapy and a vaccine delivery system.

Mechanism of neurite-like outgrowth induced by α-phenyl-N-*tert*-butylnitrone in PC12 cells

Nozomi Ito

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

α-Phenyl-N-*tert*-butylnitrone is widely used as an antioxidant against oxidative damages or spin-trapping reagent of free radicals *in vitro* and *in vivo*. Previous studies in my laboratory showed that PBN had the nerve growth factor (NGF)-like activity to induce neurite-like outgrowth in rat pheochromocytoma PC12 cells through the Ras-ERK pathway. The present study was performed to clarify the mechanism of this PBN-induced neurite-like outgrowth in PC12 cells. In the first experiment, I synthesized ^{14}C-labeled radioactive PBN ([^{14}C]PBN) from benzaldehyde-[ring-^{14}C] and N-(*tert*-butyl) hydroxylamine to examine the localization of PBN in PC12 cells. When cells were treated with [^{14}C] PBN and fractionated by 5% trichloroacetic acid and ethanol, high radioactivity was found in lipid fraction but not the other fractions, suggesting the specific localization of PBN in plasma membrane. Furthermore, PC12 cells incubated with [^{14}C]PBN were immunoprecipitated with anti-Ras antibody. This immunoprecipitate had little radioactivity. These results indicated that PBN was localized in lipid fraction, but not directly bound to Ras. Next, to examine whether nitric oxide (NO) was released from PBN and induced neurite-like outgrowth in PC12 cells, PC12 cells were preincubated with carboxy-PTIO, an NO scavenger, and treated PBN or NGF. The PBN-induced neurite-outgrowth was significantly inhibited by pretreatment of carboxy-PTIO, whereas the NGF-induced neurite-outgrowth was not affected by this pretreatment. Moreover, the treatment of NOR4, an NO donor, induced neurite-like outgrowth in PC12 cells. The activations of Ras and ERK were also observed in NOR4-treated cells. These results suggested that NO was released from PBN under intracellular oxidative environment and induced neurite-like outgrowth in PC12 cells through Ras-ERK pathway.

Inhibitory effect of a new anti-tumor drug 1-(3-C-ethynyl-β-D-*ribo*-pentofranosyl) cytosine (ECyd) against peritoneal dissemination of gastric cancer

Masato Eitaki

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

1-(3-C-Ethynyl-β-D-*ribo*-pentofuranosyl) cytosine (ECyd) is a newly developed anti-tumor cytosine nucleoside analog, and induces cell death by inhibiting RNA synthesis. This study was performed to evaluate the availability of ECyd as a drug for peritoneal dissemination of gastric

cancer. Nude mice were inoculated i.p. with 5×10^6 of MKN45 cells. Mice were treated with ECyd (i.p.) on 3, 6, 9, 12, 15, and 18 days after the innocuation of tumor cells. The formation of tumor nodules on mesenterium was observed on 19 days after the innocuation without ECyd, and the treatment of 0.2 mg/kg and 1.0 mg/kg of ECyd significantly reduced mean number and size of tumor nudules. In addition, survival times of mice were also prolonged by the treatment of 1.0 mg/kg of ECyd. Next, inhibitory mechanisms of ECyd against peritoneal dissemination were investigated *in vitro*. The viability of MKN45 cells was suppressed by 0.5 μM and 2.5 μM of ECyd concentration-dependently, and the invasive capability was inhibited by 2.5 μM ECyd but not 0.5 μM ECyd. By wound healing assay and western blotting, it was also demonstrated that 0.5 μM of ECyd suppressed mobility capability, a factor related to invasivion in tumor, through down-regulatin of phosphorelated ERK. Since angiogenesis is also believed to be important for peritoneal dissemination, the effect of ECyd on the expression of VEGF, a main factor of angiogenesis, was investigated by western blotting and RT-PCR. The expression of VEGF protein and mRNA was strongly inhibited by the treatment of ECyd. In summary, it was concluded that ECyd inhibited peritoneal dissemination of gastric cancer by cell-killing effect, invasion inhibitory effect via down-regulation of p-ERK and anti-angiogenesis effect via down-regulation of VEGF.

Radiosensitizing effects of doranidazole (PR-350) on rat glioma model

Junichi Kino

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

Hypoxia is common feature in solid tumors, and affects radiosensitivity. Numerous approaches have been tried to overcome radioresistance of solid tumor. 2-nitroimidazole derivertives are well-known as sensitizer in hypoxic condition. A new hypoxic cell radiosensitizer doranidazole ((±)-(2RS, 3SR)-3-[(2-nitroimidazole-1-yl)-methoxy] butane-1, 2, 4-triol) (PR-350) is less neurotoxic effect compared with traditional 2-nitroimidazole derivertives such as 2-nitroimidazole and misonidazole. This study was performed to investigate the radiosensitizing effect of doranidazole on C6 rat glioma. First, reproductive cell death was examined by *in vitro* clonogenic assay. The treatment with doranidazole significantly enhanced radiation-induced reproductive cell death in hypoxic condition, but not in normoxic condition. Second, the effect of X-irradiation combined with doranidazole on tumor growth was examined. T2 weighted imaging using high field MRI instrument was employed to measure the size of intracranial glioma *in vivo*. The treatment of X-irradiation with doranidazole completely inhibited the tumor growth of C6 glioma, although the growth rate of C6 glioma treated with X-irradiation alone or doranidazole alone was not significantly different from that in non-treatment group (control). Moreover, uptake and distribution of doranidazole in brain tumor were examined by autoradiography using [^{14}C] doranidazole.

Autoradiographic images revealed that [^{14}C]-doranidazole specifically accumulated in not only HIF-1α-positive living cells (hypoxic cells)

but also HIF-1α-negative living cells (normoxic cells) of glioma, though the radioactivity due to [^{14}C]-doranidazole was found to be low level in normal brain tissue and necrotic region of glioma. These results indicated that doranidazole selectively accumulated in brain C6 glioma, and enhances raditation-induced antitumor effect.

Mechanisms of warfarin resistancein *Rattus rattus*

Shima Ando

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

Warfarin is commonly used worldwide as a rodenticide. It inhibits coagulation of blood by inhibiting vitamin K 2, 3-epoxide reductase (VKOR) activity. An inadequate supply of vitamin K blocks the production of prothrombin and causes hemorrhage. However, it has been reported that repeated or long-term treatment with warfarin causes resistance in wild rodents. Recently, warfarin-resistant roof rats (*Rattus rattus*) have been found in Tokyo, Niigata, and Osaka in Japan. In this study, I focused on the clotting factor activity, mutation of VKOR gene (VKORC1: VKOR complex subunit 1), and VKOR activity in roof rats to understand the mechanism of warfarin resistance in Japanese roof rats.

Plasma was obtained from warfarin-sensitive and-resistant roof rats. Under physiological conditions, there was no difference in the coagulation times between warfarin-sensitive and-resistant rats. In addition, after warfarin administration, there was no change in the coagulation times of warfarin-resistant rats, although the coagulation times were prolonged in warfarin-sensitive rats.

Then, I determined the differences in kinetics of VKOR activities between warfarin-resistant and-sensitive rats. The hepatic microsomal VKOR-dependent activities were measured over a range of vitamin K epoxide concentrations from 6.25 to 200 μM. The V_{max} values of in resistant rats (Niigata: 160 ± 52, Osaka: 300 and 160, Tokyo: 170 ± 63 [pmol/min/mg protein]) were lower than those of sensitive rats (1,000 ± 520 [nmol/min/mg protein]). The K_m values of resistant and sensitive rats were almost identical. Thus, warfarin-resistant rats showed 2-3 fold lower V_{max}/K_m values (Niigata: 1.2 ± 0.35, Osaka: 0.89 and 1.2, Tokyo: 1.1 ± 0.35) than-sensitive rats (2.6 ± 0.60).

To clarify the mechanism of reduced VKOR activity in warfarin-resistant rats, I analyzed the sequence of the VKORC1 gene from rats. I found novel substitutions of 41st alanine to valine (Niigata), 41st alanine to threonine (Osaka), 61st arginine to tryptophan (Tokyo), and the heterozygous substitution at positions 61 and 76 of leucine to proline (Tokyo). I identified that all resistant rats possessed substitution in VKORC1 gene.

Finally, I concluded that one of the mechanisms of reduced VKOR and warfarin resistance in the Japanese roof rat might be due to VKORC1 mutation. However, further study is needed to clarify how these rats can exhibit adequate vitamin K-dependent clotting factor activities with low VKOR activity and acquire warfarin resistance at the same time.

The effects of food ingredients on the mutagenicity of benzo[a]pyrene

Marumi Ohno

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

The cytochrome P450 superfamily, notably the CYP1A subfamily, metabolically activates various chemicals, such as promutagens and procarcinogens; therefore, the activity of the CYP1A subfamily appears to be one of the most important factors in cancer initiation. Currently, various food ingredients, especially phytochemicals, are drawing attention for their alterative effects on CYP-dependent xenobiotic metabolism. In other words, it is possible that phytochemicals reduce or enhance the mutagenicity of xenobiotics via the modulation of CYP-dependent activities; however, there is little available information describing the effect of phytochemicals on mutagenicity as a consequence of metabolic activation. Therefore, in this study, the effects of two typical phytochemicals, cacao mass and astaxanthin (Ax), were studied on the mutagenicity caused by benzo[a]pyrene (B[a]P) that requires metabolic activation by CYP1A for its genotoxicity.

To clarify the effect of crude dimethyl sufoxide (DMSO) extract from milk chocolate, white chocolate and defatted cacao powder on the mutagenicity caused by B[a]P and tert-butyl hydroperoxide (t-BuOOH), the Ames test was performed using *Salmonella typhimurium* strain TA 98 and strain TA 102. B[a]P induces mutagenicity by metabolic activation and t-BuOOH induces it by generating free radicals. While white chocolate did not modulate the numbers of revertant colonies produced by B[a]P treatment, milk chocolate and cacao powder extracts did. On the other hand, surprisingly, none of the cacao products tested affected the number of revertant colonies when t-BuOOH was used as the mutagen. At maximum concentration (13.25 mg cacao powder/ml), the crude cacao powder extract reduced ethoxyresorufin O-deethylase (EROD) activity to 17.4% of the control, suggesting that whole cacao products inhibit CYP1A activity. In conclusion, inhibition of CYP1A activity by cacao products may prevent DNA damage by reducing the metabolic activation of carcinogens.

In addition, using another typical photochemical, the effects of Ax treatment (100 mg/kg body weight, for 3 days) were investigated on xenobiotic metabolizing enzymes in Wistar rats (male, 8 weeks old). In the treated group, CYP1A-dependent EROD activity was induced (2.5-fold) and CYP1A1 mRNA expressions were increased (5.6-fold) in the liver. In contrast, there was no significant induction of either glutathione S-transferase (GST) or uridine diphosphate glucuronyl transferase (UGT)-conjugating activities toward traditional substrates, 1-chloro-2, 4-dinitrobenzene and 1, 2-dichloro-4-nitrobenzene for GST and *p*-nitrophenol for UGT. These enzymes conjugate intermediates metabolized by CYP and decrease their toxicity. As a consequence, the ability to activate B[a]P mutagenicity was enhanced in Ax-treated rat livers in the Ames assay, compared to control group in the livers, indicating that Ax increases metabolic activation but does not modify the phase II-dependent detoxification of some chemicals. It is proposed that a high concentration of Ax may increase the toxicity of chemicals and drugs requiring metabolic activation.

In this study, the entirely different effects of phytochemicals, which are widely consumed as

dietary constituents or supplements with health-promoting benefits, were shown on CYP-dependent mutagenicity of promutagen.

cDNA cloning and phylogenetic analyses of cytochrome P450 1As from mammalian species

Yusuke Kawai

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

Members of cytochrome P450 subfamily 1A (CYP1As) are involved in the metabolism of toxic environmental chemicals, such as polycyclic aromatic hydrocarbons (PAHs) and aromatic amins. Thus, clarification of the CYP1A evolutionary process is essential to predict and interpret species differences in adaptation to environmental chemicals. The CYP1A gene subfamily comprises two paralogs in birds and mammals. Phylogenetic analysis of complete coding CYP1A sequences suggests that mammalian and bird CYP1A pairs (CYP1A1/2 and CYP1A4/5, respectively) are generated in each lineage; however, another explanation is that the high degree of sequence similarity between CYP1A4 and CYP1A5 is due to gene conversion, and avian and mammalian CYP1A paralog pairs resulted from a single gene duplication event. This indicates that these genes have evolved according to a noncanonical evolutionary mechanism; however, there is still a paucity of information on the evolution history of mammalian CYP1As. To understand and predict the function of CYP1As in mammals, detailed information concerning the evolutionary process is essential. In this study, novel partial CYP1A genes from four mammals were identified in addition to the published CYP1A genes. Livers were obtained from a male lion (*Panthera Leo*), male Eurasian otter (*Lutra lutra*), female cottontop tamarin (*Saguinus oedips*) and red-bellied tamarin (*Saguinus labiatus*) that had died at Maruyama Zoo (Sapporo Japan).

Although the regions were regarded as unconverted, the results of phylogenetic analysis using CYP1A genes from 43 mammalians suggested that some lineages of gene conversion might homogenize CYP1A sequences. Algorithms designed to detect recombination among CYP1A nucleotide sequences also indicate that gene conversion events have impacted the 1,000 to 1,500 bp region in mammals. The analysis also suggests a difference in the gene conversion region between marsupialia and eutheria. Focusing on the unconverted region, which might be important for generating functional differences between CYP1A1 and CYP1A2, one amino acid site was found in marine mammals. The unconverted region was also investigated to detect selection pressures. The results of these analyses suggested no positive selection in the CYP1A evolution process in mammalians. Environmental chemicals such as PAHs are metabolically activated and converted to toxic intermediates by CYP1A, and subsequently metabolized by phase II enzymes, which detoxify or accelerate the elimination of toxic compounds. It is well known that the activities of phase II reactions are markedly reduced in carnivorous species; however, in this study, selection pressure in carnivore CYP1A1 evolution may not be related to the low ability of xenobiotic metabolism in phase II reaction. Further investigations are required for accurate prediction of the evolutional process of CYP1A function in carnivorous animal species.

Morphological observation of the vomeronasal organ of the Japanese black bear (*Ursus thibetanus japonicus*)

Masahiko Befu

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

Chemical communication mediated by odorant plays an important role in social behavior such as recognition of other animals and reproductive behavior, especially in wild animals such as Japanese black bears. The vomeronasal organ (VNO) is one of the chemoreceptors that receive pheromones, but the morphology and function of VNO in Japanese black bears has not previously been investigated. In the present study, I observed the VNO of the Japanese blackbear anatomically and histologically in order to clarify the morphological characteristics. The VNO of the Japanese black bear is a bilateral tubular structure surrounded by cartilage, and it opened to an incisive duct at the rostral end while the caudal end was closed. The vomeronasal duct was lined with 4 types of epithelia: stratified squamous epithelium at the rostral part of the duct, sensory and non-sensory epithelia on the medial and lateral walls of the middle part of the duct respectively, and simple columnar cells caudally. Many neurons were present in the sensory epithelium. The VNO epithelium was thickest at middle of the sensory epithelium, while simple columnar epithelium and epithelium in the transitional area between stratified squamous and sensory areas were thin. The perimeter of the duct was longest at the middle of the sensory epithelium and decreased at the rostral and caudal area. Glands, blood vessels, nerves and connective tissue surrounded the vomeronasal duct. Vomeronasal glands were developed caudally as well, and glandular tubes opened to the VN duct at the ventral, dorsal and caudal part of the duct. In the present study, there were no morphological differences due to sex, age or season. Though the basic components of the VNO of the Japanese black bear were similar to those of other mammals, there were some morphological differences such as the thickness of the sensory epithelium and distribution of blood vessels. These differences indicate that the function of the VNO of the Japanese black bear differs somehow from that of other mammals. I elucidated the basic morphology of the VNO of the Japanese black bear in this study, but further investigation with sufficient samples is needed to further clarify differences based on sex, age and season, as well as function of the VNO.

Changes in serum pregnancy-associated glycoproteins (PAG) concentrations and expression of interferon-stimulated genes (ISG) mRNA in peripheral blood leukocytes during early pregnancy in Hokkaido sika deer (*Cervus nippon yesoensis*)

Chizuru Miyazawa

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

In this study, expression of pregnancy-associated glycoproteins (PAG) in placental extracts, serum PAG concentrations, expression of interferon (IFN)-τ in uterine flushings, and expression of IFN-stimulated genes (ISG) family mRNA in peripheral blood leukocytes (PBL) were examined, aiming at establishment of a new technique for early-pregnancy diagnosis in Hokkaido sika deer (*Cervus nippon yesoensis*).

In ruminants, PAG synthesized in the outer layers of the trophoblast is suspected of being a signal that pregnancy has occurred, which then prevents luteal regression in the maternal system. In the present study, placental extracts were obtained from a deer that had died in an accident 83 days after breeding (= day 83) and blood samples were collected from 6 pregnant deer. Western blotting was performed with anti-caprine $PAG_{55kDa+59kDa}$ antiserum. The placental proteins reacted with the antiserum. Their estimated molecular masses were 67 and 89 kDa, respectively.

However, a heterologous double antibody radioimmunoassay was used to determine PAG concentrations in serum. The serum PAG concentration increased over basal concentrations at day 25～32 and continued to increase gradually with advancing pregnancy.

During early pregnancy in ruminants, the trophectoderm of the developing conceptus secretes IFN-τ, which acts as an antiluteolysin, into the uterine lumen. In addition, IFN-τ increases expression of several ISG in the uterus. In the present study, uterine flushings were collected from 15 deer, including 6 pregnant deer and PBL samples were collected from 4 pregnant deer at day 15～26. A radioimmunoassay was used to determine IFN-τ expression in uterine flushings, but it was not successful because there might not have been any IFN-τ in these uterine flushings or deer IFN-τ might not cross-react with bovine IFN-τ. However, mRNA expression levels for ISG-15, Mx1 and Mx2 were analyzed by real-time PCR. Steady-state levels of mRNA for ISG-15 were increased at day 18～20 compared with those at day −3～0, but levels of Mx1 and Mx2 expression did not differ between pregnant and non-pregnant deer.

In the present study, a partial physiological phenomenon during early pregnancy in Hokkaido sika deer was clarified. This finding may be applicable to the diagnosis of early-pregnancy by measuring serum PAG concentrations and levels of ISG mRNA expression in PBL from pregnant female deer after day 30 and between day 18 and day 20, respectively.

Studies on the intracellular localization of PrP^{Sc} in prion-infected neuroblastoma cells

Takeshi Yamasaki

Laboratory of Prion Diseases, School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Prion diseases are fatal neurodegenerative diseases characterized by vacuolation of neurons and neuropil, astrocytosis and accumulation of an abnormal isoform of prion potein (PrP^{Sc}), in the central nervous system. The propagation of prion in neurons is believed to be tightly associated with neuronal degeneration. To clarify the mechanism of prion propagation, biosynthesis of a cellular isoform of prion protein (PrP^{C}) and PrP^{Sc} have been studied by using cells persistently infected with prion, however, it has not been fully understood yet. Clarification of the intracellular localization of PrP^{Sc} is essential for the understanding of molecular mechanism of prion propagation, therefore, at the beginning of this study, I tried to analyze the intracellular localization of PrP^{Sc} in cells persistently infected with prion using the indirect immunofluorescence assay reported by Taraboulos et al. (*J. Cell. Biol*., 110: 2117-2132. 1990). However, not only PrP^{Sc} but also PrP^{C} was occasionally detected by this method, depending on the cell condition and anti-PrP antibodies used for the detection. Thus I attempted to improve the method in terms of reliability and specificity in detecting PrP^{Sc}, and found that PrP^{Sc} could be specifically detected by using an anti-PrP monoclonal antibody 132 that recognizes the region adjacent to the most amyloidgenic region of PrP (aa112-119). Then, I extensively analyzed the intracellular localization of PrP^{Sc} by a double staining with organelle markers. Co-localization of PrP^{Sc} with lysosome markers was observed as reported previously, however, the most prominent finding was the presence of PrP^{Sc} at juxtanuclear region that was not co-localized with lysosome markers. PrP^{Sc} detected at juxtanuclear region existed very close to trans-Golgi network (TGN), and appeared to be closely associated with an intracellular transport pathway of Cholera toxin subunit B. Furthermore, I found that PrP^{Sc} was co-localized with components of retromer that is one of the machineries associated with retrograde-transport of proteins from endosome to TGN.

The amount of PrP^{Sc} decreased and the perinuclear accumulation of PrP^{Sc} disappeared when the expression of a component of retromer, vacuolar protein sorting 35, was down-regulated by small interfering RNA. These results propose a novel idea that the retrograde-transport pathway of proteins from endosome to TGN may be involved in the biosynthesis of PrP^{Sc}.