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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, 2005.

The authors summaries of their theses are as follows :

Analysis of renal renin-angiotensin system in mouse diabetic nephropathy

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The aim of this study is to clarify the roles in part of renal renin-angiotensin system (RAS) performing in the diabetic nephropathy (DN). Male CBA/N and CBA/J mice were used, in which the former Ren-1 typed mice possess single renin gene, whereas the latter Ren-1 Ren-2 typed mice carry two renin genes. In this study, dynamics of renal RAS including morphological changes of the kidney in streptozotocin (STZ)-induced diabetic mice were analyzed by using immunohisitochemical technique and laser-microdissection method.

It was noted in both strains that renal pathological changes demonstrating renal hypertrophy and tubular expansion were observed until 56 days after a STZ-injection. In diabetic kidneys, distinct immunoreactivity for renin and angiotensin Ⅱ was restricted on the luminal surface of flattened epithelial cells in dilated distal tubules and collecting ducts, whereas no immunoreactive cells were detected on these regions in control mice. Expression of renin mRNA was predominantly detected in the renal cortical artery and glomerulus, on the other hand, there was no expression of other RAS-related genes, angiotensinogen, angiotensin-converting enzyme, and two angiotensin Ⅱ receptors. Comparing these two strains, CBA/N exhibited an early and intense renal pathological change as well as numerous expression of renin and angiotensin Ⅱ, whereas CBA/J showed gradual changes in these criteria.

These findings suggest that renin is secreted from juxtaglomerular apparatus into distal tubule lumen via macula densa with stimulation of hyperglycemia, and then that angiotensin Ⅱ produced in these epithelial cells plays an important role for onset of renal pathological change. Additionally, it is likely that Ren-2 gene functions as a secretion of non-active renin, prorenin, preventing production of angiotensin Ⅱ and progression of DN in early stage.

Further more studies should be required for identification of Ren-2 gene functions, since clinical control of local RAS is a useful therapy against the diabetic complications as well as nephropathy.

Functional characterization of Na^+-HCO_3^- cotransporter (NBC) current in bovine parotid acinar cells : biophysical properties and molecular basis

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The parotid glands of ruminants significantly differ from other mammalian salivary glands in secreting a HCO_3^--rich, isotonic saliva, which is most likely driven by transepithelial HCO_3^- transport in acinar cells. Although Na^+-HCO_3^- cotransporter (NBC) is suggested to play a role in mediating HCO_3^- influx across the basolateral membrane for the eventual HCO_3^- secretion in the parotid acinar cells, its functional properties and molecular nature still remain unknown. In the present study using electrophysiological and molecular techniques, I have now examined whether electrogenic NBC is expressed in bovine parotid acinar cells. In the whole-cell patch clamp experiments, acutely dissociated bovine parotid acinar cells exhibited a membrane conductance (G_{Na^+/HCO_3^-}), which was dependent on the presence of both extracelluar Na^+ and HCO_3^- and was inhibited by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS ; 0.5mM). The reversal potential of G_{Na^+/HCO_3^-} was consistent with that predicted from a knowledge of the transmembrane HCO_3^- and Na^+ gradients, but not from either a HCO_3^- or Na^+ gradient alone. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed the presence of transcripts of the pancreatic variant of NBC 1 (pNBC) in bovine parotid cells. Human embryonic kidney-293 cells transfected with pNBC cloned from bovine parotid displayed a G_{Na^+/HCO_3^-}, whose electrophysiological and pharmacological properties were similar to those of the native G_{Na^+/HCO_3^-}. These results suggest that bovine parotid acinar cells express a functional electrogenic NBC, which may be mediated, at least in part, by pNBC.

Whole-cell patch clamp studies of Ca^{2+}-activated K^+ channel in rat submandibular acinar cell

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It is well established that Ca^{2+}-activated K^+ (K_{Ca}) channels in the basolateral membrane play a critical role in fluid and electrolyte secretion by salivary acinar cells. To date, two types of K_{Ca} channel, Maxi-K^+ (Slo 1) and IK (SK 4 /IK 1) have been identified and characterized in salivary acinar cells in various species. However, previous and recent K^+ flux and fluid secretion studies have raised the possibility that non of these K_{Ca} channels may significantly participate in Ca^{2+}-dependent salivary fluid secretion. In the present study

using the standard whole-cell patch clamp technique, I have examined whether a K_{Ca} channel that is distinct from Maxi-K^+ and IK channels would be present in freshly dissociated rat submandibular acinar (RSA) cells. When RSA cells were dialyzed with a pipette solution containing 1 μM free Ca^{2+} concentration in a bath solution containing tetraethylammonium (TEA; 10mM), they displayed a K_{Ca} conductance, which was partially inhibited by clotrimazol (CLT; 1 μM), a SK4/IK1 blocker. The residual TEA-and CLT-insensitive K_{Ca} conductance had a relative permeability sequence of $K^+ = Rb^+ >> Na^+$, and was inhibited by quinine (1 mM) and Ba^{2+} (1 and 5 mM), but not by linopirdine (100 μM) or chromanol 293 B (10μM), a blocker of KCNQ1. These results suggest that a K_{Ca} channel, which is different from Maxi-K^+ and IK channels may be present in RSA cells and might be involved, at least in part, in Ca^{2+}-dependent salivary fluid secretion in rat submandibular gland.

Nerve Growth Factor-induced membrane hyperexcitability of rat sensory neurons in culture

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Primary sensory neurons isolated from neuropathic model animals produced by an artificial nerve injury show spontaneous action potentials, and such hyperexcitability of the sensory neurons is considered to be a cause of neuropathic pain. It was reported that an expression level of Nerve Growth Factor (NGF) in the Dorsal Root Ganglion (DRG) of the neuropathic model animals was increased in hours after sciatic nerve constriction, suggesting that NGF played an important role in the pathogenesis of neuropathic pain. However, it is not clear whether the spontaneous firing of the DRG neurons was induced by a direct action of NGF to neurons. In the present study, I performed the patch clamp recording and examined the changes in the membrane excitability of DRG neurons of rats during culture in the presence of NGF.

Under a cell-attached configuration, almost all the control DRG neurons cultured without NGF showed no spontaneous spike. On the contrary, a certain proportion of DRG neurons cultured with NGF showed spontaneous spikes. The proportion of neurons showing spontaneous spikes increased with days of culture in the presence of NGF. The frequency and amplitude of each spike were stable in each neuron and voltage-gated sodium channel blockers, tetrodotoxin (TTX) or lidocaine, abolished spontaneous spikes in accordance with "all-or-none" fashion, suggesting that spontaneous spikes recorded under the cell-attached configuration were resulted from action potentials. Seventy-five percent of the neurons showing spontaneous spikes under the cell-attached configuration evoked transient spontaneous inward currents (named "I_{sp}") under a whole-cell voltage-clamped condition at −50mV. I_{sp} occurred even at the hyperpolarized membrane potentials over −80 mV, and its inward current did not reverse to the outward direction even at the depolarized membrane potential over +30mV. The ampli-

tudes of I_{sp} were not influenced by the reduction of the extracellular Ca^{2+}. These results suggested that the charge-carrying ion of I_{sp} was Na^+. Furthermore, I_{sp} was blocked by TTX and lidocaine in all-or-none fashion. K^+ channel blocker cocktail (2 mM 4-AP, 3 mM TEA, 0.5mM $BaCl_2$) prolonged the fall time, from the peak to the baseline of I_{sp}. These results suggested that I_{sp} was an echo of the action potentials that generated in the localized compartment of neurons in which the membrane potential is not clamped sufficiently because of the space-clamp limitation. I_{sp} was also observed under the outside-out voltage-clamped configuration. The kinetics and the pharmacological properties of I_{sp} in the outside-out patch membrane were similar to those in the whole-cell. The structure, which was difficult to be voltage-clamped seemed to exist even in the outside-out excised patch membrane, and showed spontaneous firing. These results indicated that the spontaneous firing in NGF-treated DRG neurons was caused by the change in the excitability of neuronal membrane itself. All neurons showing I_{sp} in whole-cell voltage-clamped mode generated spontaneous action potentials under a current-clamped mode. The pharmacological property of these spontaneous action potentials in terms of the voltage-gated Na^+ channel blocker was the same as that of I_{sp} in the voltage-clamped mode.

In the present study, it was clarified that the chronic NGF treatment caused the hyperexcitability of the DRG neurons. NGF upregulated in the DRG of the neuropathic model animals may act to the DRG neurons directly and increase the excitability, which results in the spontaneous firing causing the neuropathic pain.

5-Hydroxytryptamine potentiates vanilloid receptor-1 mediating responses in rat primary sensory neurons

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To identify the mechanism of 5-hydroxytryptamine (5HT)-induced hyperalgesia, I examined whether 5 HT changed the vanilloid receptor 1 (VR1) function in primary sensory neurons of the neonatal rats. The effects of 5 HT on increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$), changes of membrane potentials and inward currents evoked by capsaicin were examined in dorsal root ganglion (DRG) neurons. 5 HT receptor subtypes involved in hyperalgegia were determined by pharmacological characteristics, PT-PCR and immunocytochemistry.

VR1 expression was confirmed with VR1 immnoreactivity in many of DRG neurons. Application of capsaicin evoked a dose-dependent increase of $[Ca^{2+}]i$. Capsazepine, a VR 1 antagonist, inhibited the response to capsaicin. 5HT potentiated the $[Ca^{2+}]i$ response to capsaicin in a dose-dependent manner. 5HT augmented a $[Ca^{2+}]i$ increase evoked by protons (pH5.5). 5HT increased capsaicin-induced inward current and depolarization. In some cells, capsaicin-evoked depolarization was accompanied with spike discharges in the presence of 5 HT. Bradykinin or prostaglandin

E 2 potentiated [Ca^{2+}]i response to capsaicin. Similar potentiation was produced by phorbol 12 - mylistate acetate, a protein kinase C (PKC) activator, or forskolin, an activator of adenylyl cyclase. Bisindoylmaleimide Ⅰ (Bis), a PKC inhibitor, or N- [2 - (p-bromocinnamyl -amino) ethyl] - 5 - isoquinoline-sulfonamide (H-89), a protein kinase A (PKA) inhibitor, suppressed the potentiating effect of 5HT on [Ca^{2+}]i response to capsaicin. Bis itself partly inhibited the responses to capsaicin. RT-PCR indicated the expression of 5HT1B, 1D, 1F, 2A, 3, 4, 5B, and 7 receptor subtype mRNAs in neonatal rat DRG neurons. Both 5HT2A and 5HT7 receptor immunoreactivities co-existed in the same cells in many of DRG neurons. (±) - 2, 5 - Dimethoxy - 4 - iodoamphetamine (DOI), a 5HT2A agonist, or (±) - 8 - hydroxy -2 (di-n-propylamino) tetralin hydro-bromide (8-OH-DPAT), a 5HT7 agonist, potentiated the [Ca^{2+}] i response to capsaicin. Ketanserin, a 5HT2A receptor antagonist, or SB269970, a 5HT7 receptor antagonist, inhibited the potentiating effect of 5HT. An inflammation response was evoked by the injection of complete Freund's adjuvant into the hemirateral hind paw of neonatal rat. In DRG neurons of the L 4 - L 6 segments ipsilateral to the CFA injection (inflammation side), expression levels of 5HT2A and 5HT7 receptor subtype mRNAs increased, and the potentiating effect of 5HT on the responses to capsaicin was more prominent compared with that in the contralateral control side.

These results indicate that 5HT potentiates the response to capsaicin via VR 1 in the rat DRG neurons. It is suggested that the PKC-and PKA-mediated signaling pathways are involved in the potentiating effect of 5HT through the activation of 5HT2A and 5HT7 receptor subtypes. Under inflammatory conditions, the increases of the biosynthesis of some 5HT receptor subtypes may lead to the potentiation of VR1 function, and these changes may participate in the generation of inflammatory hyperalgesia.

Intracellular Ca^{2+}increase induced by bradykinin in cultured rat myenteric neurons

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In order to clarify the actions of bradkinin (BK) in the enteric nervous system, changes of intracellular Ca^{2+} concentration ([Ca^{2+}]i) and membrane potentials in response to BK were studied in cultured rat myenteric neurons. Analysis with RT-PCR, pharmacological and immunohistochemical studies were carried out to identify receptor subtypes mediating responses to BK. Involvement of cyclooxygenase (COX) - prostaglandins (PGs) system in BK responses was examined. In addition, the effects of long-term exposure of BK cultured myenteric neurons on [Ca^{2+}]i responses to several receptor agonists were investigated.

In primary cultured myenteric plexus, BK increased [Ca^{2+}]i in 92.9% of neurons and 62.2% of non-neurons. Less than 5 % of neurons were characterized as AH-type neurons, which exhibited an action potential with after

-hyperpolarization. Neurons immnoreactive to calbindin, a marker of AH-type neuron, were less than 10% of neurons. BK increased $[Ca^{2+}]i$ in both calbindin-positive and-negative neurons. BK (100 pM～1 μM) evoked concentration-dependent $[Ca^{2+}]i$ increases in neuron. Repetitive stimuli with high concentrations of BK showed a profound desensitization. Both B1 and B2 receptor mRNA were detected in cultured myenteric plexus. BK-induced $[Ca^{2+}]i$ increase was inhibited by a B 2 antagonist, HOE 140, but not by a B1 antagonist, [Lys-des-Arg^9]-Hoe140. A B1 agonist, [Lys-des-Arg^9]-BK, did not mimic BK-induced $[Ca^{2+}]i$ responses. Immnohistological staining revealed the expression of B2 receptors in most myenteric neurons. Ca^{2+} sources contributing to BK-induced $[Ca^{2+}]i$ increases were determined. $[Ca^{2+}]i$ response to BK was reduced in the presence of a non-selctive voltage-dependent Ca^{2+} channel blocker, Cd^{2+}. In the Ca^{2+}-free solution, BK-induced $[Ca^{2+}]i$ increase was greatly reduced, but not abolished. After treatment with thapsigargin to depletes Ca^{2+} in intracellular stores, BK-induced $[Ca^{2+}]i$ increase was partly declined. $[Ca^{2+}]i$ increase induced by BK was inhibited by a non-selective COX-inhibitor, indomethacin or a selective COX 1 inhibitor, piroxicam. Application of PGE_2 but not PGI_2, potentiated BK-induced $[Ca^{2+}]i$ increase. The suppressed response to BK by indomethacin was recovered by the application of PGE_2. EP_3 agonist, sulprostone, did not mimic the potentiating effect of PGE_2. EP_1 antagonist, SC 19220, inhibited BK-induced $[Ca^{2+}]i$ increase. Neither indomethacin nor PGE_2 affected BK-induced $[Ca^{2+}]i$ increase in the Ca^{2+}-free solution, of which response was mediated by Ca^{2+} release from intracellular stores. On the contrary, in cells treated with thapsigargin, BK-induced $[Ca^{2+}]i$ responses was suppressed by indomethacin and potentiated by PGE_2, suggesting that COX-PGs system was related to BK-induced Ca^{2+}-influx. BK significantly augmented PGE_2 release from cultured myenteric plexus.

In cells cultured at low density, the amplitude of $[Ca^{2+}]i$ increase by BK was smaller than that at high density. Indomethacin inhibited BK-induced $[Ca^{2+}]i$ increase at high density, but not at low density. BK caused persistent depolarization which was accompanied with spike discharges in some neurons. BK lowered a current intensity required for evoking action potentials. In myenteric neurons cultured in the presence of BK for 2 days, $[Ca^{2+}]i$ response to BK was increased, but those nicotine was decreased.

These results suggest that BK increases $[Ca^{2+}]i$ through activation of B2 receptors in rat myenteric neurons, which is maily due to Ca^{2+} influx of extracellular Ca^{2+}. BK-induced Ca^{2+} influx is partly mediated by PGE_2 which is released through COX 1 activation and acts on EP 1 receptor in a paracrine fashion. In addition to $[Ca^{2+}]i$ responses, BK depolarizes membrane potential so as to modulate the electrical excitability in rat myenteric neurons.

Involvement of adenosine in synaptic depression induced by hypercapnia in isolated spinal cord of neonatal rat

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In order to determine the mechanisms of synaptic depression and antinociception by carbon dioxide(CO_2), we examined the effects of the artificial cerebral spinal fluid gassed with 20% CO_2 (hypercapnic ACSF) on spinal reflexes in isolated spinal cord of neonatal rat *in vitro*. The pH of the ACFS gassed with 5 % CO_2 (normal ACFS) and hypercapnic ACSF was 7.3 and 6.7, respectively. The superfusion of hypercapnic ACSF (pH 6.7) inhibited the monosynaptic reflex potential (MSR) and the slow ventral root potential (sVRP). The solution adjusted to pH 6.7 with 5 % CO_2 (5 % CO_2 solution) also caused the similar inhibition of MSR and sVRP. Hypercapnic ACSF (pH 6.7) and 5 % CO_2 solution (pH 6.7) had no effect on the compound action potentials derived from A- and C-fibers in the L3 dorsal root fiber. Hypercapnic ACSF (pH 6.7) did not affect the depolarization elicited by glutamate.

A $GABA_A$ receptor antagonist (bicuculline) and glycine receptor antagonist (strychnine) enhanced sVRP, but had no effect on the inhibition of MSR and sVRP by hypercapnic ACSF (pH 6.7). An opioid receptor antagonist (naloxone) and α_2 adrenoceptor antagonist (atipamezole) also had no effect on it. An adenosine A_1 receptor antagonist (8-cyclopentyl-1,3-demethylxanthine) significantly decreased them in response to hypercapnic ACSF (pH 6.7) and 5 % CO_2 solution (pH 6.7).

An ecto-5'-nucleotidase inhibitor (α,β-methylene ADP) increased the frequency of spontaneous depolarization, but had no effect on the inhibition of MSR and sVRP by hypercapnic ACSF (pH 6.7). While an adenosine deaminase inhibitor (EHNA) depressed sVRP but not MSR, an adenosine kinase inhibitor (5'-amino-5'-deoxyadenosine) depressed MSR and sVRP. The depression by the adenosine kinase inhibitor was reversed by and A_1 receptor antagonist.

The rise in extracelluar Ca^{2+} concentration enhanced MSR and sVRP. Under this condition, the inhibition of MSR and sVRP by hypercapnic ACSF (pH 6.7) was significantly reduced.

Extracellular adenosine concentration was increased by the incubation of isolated spinal cords for 10 min with hypercapnic ACSF (pH 6.7).

In behavior experiments, inhalation of 20% CO_2 for 1 min significantly decreased body movement induced by capsaicin in comparison with inhalation of 5 % CO_2.

Theses results suggest that the synaptic depression by hypercapnia is mediated by the reduction of pH resulting in the release of adenosine. An activation of adenosine A_1 receptor by endogenous adenosine may causes the inhibition of transmitter releases, which mediate spinal cord reflexes (MSR and sVRP) in isolated spinal cord of neonatal rat. Inhalation of 20% CO_2 causes the antinociception, suggesting the involvement of endogenous adenosine in the antinociception by hypercapnia.

Analysis of the inhibition mechanism of the interferon production by the pestivirus infection

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Members of the genus *Pestivirus* of the family *Flaviviridae* such as classical swine fever virus (CSFV) and bovine viral diarrhea virus are characterized by the existence of two biotypes, cytopathogenic (cp) and noncytopathogenic (ncp) virus populations. Most of the ncp pestiviruses show END phenomenon that is based on enhancement of the growth of Newcastle disease virus (NDV) by pestivirus infection in cell cultures. It is postulated that the END phenomenon is caused by the inhibition of type 1 interferon (IFN) production due to pestivirus infection, but the mechanism remains to be elucidated. Recently, it has been revealed that transcriptional factor IRF-3 (IFN regulatory factor 3) is the target to prevent IFN production by other viruses such as NDV and influenza viruses. In the present study, the amount and the location of the IRF-3 protein in the cells during virus infection were analyzed.

Swine and bovine IRF-3 genes were cloned, and eleven monoclonal antibodies against recombinant IRF-3 protein expressed in *Escherichia coli* were produced. Of these three clones, especially clone 34/1, specifically detected swine IRF-3 protein by immunofluorescence assay and western blotting analysis in cultured cells of swine origin.

By the infection of CSFV strain ALD, which shows END phenomenon, IRF-3 protein was downregulated and IFN was not detected. On the other hand, IRF-3 protein was translocated into the nucleus and IFN was produced by the infection of CSFV strain GPE$^-$, which was cloned in the laboratory from the END phenomenon positive virus population, does not induce END phenomenon, and interfere with the growth of NDV. Then it is suggested that IFN production was inhibited by the pestivirus infection causing downregulation of IRF-3 protein.

Analysis of the mechanism of the IRF-3 protein downregulation and identification of virus protein responsible for the phenomenon is underway.

Analysis of pathogenicities of highly pathogenic avian influenza virus A/chicken/Yamaguchi/7/04 (H5N1) in chickens, quails, starlings, parakeets, mice, and miniature pigs

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Recent outbreaks of highly pathogenic avian influenza (HPAI) in chickens and ducks that occurred in 9 Asian countries including Japan alarmed to realize that there is no bor-

der for infections and gave a rise to great concern for human health as well as for livestock industries. In Japan, the outbreaks were effectively eradicated by proper control measures, though the route of transmission of this virus infection is still unknown. It is important to prepare for the next outbreaks to minimize the damage. This study was aimed to clarify the host range and pathogenicity of HPAI viruses in different species of animals and birds and to develop effective vaccine for the control of next HPAI outbreak.

A/chicken/Yamaguchi/7/04(H5N1)[Ck/Yamaguchi/04], which was isolated from chickens in Japan, had the ability to replicate and cause disease in chickens, quails, starlings, and parakeets in the laboratory and showed high pathogenicity for the 4 avian species. On the other hand, mice were susceptible to infection with Ck/Yamaguchi/04 but with a mild pathogenicity. In contrast, miniature pigs were not susceptible to Ck/Yamaguchi/04. These results demonstrate that Ck/Yamaguchi/04 is much highly virulent in avian species but has low or no pathogenicity for mammalian species.

To establish vaccine strain against HPAI virus, Ck/Yamaguchi/04, R(A/duck/Mongolia/54/01-A/duck/Mongolia/47/01) (H5N1) was obtained as reassortant. Inactivated test vaccine was prepared from the allantoic fluid of embryonated chicken eggs infected with the reassortant virus. This reassortant showed a low pathogenicity for chicken embryos and chickens. The chickens immunized subcutaneously or intranasally with the test vaccine, did not show severe disease signs or death upon the challenge with Ck/Yamaguchi/04. Therefore, this vaccine strain candidate should be ideal against Asian H5N1 HPAI virus including Ck/Yamaguchi/04. It is needed to determine the dose and route of vaccination, for the practical use against HPAI outbreaks.

Establishment of seed vaccine strains of H 5 and H 7 influenza viruses against highly pathogenic avian influenza

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Since December 2003, outbreaks of highly pathogenic avian influenza (HPAI) caused by infection with H5N1 virus have occurred in 9 Asian countries. In Japan, three different outbreaks were successfully controlled by proper measures for the prevention of the disease spread. However, it is important to prepare for the reintroduction of the H5N1 HPAI virus in poultry and for the emergence of human pandemic influenza. The aim of the present study is to establish H5 and H7 influenza virus strains for vaccine use. Therefore, 19 H5 and a H7 influenza virus strains were analyzed for their antigenicity, genome structure, and pathogenicity.

To analyze the antigenicity of the H5 HA of virus isolates, monoclonal antibodies against A/duck/Pennsylvania/10128/84(H5N2) virus were generated. A panel of monoclonal antibodies recognizing 6 different epitopes on the H5 hemagglutinin (HA) molecule was established and used for the analysis of the an-

tigenicity of 19 H5 virus isolates. It was found that no difference in the antigenicity between the currently circulating H5 HPAI viruses and low pathogenic avian influenza (LPAI) viruses of duck origin.

To enhance the growth capacity of the vaccine strain candidates of H5N1 and H7N7 viruses in embryonated eggs, reassortant H5N1 and H7N7 viruses were generated by the introduction of the internal protein genes from a high-growth H9N2 LPAI virus strain. Three vaccine candidate strains, R(Duck/Mongolia/54/01-Duck/Mongolia/47/01-Duck/Hokkaido/49/98) [R(MM-Hok49)](H5N1), R(Duck/Pennsylvania/10128/84-Duck/Mongolia/47/01-Duck/Hokkaido/49/98) (H5N1), and R(Duck/Mongolia/736/02-Duck/Hokkaido/49/98) [R(Mong736-Hok49)](H7N7) showed that their growth capacities in embryonated eggs were higher than those of their parental H5N1 and H7N7 viruses.

The results of the pathogenicity tests of the high-growth strains in chickens and chicken embryos showed that R(MM-Hok49) (H5N1) and R(Mong 736-Hok 49) (H7N7) were low pathogenic, indicating that these viruses should be useful for vaccine strains against H5 and H7 influenza virus infections.

The effect of colostrum feeding on vertical transmission of bovine leukemia virus

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Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leukosis. The major preventive method is "test and slaughter" because no valid vaccine is available. BLV is mainly spread through iatrogenic transfer of BLV-infected lymphocytes. However, in the present time, the transmission via milk contaminated with BLV-infected lymphocytes and *in utero* transmission have been suspected as alternative transmission routes of BLV infection. However, the detailed frequency of *in utero* and milk-borne transmission of BLV remains unclear. Thus, in this study, BLV infection was surveyed by nested PCR in neonatal calves in a herd with the BLV prevalence of 41.1% to evaluate the frequency of vertical transmission of BLV.

In a group of dams with the proviral load of over 10%, 2 out of 6 neonates (33%) were BLV-positive at birth determined by nested PCR, showing high frequency of *in utero* transmission. Furthermore, a follow-up survey was also done for a period of 3 months to estimate the relative importance of colostrum feeding and the risk of milk-borne transmission. When 4 calves born from BLV-positive dams were fed with a milk replacer at the first suckling, and subsequently fed with pooled milk contaminated with milk from BLV-positive dams, one of them turned out to be BLV-positive at 3 months after birth. These results suggest the importance of colostrum-acquired maternal antibody for the protection against milk-borne transmission of BLV. Although both BLV proviruses and infected lymphocytes which can transmit BLV were detected in colostrum from a considerable number of BLV-positive dams, maternal antibody which was also present in the colostrum significantly suppressed the infectivity of BLV

from these infected lymphocytes as evidenced by the inhibition of syncytium formation *in vitro*. In addition, the positive correlation in antibody titers was found between maternal antibody titers in colostrum and serum antibody titers of dams. This observation indicates that the risk of milk-borne transmission of BLV can be estimated by measuring the serum antibody titers of dams.

Preparation of anti-ganglioside GM3 monoclonal antibody for therapeutic application against canine melanoma

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Over 400 kinds of glycolipids are synthesized in human and animals, and a wide variety of biological functions of these glycolipids have been reported. Until recently, in addition to physicochemical tools, only lectins have been used as probes to analyze the biological properties of glycolipids. However, these methodologies are insufficient since they are not specific enough to differentiate each of glycolipids nor convenient for *in vivo* analysis. To overcome these problems, highly specific anti-glycolipid monoclonal antibodies (MoAbs) may be useful for both *in vitro* and *in vivo* analyses. In this study, an anti-ganglioside GM3 MoAb (No. 2-14) was successfully produced by immunizing mice with liposomes in which GM3 was inserted, though glycolipids such as GM3 are not so immunogenic to mice.

Recently, GM3 has been shown to be abundantly expressed on the surface of human and mouse melanoma cells, and development of anti-melanoma therapy using anti-GM3 MoAbs is now in progress in human. Thus, GM3 may also be a candidate tumor-associated antigen (TAA) as a target for anti-melanoma therapy in other animals including dogs. When glycolipids extracted from dog melanoma cell lines were analyzed on thin-layer chromatography and subsequent immunostaining with anti-glycolipid MoAbs, GM3 was highly expressed in these melanoma cell lines. In addition, by immunofluorescence test, GM3 was found to localize on the surface of the cell line, but not detected in normal blood cells of dogs. These results suggest that, as reported in human and mouse melanomas, the high level of the GM3 expression is also specific to dog melanomas though all of normal tissues were not analyzed in dogs. When the therapeutic effect of the anti-GM3 MoAb (No. 2-14) on dog melanomas was examined, this MoAb showed complement-dependent cytotoxicity *in vitro* against dog melanoma cells. Furthermore, the growth of a melanoma transplant in BALB/c nu/nu mice was partially suppressed when these mice were treated with the anti-GM3 MoAb (No. 2-14). These results raise the possibility that anti-GM3 MoAb could be used for anti-melanoma therapy in dogs.

In the future, it would be necessary to further examine whether GM3 is a suitable as a candidate TAA in dogs. For the clinical application of anti-GM3 MoAb, artificial immunotoxins consisting of single-chain antibody

and antitumor compounds can be developed, or chimeric antibody can be produced by joining the variable regions of this MoAb with the constant regions of canine antibodies.

Comparative analysis of the genomes of Marek's disease virus derived from chickens and white-fronted geese

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Marek's disease (MD) can be almost completely controlled by vaccination with nonpathogenic strains of Marek's disease virus (MDV). However, the use of more effective vaccines to control MD resulted in a notable increase in the virulence of MDV field isolates. In the 1990s, several MDV strains more virulent than very virulent MDV (vvMDV) strains were isolated in many countries, and they were classified into a new category of pathogenic MDV, very virulent plus MDV (vv+MDV). In 1997, an outbreak of MD at the rate of 7 ～ 8 % occurred in a poultry farm in Hokkaido. This MD incidence was unusually high whereas chickens had been appropriately vaccinated. When some genes of this MDV strain were compared with those of another strain isolated from an MD case in Sapporo, Hokkaido in 2003, the nucleotide sequences of these genes were exactly same between these two Hokkaido strains. These two MDV strains showed apparent differences in the genes such as *meq, glycoprotein* B and L genes compared to other known MDV strains. However, no mutations, which were suggested to be consistent with the increase in MDV virulence, were identified in the two Hokkaido strains.

In 2001, an MD case was reported for the first time in the world in a white-fronted goose (*Anser albifrons*), a migratory bird to the Hokkaido area, raising the possibility that MDV carried by migratory birds could potentially spread over poultry farms in Hokkaido. Thus, epidemiological survey of MDV was performed, and the high rate of MDV infection was confirmed in white-fronted geese. The most of the *meq* gene sequences detected in these birds were identical to those of a vv+MDV strain, W, and a vvMDV strain, Md 5. However, the *meq* gene derived from one white-fronted goose was partially identical to that of the two Hokkaido MDV strains. These results suggest the relationship between MDV strains which cause MD in chickens and MDV strains which white-fronted geese carry.

I dentification of host animals of feces by multiplex-PCR, for epizootiological study of echinococcosis

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Echinococcus multilocularis is a zoonotic parasite which causes human alveolar echinococcosis. It is important to assess the prevalence among the definite hosts because the prevalence is related to the risk of the infection in humans. Epizootiological studies of echinococcosis in the definitive hosts have been conducted using feces collected in the field. However, host animals of feces are usually judged by their appearances and other characteristics, which may not be always correct. To overcome this problem, multiplex-PCR was developed for the identification of host animals of feces.

First of all, methods of DNA extraction from feces were evaluated. Extraction was more efficient by using washings of the fecal surface with buffer than mixture of feces with buffer, because the washing method enabled the collection of intestinal mucosal cells which were allocated on the surface of feces and the minimal inclusion of PCR inhibitors originated from feces. Moreover, QIAmp DNA Stool Mini Kit was shown to be more efficient to remove PCR inhibitors during DNA extraction than the other methods using potato flour or GuSCN. When two methods for sterilization of the parasite eggs, freezing feces at －80℃ for 2 weeks and heating feces at 70℃ for 12 hours, were compared, higher DNA concentration and more number of successful PCR results were obtained in frozen samples. D-loop region in mitochondrial DNA was targeted for the multiplex-PCR. PrH, which sequence is conserved among most mammalian species, was used as a reverse primer. Six forward primers, spFox, spRdg, spDog, spCat, spRcn and spWsl, were designed so that the following conditions were satisfied : the six primers were specific to fox, raccoon dog, dog, cat, raccoon and weasels, respectively ; the sizes of PCR products were about 160 bp for fox and cat, about 240 bp for raccoon dog and raccoon, and about 330 bp for dog and weasels. In order to distinguish animals by the size of PCR products, two primer mixtures, Mix 1 (spFox, spRdg, spDog and prH) and Mix 2 (spCat, spRcn, spWsl and prH), were used in the multiplex-PCR. The PCR system showed no cross reaction among the carnivores. Moreover, the system showed no reaction to DNA from rodents, which are probably preyed on by carnivores in Hokkaido. The minimum amount of DNA required for positive reaction in the PCR was less than 10 pg per 20μl reaction volume. In addition, feces could be used for successful DNA extraction and following PCR amplification even after exposure to outside environment for 8 weeks although the amount of extracted DNA decreased.

Host animals could be identified by the multiplex-PCR in 140 of 147 feces (95%) collected in the field of Hokkaido, Japan.

These results suggested that the multiplex-PCR identification would provide precise evaluation of prevalence in specific animals in epizootiological studies of *Echinococcus multilocularis*.

Genetic analysis of the congenital hypothyroid mouse DW/J-*grt*

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The DW/J-*grt* mouse has an autosomal recessive, fetal-onset, severe hypothyroidism related to a thyroid-stimulating-hormone (TSH) hyporesponsiveness and associated with an elevated TSH. We have previously assigned the *grt* locus on 60 centimorgan (cM) distal from the centromere of Chr 5 using a linkage analysis. In this study, one additional marker *D5Mit24* was genotyped relative to the *grt* gene in 1,084 backcross progenies, showed no recombination with the *grt*, indicating linkage of <0.1 cM. Therefore, we performed expression and sequence analyses of the genes closely located to *D5Mit24* and identified a point mutation in tyrosylprotein sulfotransferase 2 (*Tpst 2*), which leads to the replacement of a highly conserved histidine with a glutamine at amino acid position 266 in the catalytic domain of the enzyme. TPST transfers a sulfonyl group from the donor 3'-phosphoadenosine 5'-phosphosulfate onto a tyrosine residue within a target protein, and tyrosine sulfation is a widespread post-translational modification, which intensifies protein-protein interactions. The recent study showed that tyrosine sulfation of TSH receptor (TSHR) was required in TSH-TSHR binding that is essential for thyroid hormone secretion and thyroid development. The *in vitro* TSPT assay revealed that the substitution in the Tpst 2 resulted in a loss of tyrosylprotein sulfotransferase activity. Further, Tpst 2 in the two Tpst isoforms is predominantly expressed in thyroid and has a high substrate specificity for the peptide sequence from TSHR. Therefore, I concluded that the hypothyroidism in the *grt* mouse was caused by a mutation of *Tpst 2*, which leads to the defect in TSH signaling and thyroid development.

Genetic linkage analysis of resistance to Sendai virus infection in inbred mice

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Sendai virus, a parainfluenza type 1 virus, is a serious respiratory pathogen of rodents. The differences in resistance of various inbred strains of mice to Sendai virus have been known. For example, C57BL/6 mice are much more resistant to Sendai virus infection than DBA/2 mice. However, genetic factors of resistance/susceptibility to this virus are unknown. In the present study, we examined a resistance to Sendai virus infection using C57BL/6, DBA/2, (C57BL/6X DBA/2) F_1 (BDF_1) and BDF_2 mice. C57BL/6 mice had a longer survival time than DBA/2 after inoculation of Sendai virus, and BDF_1 mice showed similar

result to that of C57BL/6. In contrast, there was a remarkable difference in survival time among BDF_2 mice, suggesting that survival times against Sendai virus infection are controlled by multiple genetic loci. A genome-wide analysis of linkage with survival time revealed two weak quantitative trait loci (QTL) on chromosome 11 between D*11Mit21* and *D11Mit4* (20 cM-37 cM) loci and on chromosome 1 around *D1Mit291* (101.5 cM) locus. Comparing genotype with phenotype on each marker suggested that C57BL/6 have a resistant allele on *D11Mit21-D11Mit4* loci and a susceptible allele around *D1Mit291* locus. There are some genes that effect host antiviral activity, such as *Il12b*, *Irf1* and *Hlx*. Our results suggest that there are polymorphisms in these genes as providing strain difference in resistance to Sendai virus.

Production of neomycin-resistant transgenic mice and analysis of their phenotype

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In 2002, genome sequence of the human malaria parasite *Plasmodium falciparum* clone 3D7 was reported. Moreover, the strategy to produce specific gene knockout malaria parasites has been established and the analysis of the gene function has been facilitated. Genome project of the other protozoas such as *Babesia* and *Theileria* parasites, which are responsible for lots of cases of babesiosis in a wide variety of wild and domestic animals, is also in progress. However, some *Babesia* and most *Theileria* parasites were not succeeded to produce knockout parasites, since they could not be cultivated *in vitro*. To overcome this problem, *in vivo* screening system for the knockout parasites is necessary. In this study, as the first step to establish *in vivo* screening system of the hematozoa, I attempted to produce neomycin-resistant transgenic mice.

After a neomycin-resistant gene was injected to approximately 5,400 fertilized eggs, 3,000 eggs were transferred to 100 recipient ICR mice and 202 mice were born. Among them, only 1 mouse possessed transgene. The systemic expression of the transgene in all tissues and *in vivo* resistance to the drug were observed in this transgenic mouse and its progenies. A neomycin-resistant transgenic mouse produced in this study will be useful to establish *in vivo* screening system for gene-knockout hematozoa.

The role of lipid-raft protein stomatin for remaining the Na, K-ATPase of canine erythrocytes, characterized with hereditary high Na, K-ATPase activity

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In the nomal canine erythrocytes containing low potassium (LK) and high sodium concentrations (ie, LK erythrocytes), it is known that Na, K-ATPase disappears during their maturation. It has also been reported that stomatin, an erythrocyte membrane protein, disappears with Na,K-ATPase. In contrast, some dogs have erythrocytes containing high potassium (HK) and low sodium concentrations as a result of an inherited high Na,K-ATPase activity (HK erythrocyte). In HK erythrocytes, it has also been suggested that a large amount of stomatin remains along with Na, K-ATPase. Accordingly, in the present study, quantitative and qualitative analyses of stomatin was performed in HK erythrocytes and LK erythrocytes to compare the too. I also investigated the role of stomatin in the decrease of Na, K-ATPase. The results showed the amount of stomatin was well correlated with that of Na, K-ATPase. This suggested that stomatin might interact directly with Na, K-ATPase. There were no structural differences in stomatin between HK and LK erythrocytes, and HK reticulocytes contained a larger amount of $mRNA^{-}$ coding stomatin than LK reticulocytes. Moreover, in HK reticulocytes, stomatin and Na, K-ATPase decreased during its maturation into erythrocytes, the same as in LK reticulocytes. These results indicated that the over expression of stomatin and Na,K-ATPase in the HK reticulocytes might cause these to remain in HK erythrocytes. Furthermore, in the reticulocytes culture in vitro, stomatin and Na, K-ATPase disappeared from their membranes and were detected in the vesicle which was released into the supernatant. Some of them were also detected in the same lipid-raft fraction. These results showed that they might relate to each other in the same lipid-raft, and they might be released as vesicle during the maturation into erythrocytes. Although the role of stomatin was still unclear, it seemed to be related to the disappearance of Na, K-ATPase by the vesicle. In HK reticulocytes, the over expression of Na, K-ATPase might cause that of stomatin secondarily.

The effects of an ionophore, salinomycin, against *Babesia gibsoni* and canine erythrocytes

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The objective of the present study was to investigate the effects of salinomycin, a neutral ionophore which catalyzes the diffusion through membranes of alkaline cations, gen-

erally potassium ions according to the membrane potential, on *Babesia gibsoni in vitro*. The toxicity of salinomycin against canine erythrocytes was also examined.

In this study, the effects of salinomycin against *B. gibsoni* were examined using two types of canine erythrocytes, HK and LK erythrocytes. HK dog erythrocytes have high intracellular K^+ and low Na^+ concentrations through the function of Na, and K-ATPase being present genetically in their membranes. LK cells are normal dog erythrocytes containing a high Na^+ and low K^+ in their cytosol, lacking the ATPase. *B. gibsoni* was cultured with HK and LK cells, respectively.

When salinomycin was added to each of the cultures, the number of *B. gibsoni* within LK cells markedly decreased, while the number of the parasites in HK erythrocytes was not changed. On the other hand, the intracellular concentrations of Na^+, K^+ and adenosine 5'-triphopahate in HK erythrocytes were all changed after the treatment of the cells with salinomycin, though the concentration of intracellular K^+ was still higher and Na^+ was lower than those in LK cells. The concentrations of potassium and sodium in LK erythrocytes was almost unchanged.

These results suggested that salinomycin might directly injure *B. gibsoni* within canine LK erythrocytes by the function of changing cation concentrations in the parasite cytosol, as was shown in HK erythrocytes treated with salinomycin. They also suggest that the cytosol of *B. gibsoni* may contain relatively high potassium and low sodium concentrations.

Rapid screening of GM1 gangliosidosis in dogs using matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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GM1 gangliosidosis is a lysosomal disease which develops from a deficiency of β-galactosidase and is inherited as an autosomal recessive trait. GM1 ganglioside, the major substrate of the enzyme, accumulates in the brain and other visceral organs resulting in progressive neurologic dysfunction. The concentration of GM1 ganglioside in cerebrospinal fluid (CSF) is markedly increased in Shiba dogs with GM1 gangliosidosis due to GM1 ganglioside accumulation in the central nervous system and the leakage to CSF. In the present study, we directly detected GM1 ganglioside in CSF of affected Shiba dogs with GM1 gangliosidosis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and determined the utility of this method as rapid screening in domestic animals with GM1 gangliosidosis.

Standard solutions of GM1 and GM2 gangliosides, and CSF of Shiba dogs with GM1 gangliosidosis were analyzed. One μl of the specimens and 1.5μl of a matrix solution containing 2,5-dihydroxybenzoic acid were mixed, and 1 μl of the mixture was spotted onto the sample plate, and then allowed to dry. The analysis of CSF required a deionizing

step which involved adding 2 μl of 0.1% trifluoroacetic acid to the sample spot and then removing the supernatant. We used 3 different sample plates containing an AnchorChip plate designed so that sample droplets shrink and become concentrated during solvent evaporation.

Signals of GM1 and GM2 gangliosides could be detected in the standard solutions at over 50 nmol/l using an AnchorChip plate which showed approximately 10-fold sensitivity compared with the other plates. GM1 gangliosides in CSF (131-618nmol/l) of 4-11month-old affected dogs was also detected after the deionizing step. On the other hand, GM2 gangliosides in CSF could not be detected although the concentrations of CSF GM2 gangliosides were 80-127 nmol/l, which were enough high to be detected in the standard solution.

These results showed CSF GM1 gangliosides of Shiba dogs with GM1 gangliosidosis can be detected easily using MALDI-TOF-MS under appropriate conditions as employed in the present study. This MALDI-TOF-MS method was much more rapid than the thin-layer chromatography/enzyme immunostaining method which had been employed for measurement of gangliosides. Therefore, this method will be useful for the rapid screening of canine GM1 gangliosidosis.

A study on the effectiveness of tyrosine kinase inhibitor and on the gene mutation in canine mast cell tumor

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Mast cell tumor (MCT) is one of the most common malignancies encountered in dogs. Only minor progress has however been made in its medical intervention so far. Recently, it was reported that KIT, one of the tyrosine kinase (TK) receptors, was activated ligand-independently and that the activation was a main mechanism of carcinogenesis in canine MCT.

Imatinib mesilate, TK inhibitor, dramatically inhibited tumor progression in tumors with ligand-independent receptor TK activation. Therefore, examination on the effectiveness of TK inhibitor was expected to be beneficial to evaluate to antitumor ability.

In the present study, mutation of c-kit gene was firstly investigated in three canine MCT cell lines. Secondly, TK phosphorylation was evaluated in a canine MCT cell line with non-c-kit mutation. Finally, existence of c-kit mutation in spontaneously developed canine MCTs was investigated.

It was made apparent in the present study that TK phosphorylation was correlated with c-kit mutation in canine MCTs. All of reported TKs were not phosphorylated in VI-MC cell line. Considering this outcome, there is a possibility that other protein kinase has some abnormalities. In clinical cases, c-kit mutation was observed at the incidence rate reported previously.

Therapeutic effects of oral administration of green lipped mussel extracts on chymopapain-induced osteoarthritis in rats

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Osteoarthritis (OA) is a disease of synovial joints characterized by destruction of articular cartilage and osteophyte formation. In recent years, non-steroidal anti-inflammatory agents and disease modifying OA products, such as neutraceuticals containing glucasamin and/or chondroitin sulfate, and intraarticular hyaluronic acid, are now available as therapeutic tools to modify disease process of OA, while mechanisms of these agents are not fully understood. Omega-3 fatty acids and glucosamine are two major components of green lipped mussel, which would be one of neutraceuticals for OA treatment.

In this study, 24 rats were randomly assigned to four groups based on following conditions: oral administration of green lipped mussel ethanol extracts (n=6), oral administration of meloxicam (n=6), no food supplement given (no-treatment; n=6) and arthrocentesis for saline injection only (control; n=6). From 2 weeks before the injection of 0.1mg of chymopapain through the end of the experiment, food preparation containing 300 mg/kg of green lipped mussel extracts were given in 6 rats. Synovial hyarulonic acid concentrations were measured. Histological findings of articular cartilage and synovium were also examined when animals were sacrificed, at 3 or 6 weeks after the enzyme injection.

In rats given green lipped mussel ethanol extracts, histological changes of the enzyme-induced destruction of articular cartilage were relatively mild, compared with no-treatment or meloxicam given rats. The highest concentration of hyaluronic acid in synovial fluid was seen in mice given green lipped mussel ethanol extracts.

In conclusion, histopathological changes responsible for synovitis in artificially induced OA might be anyhow suppressed by oral administration of gleen lipped mussel ethanol extracts. Synthesis of endogenous hyaluronic acid might be accelerated by food supplementation of this extracts. Oral administration of green lipped mussel ethanol extracts might therefore be suggestive as one of effective disease modifying agents in the course of OA treatments in dogs.

The effect of photodynamic therapy with Benzoporphyrin derivative monoacid ring A (BPD-MA) in three different murine tumors

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Photodynamic therapy (PDT), which destructs a neoplastic tissue by the excitation light delivery to the tumor after administration of tumor-specific photosensitizers, is expected an effective local anti-tumor treatment. Recently, various new agents have been developed for improving their tissue penetration potential and for decreasing side effects, such as photosensitive dermatitis. In this study, *in vitro* cellular uptake of BPD-MA was examined in cell lines of three different murine tumors including B16F1 (melanoma), LM8 (osteosarcoma with high metastatic potential to the lung) and KLN205 (squamous cell carcinoma). Photodynamic *in vivo* effects of BPD-MA, which was activated by the light at the wavelength of 690nm, were also evaluated at two different timing, 15 minutes and 3 hours, after the intravenous injection of the drug into mice bearing one of these three tumors.

Intracellular concentrations of BPD-MA in three cell lines were measured before and 1, 3, 6, 12 and 24 hours after incubation in the media with BPD-MA at the concentration of 1, 2 or 4 mg/ml. Of these cell lines, the largest amount of cellular uptake of BPD-MA was seen in B16F1. That of KLN205 was likely to be larger than that of LM8.

Growth of tumorous tissues in tumor-implanted mice, which were treated with photodynamic therapy with BPD-MA, was monitored. Maximum anti-tumor effects were seen in KLN205 treated by either drug-light intervals of PDT. LM8 tumor seemed to be more tolerant to PDT than that of B16F1. In mice with these tumors, 15 minutes drug-light interval PDT, which would target on angiogenesis of tumorous tissue, was more effective than 3 hours interval PDT, which should be standard and had direct effects on the tissue.

In conclusion, effects of PDT were obviously different in three tumor cell lines. Simple correlation between *in vitro* cellular uptake of the drug and *in vivo* anti-tumor effect was not detected. These results may suggest that photodynamic effects in anti-tumor treatment would largely depend on features of tumor cells and not on intracellular concentration of the drug. Angiogenic PDT protocol would be much more effective than PDT against tumor cells in destruction of tumor tissues.

The effects of immunization using fragmented tachyzoite antigen on *Neospora caninum* infection in mice

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Neospora (*N.*) *caninum* is maintained in cattle herds by vertical transmission for several generations. Horizontal transmission of the disease by the ingestion of oocysts may also occure in the herds. So far, effective vaccination to prevent *N.caninum* infection is not established.

In the present study, the effects of vaccination on acute or chronic *N.caninum* infection were examined. In immunized group, mice were inoculated subcutaneously with the fragmented tachyzoites of *N.caninum* incorporated with Freund's complete adjuvant. Both immunized and non-immunized mice were challenged by an intramuscular inoculation of *N.caninum* tachyzoites and severities and distributions of the lesions were compared between the groups. The immunization enhanced both acute and chronic lesions in the mice, leading the conclusion that immunization using fragmented *N. caninum* tachyzoite antigen incorporated with Freund's complete adjuvant may not protective against *N. caninum* infection.

Neospora caninum spreads hematogenously in the infected host, however there are some evidences indicating transneural spread of the protozoa. In the present study, distributions and development of the lesions in peripheral and central nervous system were examined. The results indicated the parasites and inflammations ascended from the peripheral nerves to central nervous system, and they tend to be localized in sensory nerve routes. These results supported the transneural spread of *N.caninum*. Moreover, the results from in vitro experiment suggested that *N.caninum*. could spread extra-axonal routes other than intra-axonal routes.

Epidemiologial study on retrovirus-induced astrocytoma －Prevalence in Japan and the virus genome mutations－

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Retrovirus-induced astrocytoma (so-called fowl glioma) is suggested to be a neoplasm caused by an avian leukosis virus subgroup A (ALV-A). ALV commonly induces bursal lymphoma, while the fowl glioma-inducing virus (FGV) is distinct from these ALV-A in that it has neurotropic oncogenesis. Approximately 40% of Japanese fowl in a zoological garden A in Toyama has been suggested to be infected with FGV. Since the zoological garden A is a

major supplier of Japanese fowl and the fertile eggs for other domestic gardens, FGV infection is suspected to be spread among Japanese fowl in Japan by the transportation of the affected chickens and fertile eggs from that garden.

In this study, the prevalence of this desease was examined in 73 Japanese fowls of a zoological garden B in Hokkaido, 36 fowls in a zoological garden C in Tokyo and 24 fowls in a zoological garden D in Tokyo by nested polymerase chain reaction (PCR), reverse transcriptase (RT)-nested PCR and enzyme-linked immunosorbent assay (ELISA). These fowls in the three zoological gardens were originated from the fowls of zoological garden A. In addition, the nucleotide sequence of the 3' untranslated region (3'UTR) and the *env* gene of the 5 ALV isolates were determined because the FGV-specific sequence has been demonstrated in 3'UTR and because the viral envelope has been reported as a major determinant for the induction of neoplasm. Forty seven (64%) of 73 fowls in the zoological garden B were positive by nested PCR, 16 (44%) of 36 birds in the zoological garden C, and three (13%) of 24 birds in the zoological garden D. A phylogenetic tree was made based on the *env* sequences of the 5 viruses and other ALVs. 3' UTR of the 5 ALV isolates showed exceptionally high sequence homology and a close phylogenetic relationship with FGV. In contrast, *env* of the isolates frequently showed mutations in their genomes. These results suggest that FGV mutants of FGV are prevalent among Japanese fowls kept in zoological gardens in Japan. Genome mutation and recombination of the *env* region may frequently occur among other ALSVs, including FGV, in the infected fowls of these zoological gardens.

Effect of estradiol-benzoate in estrus synchronization treatment with intravaginal progesterone releasing device on follicular dynamics in dairy cattle

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The objective of the present study was to examine the effect of standard (1 mg) and high (5 mg) doses of estradiol-benzoate (EB) in intravaginal progesterone releasing device (controlled internal drug release; CIDR) treatment on follicular wave dynamics in dairy cattle. Four non-lactating Holstein cows in 5-7 days after ovulation were treated with CIDR for 9 days. Estradiol-benzoate and $PGF_{2\alpha}$ were administered intramuscularly on the day of CIDR insertion and removal, respectively. All cows were received two CIDR treatments with a standard (1 mg) and a high (5 mg) dose of EB (EB1 and EB5 treatment, respectively). Once or twice a day, number and size of follicles and corpora lutea were examined using transrectal ultrasonography, and blood samples were collected for estradiol (E_2) and progesterone (P_4) assays.

Plasma E_2 concentrations reached a peak level on Day 0.5 (Day 0 = EB administration) and then declined in both EB1 and EB5 treatment. In EB5 treatment, peak concentrations of E_2 were higher than that of EB1

treatment, and high concentration was delayed. Plasma P_4 concentrations and corpus luteum diameters changed similarly between two treatments. In EB5 treatment, the number of medium-sized follicles (5-10mm), but not small follicles (< 5 mm), delayed to increase for 2 days compared with EB1 treatment. In EB1 treatment, follicular wave emerged, and dominant follicle was determined on 4.9 and 7.6 days after EB administration, respectively. Ovulation was occurred 71.5 hr after $PGF_{2\alpha}$ administration. In EB5 treatment, the emergence of follicular wave and detection of dominant follicle (7.5 and 9.2 days after EB, respectively) delayed compared with EB1 treatment. Consequently, diameter of the dominant follicles at the time of $PGF_{2\alpha}$ administration was smaller, time between $PGF_{2\alpha}$ administration and ovulation (86.7 hr) was extended. The duration between dominant follicle determination and ovulation (4.5 vs. 3.8 days), diameter of dominant follicles before ovulation (15.7 vs. 15.1 mm) and first follicular wave dynamics and luteinization in the next estrous cycle to CIDR treatment were similar between EB1 and EB5 treatment.

The present results indicate that use of a high dose of EB in CIDR treatment delays emergence of follicular wave. However, degree of synchrony in the time of ovulation and the period of dominance of the ovulating follicles, which may affect fertilizability and developmental competence of oocytes, were similar to the CIDR treatment with a standard dose of EB and within the normal range.

In vitro culture of mouse secondary follicles using membrane inserts : the effects of serum and FSH on the development and ovulation of follicles, and the maturation of oocytes derived from cultured follicle

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The objective of this study was to examine the effect of serum and follicle stimulating hormone (FSH) dose supplemented to culture media on the survival, growth, antrum formation, ovulation, estrogen production of cultured mouse follicles and nuclear maturation of oocytes from cultured follicles using membrane inserts. Secondary follicles were allocated to one of two size-categories : 175-200 μm (large) and 150-174 μm (small) in diameter. Culture medium was supplemented with 5 % mouse serum (MS group) or 5 % fetal calf serum (FCS group). In the MS group, the culture medium was containing 1 IU/ml FSH. In the FCS group, the culture medium was containing 1 or 0.1 IU/ml FSH (FSH-1 or FSH-0.1 groups, respectively). After 3-6 days of culture, follicles were transferred to new inserts of culture medium containing 5 IU/ml human chorionic gonadotropin (hCG) for inducing ovulation.

The timing of antrum formation of follicles in MS group was earlier than those in FCS group, and the diameter of cultured follicles in MS group was larger than FCS group until day 3 of culture. However, most of the follicles in MS group had degenerated at day 4 of culture. The follicles in MS group produced few amount of estrogen, in spite of the diameter of follicle was more than 400

μm. The concentration of testosterone in MS (0.988ng/ml) was about ten times higher than that in FCS (0.085ng/ml).

When the follicles cultured in the medium supplemented with FCS, there were no differences in the survival, follicle growth and timing of antrum formation nevertheless of FSH dose. But the ovulation rate of FSH-0.1 group seemed to be higher than that of FSH-1 group (P=0.1288). Most of the ovulated oocytes in FSH-0.1 group were at the metaphase Ⅱ (60%). However, in FSH-1 group, there was no oocyte reached to metaphase Ⅱ and most of the ovulated oocytes remained at the germinal vesicle stage. In both group, estrogen production of ovulated follicles increased after antrum formation. In FSH-0.1 group, estrogen production of ovulated follicles decreased after induction of ovulation. But in FSH-1 group, estrogen production also increased after induction of ovulation.

These results suggest that the development of the secondary follicle is inhibited in the culture media with the serum containing high concentration of testosterone, and that the high dose of FSH inhibits ovulation and oocyte maturation.

Efficiency and accuracy of estrus detection using radio-telemetric pedometer in dairy cows housed in free-stall and tie-stall

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It is suggested that the efficiency of estrus detection by a visual observation is decreased because individual cow management become inadequate in the free-stall barn. When cows are tied in tie-stall barn, estrus behaviors (standing etc.) are unobservable. To develop the effective estrus detection methods, this study examined the efficiency and accuracy of estrus detection using a radio-telemetric pedometer in Holstein cows housed in free-stall and tie-stall barns.

The pedometers were attached to the front and rear legs of lactating cows housed in free-stall (n=23), and to the front, rear legs and neck of heifers housed in tie-stall (n=9). The activity ratio (the count for the last 24 hr to the average count for corresponding 24 hr for the last 5 days (contrast terms)) was calculated hourly.

There was a high correlation between front leg and rear leg counts of pedometer that attached simultaneously to a cow, and the activity ratios of front and rear legs increased during estrus in the cows housed in free-stall and tie-stall. There was a low correlation between front or rear leg and neck counts of pedometer, and the activity ratio calculated by neck pedometer counts did not increase during estrus.

Some cows housed in the free-stall were pastured to a grazing field for 4 hr, and it was examined whether grazing affects pedometer counts and activity ratio. The mean value of pedometer counts was increased, and activity ratio was reduced by grazing in the field. Consequently, the efficiency of estrus detection reduced. However, the efficiency of estrus detection increased, when the data were analyzed

without pedometer counts during grazing and walking to a grazing field.

For developing the efficacy of estrus detection using pedometer, the proper contrast term and the threshold (detecting the estrus when the activity ratio overcome) were analyzed. The maximum estrus detection indexes (efficiency × accuracy) (100, 76, 83 and 75) were obtained from cows housed in the free-stall, free-stall with grazing, free-stall with grazing (only calculated by pedometer counts in the free-stall) and the tie-stall, when the thresholds of activity ratios were 1.7～2.1, 1.3, 1.5 and 1.4, respectively, and their contrast terms were set to 10 days. Efficiency of estrus detection using a radio-telemetric pedometer in the optimum condition was equal to or more effective than that by visual observation.

Molecular interactions involved in localization of Claudin-16 to the tight junction

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Claudins are the major components of the tight junction in epithelial cells and participate in intercellular adhesion and paracellular transport of ion and solutes. Claudin-16 is a member of claudin family and distributes specifically to the thick ascending limb of Henle's loop in the kidney. Inherited deficiency of claudin-16 causes renal tubular dysplasia in cattle although other claudins including claudin-3 are also present at the same tubular segment, suggesting a pivotal and novel role of claudin-16 to form and maintain renal tubules. It has been anticipated that the PDZ(PSD95/Dlg/ZO-1)domain-binding motif at the C-terminus plays an essential role to localize claudin proteins to the tight junction through association with membrane skeletal and cytoskeletal proteins. The purpose of the present study is to identify some proteins that associate with bovine claudin-16 and to verify if wild-type claudin-16 could rescue claudin-16 lacking the PDZ motif (claudin-16ΔTRV) to localize to the tight junction. Screening by yeast two-hybrid system using C-terminal cytoplasmic tail of claudin-16 as the bait found 8 identified and 7 unidentified proteins derived from kidney cDNA as candidates for partner to claudin-16. Claudin-16 and EGFP-claudin-16 showed localization to the tight junction when transfected into wild-type MDCK cells, whereas EGFP-claudin-16ΔTRV exhibited transport to the plasma membrane but had broad distribution at basolateral membranes. In contrast, deletion of the PDZ motif from claudin-3 (claudin-3ΔYV) caused no change in its localization to tight junction of MDCK cells. Stable MDCK transfectants of claudin-16ΔTRV represented trafficking to basolateral regions even in the presence of wild type claudin-16. These findings suggest that claudin-16 but not some other claudins including claudin-3 absolutely requires PDZ motif-dependent transport to be localized to tight junction.

Mechanism of the promoter activation and the role of erythroid Krüppel-like factor in bovine γ- to β-globin gene switching

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Hemoglobin switching of the birth period is a physiological response that globin chains change from fetal γ-chains into adult β-chains. This process is controlled mainly by erythroid Krüppel-like factor (EKLF), an erythroid-specific transcription factor. Anemia of newborn cattle with unknown etiology has been suspected of some defects in hemoglobin switching, despite of the lack of pathobiological bases. The purpose of the present study is to analyze mechanisms for promoter activation of γ- and β-globin genes and to reveal a role of bovine EKLF (bEKLF) in γ- to β-globin switching in cattle. Bovine γ- and β-globin promoter regions isolated from genomic DNA had nucleotide sequences very similar to each other and shared the CACCC box, contrary to that β- but not γ-globin promoter possessed this EKLF-responsive element in human. bEKLF cDNA was isolated and was shown to be expressed specifically in hematopoietic organs. Reporter assay and electrophoretic mobility-shift assay demonstrated that bEKLF activated reporter gene transcription through binding to the CACCC box at -108 bp of γ- and β-globin promoters and also to a downstream CACCC-like sequence in γ-globin promoter. Interestingly, reporter assay in 293 cells and MEL cells for various deletion mutants of γ- and β-globin promoters showed that the cells distinguished these promoters in transcription activation: basically, γ-promoter was activated while β-promoter was suppressed, suggesting that some transcription factors other than EKLF play critical roles in controlling promoter activation. In conclusion, these findings indicate that bEKLF is an important factor in efficient expression of γ- and β-globin genes, but not essential to nor sufficient for γ- to β-globin gene switching in cattle.

Molecular identification of a major transmembrane protein, gp155 in ruminant red cells as the MRP4, a member of ATP-binding cassette (ABC) transporter subfamily C (ABCC 4)

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Ruminant red cell membranes contain a unique transmembrane glycoprotein, gp155 as a major constituent in addition to other membrane proteins including band 3. Goat gp155 with an apparent molecular mass of 155,000 has multiple membrane spans and appears to

be associated with band 3 and other membrane skeletal components to form a macromolecular complex. The present study describes molecular identification of gp155 as a member of ABC transporter superfamily. Proteolytic peptide of gp155 isolated from ovine and goat red cell membranes were analyzed for their N-terminal amino acid sequences. Homology search revealed that the sequences of 5 out of 12 peptides were highly homologous to those of multidrug resistance protein 4 (MRP4/ABCC4), a member of ATP-binding cassette transporter subfamily C. MRP4 cDNA was isolated from bone marrow cells of a sheep by combination of PCR and 5' and 3' rapid amplification of cDNA ends. The deduced amino acid sequence of ovine MRP4 was similar to that of human homologue consisting of two each sets of the transmembrane domain with 6 membrane spans and the nucleotide-binding domain, and contained amino acid sequences corresponding to 8 out of 12 peptides derived from gp155. These data indicated that gp155 is identical to the MRP4 protein. Relatively high levels of expression of MRP4 transcripts and the protein were found in erythroid cells in bone marrow, lung, liver, spleen, kidney, adrenal gland, intesitine, and colon. Comprising of 10% of total red cell membrane proteins and distribution at the apical membrane of intestinal epithelial cells and renal tubules suggested that MRP4 participates in transport of some metabolites characteristic to physiology of ruminant amimals. Contribution of C-terminal PDZ (PSD-95/Dlg/Zo-1) domain-binding motif might be possible in macromolecular complex formation in red cell membranes.

Physiological and pathological roles of LYST, a product of the causative gene for Chediak-Higashi syndrome in formation of secretory granules

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The LYST (lysosome trafficking protein) is a protein product of causative gene identified for Chediak-Higashi syndrome (CHS) in human, *beige* mice, and cattle. LYST has been anticipated to play key roles in formation, transport, and secretion of intracellular granules, lysosome-related vesicles. In addition, studies on mutations and pathobiology of CHS in *beige* mice and Japanese black cattle suggested that distinct structural domains consisting of LYST (HEAT repeat, BEACH, and WD-40) had different functions in formation of secretary granules: *beige* mice with the deletion mutation of BEACH and WD-40 domains exhibited immunodeficiency and bleeding, while Japanese black cattle with a single amino acid substitution at the C-terminal part of the HEAT repeat had only abnormality in blood coagulation. The purpose of the present study is to establish antibodies that specifically react with LYST proteins, and to establish cell culture system to investigate roles of LYST. Antibodies were raised against recombinant proteins consisting of N- and C-terminal regions of bovine LYST tagged with 6xHis motif and the antibody recognizing C-terminal part of LYST (anti-LYST-Ct) was obtained. Immunofluorescence microscopy using

anti-LYST-Ct showed granular signals in the cytoplasm of myeloblasts and myelocytes from bovine bone marrow cells but not in erythroid progenitors. Megakaryocytes also possessed LYST although signals were week. In K562 cells that were induced into megakaryocytic but not erythroid differentiation showed an increase in expression of the LYST gene. To study LYST function in formation of melanosomes, a subclone B16 cells (B16mel＋) was established. The vast majority of B16mel＋ cells showed differentiation into melanocytes in response to an inducer and revealed enhanced expression of the LYST gene and increased the level of LYST proteins. The cells established would provide useful information on the mechanisms for physiotogical function of the LYST.

The function of mosquito JNK in the WNV infection

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West Nile virus (WNV) has become a significant problem all over the world. An outbreak of WN encephalitis occurred in New York City on late August 1999, and more recently, the WNV epidemic is spreading throughout the United States, and there is a possibility that WNV is introduced to Japan. Mosquitoes are important key vector for spread of WNV infection to humans. *Aedes* (*A.*) *albopictus,* which is spreading in Japan and a vector of Dengue virus and Japanese encephalitis virus, has a capability to be a vector of WNV when WNV invades Japan. Because there is no vaccine for WNV right now, control of mosquitoes is the only way for preventing the spread of the disease. For the purpose of it, accumulating the information on the mosquito cells is also important to know the target molecules of mosquito cells to WNV infection. C6/36 cells derived from *A. albopictus* are widely used for flavivirus research even though the characteristics of the cells are poorly understood. In this study I characterized the cells and studied the WNV infection in the cell.

It has been shown that C6/36 cells can phagocytose the bacteria and artificial microspheres, and c-Jun amino-terminal kinase (JNK), one of the mitogen-activated protein kinases (MAPKs), is associated with the phagocytosis of the cell. Firstly, to clarify whether new activation of JNK by the stimulation (e.g. bacteria) is required for phagocytosis, I incubated C6/36 cells with FITC-labelled Escherichia (*E.*) *coli* or fluorescent microspheres, and detected activated JNK by Western blotting. New activation of JNK was observed only when the cells were incubated with *E.coli*. This indicates that phagocytosis of the cell is occurred by spontaneously activated JNK and new activation of JNK is not required for phagocytosis. I also found that phagocytosis of *E.coli* increased with time and there was a trend that bacteria were phagocytosed strongly compared with microsphere. This indicated that phagocytosis was enhanced by newly activated JNK.

It has been shown that JNK of C6/36 cell is activated by the stimulation of lipopolysaccharides (LPS) or 45℃. I incubated the

cells with fluorescent microspheres after the stimulation by LPS or 45℃, and observed the phagocytosis activity. Compared with them, I found that phagocytosis increased slightly after the LPS stimulation but not 45℃ treatment. Then, I detected activated JNK by the LPS or 45℃ stimulation with Western blotting, respectively. I found that JNK activated by the LPS stimulation had a higher molecular weight than that by 45℃ stimulation.

It has been shown that MAPKs such as ERK 1, 2, ERK 5, p 38 are associated with the cell proliferation. To know whether JNK is associated with the cell proliferation, I incubated C 6/36 cells with SP600125, a specific inhibitor of JNK. I found that the cells incubated with S9600125 did not increase in number. This indicates that JNK is associated with the cell proliferation.

It has been shown that JNK of the C 6/36 cell is associated with endocytosis, and WNV enter the C 6/36 cells by endocytosis. Therefore, the JNK inhibitor can protect the WNV entrance to C 6/36 cells. In this study, it is revealed that JNK of the C 6/36 cell is associated with the cell proliferation. To clarify whether JNK of C 6/36 cell is associated with the WNV replication, I incubated C 6/36 cells with SP600125 after the WNV infection and observed the WNV replication. WNV replication was inhibited by SP600125 in 24 hours post infection (hpi) but no inhibition in WNV replication was observed in 36 and 48 hpi. This indicates that JNK of C 6/36 cell is associated with the WNV entrance or early events of infection to the C 6/36 cells but not with the later events of WNV replication such as virus release.

Development of West Nile virus detection test using real-time PCR

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West Nile virus (WNV) appeared in New York City for the first time in the western hemisphere in late August 1999, and rapidly expanded to the whole country of the U.S.. It is anxious that WNV may invade Japan due to the frequent trade and traveling.

On the other hand, Japanese encephalitis virus (JEV) which belongs to flavivirus of the JEV serocomplex exists in Japan. The diagnostic methods to distinguish the both viruses will be necessary when WNV invades Japan. It is difficult to differentiate the two virus infections by serological diagnostic tests. Molecular diagnostic methods are therefore preferred and reverse transcription (RT)-PCR has been used to develop as sensitive and specific assay for the identification of WNV. Recently, more sensitive assays, such as fluorogenic real-time (TaqMan) PCR, SYBR Green -based real-time PCR, and loop-mediated isothermal amplification (LAMP), have been developed for the diagnostic test to detect WNV genome. However, the diagnostic methods described above were designed to detect only WNV or, more specifically, the New York strain of WNV. In this study, we developed diagnostic method that can detect and distinguish WNV and JEV using TaqMan RT-PCR.

It has been reported that only genotypes 1, 2, and 3 of JEV are widely distributed in

Southeast and East Asia. Therefore, we used the sequences of WNV lineage 1 strains (NY 99-382-99, Eg101), WNV lineage 2 strain (FCG), JEV genotype 1 strain (K04 P05), JEV genotype 2 strain (FU), and JEV genotype 3 strain (JaGAr 01) to design the TaqMan probe and primers.

To determine the cycles of threshold (Ct) values for the TaqMan assay, we performed the assay with serially diluted cDNA from the NY99-6922 strain and decided that a Ct value of <40 and a ΔRn signal of >0.5 were considered positive. The specificity of the primer sets was tested using various flaviviruses. The results indicated that the primer set for WNV could detect only WNV strains. The primer set for JEV could detect only JEV strains. Other flaviviruses were also not detected by either primer sets.

Next, to measure the sensitivity of the TaqMan assay for WNV and JEV, we performed the assay using serially diluted cDNA samples from titrated virus stocks. At least 10^{-1} PFU of virus was required for fluorescence detection by the TaqMan assay with the WNV or JEV primer sets. And 10^{-2} PFU of virus was required to detect FCG strain of WNV.

Finally, we examined whether the primer sets used in this study could detect viruses in animal tissues. We performed the TaqMan assay for tissues from experimentally infected mice. BALB/c mice were infected with 10^3 PFU of WNV (BC787, B-LP, B-SP, Eg101) via the i.p. route. Consequently, the assay detected viral RNA in the blood, spleen, brain samples.

In addition, our method used only a single probe. Thus, our TaqMan assay was cost-effective for the detection of WNV and JEV.

In summary, we developed a diagnostic method that is able to detect WNV and JEV using real-time PCR assays with a single probe and specific primer sets. We believe that the TaqMan assays presented here will be useful for the detection of WNV in JEV-endemic regions.

Application of tick-borne encephalitis virus recombinant proteins to ELISA antigen and immunogen.

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In October 1993, a human case of encephalitis was diagnosed as tick-borne encephalitis (TBE) in Kamiiso, Hokkaido. Far-Eastern subtype TBE virus strain Oshima U-5-10 was isolated from a sentinel dog in the same area. Since the suspected vector ticks and reservoir rodents are commonly found in Japan, TBE virus may be endemic not only in the area where a patient was found but also in other parts of Japan. Seroepidemiological survey among field rodents is an effective mean to detect TBE endemic area. Now, virus-neutralizing test (NT) is used for serological survey of wild rodents. NT is highly specific, but it has some disadvantages because it is needed to use hazardous live virus and many samples can not be tested at once. Therefore, enzyme-linked immunosorbent assay (ELISA) system which can detect TBE virus-specific antibodies from rodents sera was es-

tablished by using recombinant TBE E protein domain Ⅲ as antigen. The cut-off value of ELISA was determined on the basis of the NT of field rodents of Kamiiso. When the cut-off value was set at 0.59, sensitivity and specificity of the ELISA was 77.3% and 77.0% respectively. This result indicates that the ELISA is able to detect TBE specific antibodies by high probability. In order to check the usefulness of this ELISA, we tried a screening of the field rodent sera of Khabarovsk, endemic area of TBE. Among 28 serum samples, 3 samples were positive In the ELISA and all of them were also confirmed to be positive in NT. From these results, it was shown that this ELISA combined with NT as confirmatory test is useful to specify TBE endemic area rapidly.

For immunoprophylaxis against TBEV, inactivated TBE vaccine is now commercially available in Europe. However, the vaccine has not been approved in Japan and TBE vaccination to Japanese is restricted. Therefore, it is necessary to develop new vaccine against TBE virus existing in Japan. In order to assess the ability of VLPs and recombinant DNA expressing VLPs to induce protective immune response, we evaluated the neutralizing antibody response after inoculation of these immunogen. All the mice inoculated 1 or 10μg of VLPs and a part of mice inoculated DNA vaccine developed neutralizing antibodies. Results of this study indicate that VLPs and DNA vaccines can be possible measures for the future TBE vaccine development. And it is suggested that DNA vaccine may become effective measure of immunization by improving the inoculation procedure of plasmid DNA. In future, it is expected that the protective effect will be evaluated against challenge by live virus and usefulness of these vaccines will be established.

Expression and antigenic analysis of Amur type hantavirus glycoproteins

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Hantavirus causes two human illnesses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Variety of hantaviruses are carried by rodent species and distributed in the world. In the Far East Russia, it was revealed that two hantavirus serotypes, Amur type (AMR) and Hantaan type (HTN), cause severe HFRS. Although HTN and AMR are carried by different species and genetically distinct, the antigenic properties of HTN and AMR are extremely close. To make the differential diagnosis between HTN and AMR, cross neutralization test (NT) should be carried out. However, live hantavirus is used in NT and 7 to 14 days require to obtain the result.

In this study, therefore, pseudotype vesicular stomatitis virus (VSV) bearing the AMR H5 strain envelope glycoprotein (VSVΔG*AMRG) was generated to develop a simple, safe and rapid NT for differential diagnosis. The pseudotype VSV (VSV⊲G*G), in which the enveloped protein (G) gene is replaced by the green fluorescent protein (GFP) gene, has advantages in safety and time because of its impossibility of budding and rapid expression

of GFP in infected cells.

The plasmid for the expression of the full length glycoprotein precursor of the H 5 strain (pCAG-H5M) in mammalian cells was constructed to generate pseudotype VSV. Moreover, two plasmids, pCAG-G1 and pCAG-G2, which express G1 and G2 glycoprotein, respectively, were constructed to investigate the antigenicity, expression and distribution of glycoproteins in mammalian cells. In indirect fluorescent antibodies assay (IFA) by using a panel of monoclonal antibodies (MAbs) to HTN glycoproteins, AMR recombinant glycoproteins and the authentic virus glycoproteins reacted with each MAb in the same way. It is known that hantavirus glycoproteins accumulate in the Golgi complex following virus infection. However, when G1 and G2 were expressed separately, each protein did not accumulate in the Golgi complex. This result suggests that the Golgi localization of hantavirus glycoproteins needs the coexpression of G1 and G2.

In addition, 293T cell were transfected with pCAG-H5M and infected with VSVΔG*G to generate AMR pseudotype (VSV⊲G*AMRG). HTN pseudotype (VSVΔG*HTNG), which is bearing HTN glycoproteins was kindly provided by Dr.Yoshimatsu. The pseudotype VSVs were neutralized by HTN or AMR antisera. The neutralization titer of antiserum to the homologous pseudotype VSV were more than 4 times higher than that of the antiserum to heterologous pseudotype. This result shows that VSVΔG*AMRG and VSVΔG*HTNG are used for a rapid, safe and specific NT for differentiating HTN and AMR infections.

Suppression of cell proliferation in colon 26 solid tumor by a treatment combining X irradiation and local injection of T34A mutant survivin fused with membrane permeable TAT peptide.

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Survivin is known to be a member of inhibitor of apoptosis proteins (IAP) and has an activity to inhibit apoptosis induction. The inhibition of apoptosis is reported to be induced through the binding of survivin phosphorylated at 34 threonine with caspase-9. Since there are many reports that overexpression of survivin occurs in many types of tumors, the overexpression of survivin is considered to be responsible for the resistance of tumor cells to chemotherapy and X irradiation. In this study, antitumor efficacy of radiation treatment was investigated in the case that the antiapoptotic function of survivin was targeted in the solid tumor. For this purpose, a wild type survivin (TAT/WT survivin) and a dominant negative T34A survivin protein (TAT/T34A survivin) fused with membrane permeable TAT peptide were constructed by *E. coli* protein expression system using T7-promotor-based expression vector, and the TAT-fused proteins were locally injected to solid colon 26 tumor implanted in foot pad of mouse. The distribution of the TAT/WT and TAT/T34A survivin in colon 26 solid tumor was immediately examined within 30 min after injection by immunostain-

ing with an antibody for survivin. The results indicated that the fusion with TAT peptide gave the high efficacy of intracellular delivery of survivin. TAT/T34A survivin injection alone or X irradiation alone did not influence the growth rate of tumor, but the combination of the local injection of TAT/T34A survivin and X irradiation significantly suppressed it. In histological examinations, nuclear fragmentation, chromatin condensation and TUNEL-positive cells, characteristic for the increase of apoptosis, were observed in the tumor with the combination treatment. Furthermore, prolifelative cells, which were detected by immunostaining with an antibody for Ki-67, decreased in the tumor suggesting that apoptotic cell death was predominantly induced in the prolifelative cells. In addition, it was demonstrated that the combination treatment of injection of TAT/T34A survivin and X irradiation did not change the radiation-induced increase of cyclin B1, which was known to increase around the G2/M phase. From these results, it was suggested that the dysfunction of anti-apoptotic activity of endogenous survivin by the injection of TAT/T34A survivin decreased the prolifelative cells in tumor by inducing apoptosis during radiation-induced G2/M checkpoint. The present study revealed that the fusion of protein with TAT peptide was useful to deliver protein to solid tumor cells and that the combination treatment of injection of TAT/T34A survivin and X irradiation gave strong suppressive effects on tumor growth.

Overexpression of survivin mutants enhances radiation-induced apoptosis in mouse fibroblast NIH3T3 cells

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Survivin is a member of IAP (inhibitor of apoptosis proteins) that has been implicated in both apoptosis inhibition and cell cycle control. Majority of tumors expresses survivin at high levels, and this is one of causes that tumors resist radiation and chemotherapeutic agents. This protein is reported to be phosphorylated at threonine 34 (T34) by Cdc 2 and to suppress apoptosis by inhibiting caspases through cysteine 84 (C84) residue. Recently, aspartic acid 53 (D53) residue was also demonstrated to be important for the inhibition of apoptotic signaling. Moreover, survivin was shown to keep mitosis accurate by the formation of complex with Aurora-B kinase and INCENP. In this study, we examined effects of survivins with point mutation (T34A, C84A and D53A) on mitotic control and radiation-induced apoptosis. In addition, the phosphomimic mutants, T34E and T34D, were also used to clarify the role of negative charge at these residues. The expression vectors were transfected to mouse fibroblasts NIH3T3 cells by using LipofectAMINE PLUS. After irradiation of cells with 20 Gy, they were incubated in medium with or without caspase inhibitors for 48h. The apoptosis induction was evaluated by observing cells after nuclear staining with propidium iodide. Overexpression of survivin-T34A, -C84A and-D53A en-

hanced the radiation-induced apoptosis in comparison with that of wild type survivin. Moreover, this enhancement was suppressed by caspase-9 and caspase-3 inhibitors. The amount of radiation-induced apoptosis in T34E- and T34D-transfected cells was similar to that in wild type survivin-transfected cells. These results suggested that the point mutations at T34A, C84A and D53A abolished the antiapoptotic activity of survivin and enhanced the induction of caspase-dependent apoptosis in X-irradiated mouse fibroblasts NIH3T3 cells.

Enzymatic characteristics of Cytochrome P450 in livers of horse and deer

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Cytochrome P450 (CYP) comprises a super family of monooxygenases that catalyze the metabolic activation and detoxification of substrates, which can be found in a broad spectrum of structurally unrelated compounds including ; drugs, chemical carcinogens, environmental pollutants. The characterization of CYP-dependent drug metabolisms is important from the clinical point of view. Although the variations in expression and activity profiles of CYP related to sex, species, strains and genetic polymorphism are reported, most of the knowledge on drug metabolism is based on studies in man and in experimental animals. The information of drug metabolisms in domestic animals, such as bovine, swine and sheep is less known compared to laboratory animals. Especially, there is little report on the drug-metabolism of horses or deer. Thus, I studied on the enzymatic characteristics of hepatic CYPs of horses and deer.

In present study, I measured catalytic activities of CYP isoenzymes in liver microsomes from horses and deer. The metabolic activities of alkoxyresorufins, diazepam, and bunitrolol were measured which were typical substrates for CYP1A, 2B, 2C, 2D and 3A subfamilies of experimental animals. Since the kinetic study is fundamental to profile the patterns of drug-metabolism, I attended to the substrate concentrations and calculated kinetic parameters in each metabolic assay. I found that most CYP isoenzymes in horse liver showed low substrate affinities compared to those of rats. The liver microsomes of horses showed higher catalytic activities in ethoxyresorufin O-deethylation (typical substrate of CYP1A in rat) and methoxyresorufin O-demethylation (CYP1A) than those of rats. On the other hand, activity of pentoxyresorufin O-dealkylation (CYP2B) was low in horse liver.

I detected higher affinities of CYP isoforms to substrate in deer liver than those of rats. Deer's CYPs showed equal or higher levels of metabolic activities in diazepam N-demethylation (catalyzed by CYP2C in rat) and 3-hydroxylation (CYP3A) than those of rats. In deer, I also found existence of the extensive and poor metabolizers in the catalytic activity of diazepam p-hydroxylation.

Furthermore, I isolated cDNA fragments of CYP1A and CYP3A from liver of horses and deer, using the degenerate RT-PCR method. I

sequenced CYP isoforms of horse and deer, and aligned with corresponding CYPs of other mammalian species. Phylogenetic trees were constructed from sequences of fragments of CYP1A and CYP3A of horses and Hokkaido sika deer. Both of horses and deer CYP1A sequences were similar to rabbit CYP1A2. Isolated horse and deer CYP3A were closely related to the bovine CYP3A28, and to sheep CYP3A24, respectively.

The genetic polymorphism of diazepam metabolism in rats

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The previous studies reported inter-or intra-strain differences in diazepam metabolism among Sprague-Dawley (SD), Brown Norway (BN), Dark Agouti (DA) and Wistar rats. In particular, there were marked inter-or intra-strain differences in diazepam *p*-hydroxylation activity at low concentrations of substrate. However, the enzyme which catalyzed diazepam *p*-hydroxylation was still unclear. Then, to identify the diazepam *p*-hydroxylase, the assay of diazepam metabolism was carried out using rat-cytochrome P 450 (CYP) 2 D isoforms expressed in the yeast as recombinant enzymes. I found that CYP2D3 was responsible for diazepam *p*-hydroxylation. This result was in good agreement with the observation that polymorphic expression of CYP2D3 was consistent with phenotype of diazepam *p*-hydroxylation. In addition, diazepam *N*-desmethylation was partially catalyzed by CYP2D4, which was highly expressed in DA rats compared to other strains. Thus, the polymorphic expressions of not only CYP2D3 but also CYP2D4 are involved in strain differences among four strains.

Furthermore, to determine the mechanism of polymorphic expression of CYP2D3, I investigated genetic polymorphism among four rat strains. Although there was no difference in the expression level of CYP2D3 mRNA between extensive and poor metabolizer (EM and PM) rats, I identified a single base insertion in the sequence of CYP2D3 of PM rats. It resulted in a frameshift and the creation of premature termination codon in the upstream of heme-binding region, which is essential to maintain proper heme binding and active P 450. The deletion of heme-binding site produced a non-functional CYP2D3, and was associated with low activity of diazepam p-hydroxylation. In contrast, EM rats had no point mutation. This is the first report that genetic polymorphism of CYP2D3 causes the inter-or intra-strain differences in diazepam metabolism.