HOKKAIDO UNIVERSITY

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Description	Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 46 graduates of the School of Veterinary Medicine on March 25, 2008.
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INFORMATION

Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 46 graduates of the School of Veterinary Medicine on March 25, 2008.
The summaries of their theses are as follows:

Molecular cloning of TRPM6 and TRPM7 in bovine parotid gland: electrophysiological characterization of heterologously expressed TRPM7 current

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In this study, I examined mRNAs expression of divalent cation permeable channels TRPM6 and TRPM7 in bovine parotid gland and characterized electrophysiological properties of TRPM7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed the presence of the transcripts of TRPM6 (bpTRPM6) and TRPM7 (bpTRPM7) in bovine parotid cells. Subsequent studies indicated that the deduced amino-acid sequence of bpTRPM 7 was homologous to those of human and mouse orthologues, and suggested that some basic electrophysiological properties of bpTRPM7 current heterologously expressed in human embryonic kidney 293 (HEK293) cells were similar to those previously reported for human and mouse orthologues.

Expression and production of hepatocyte growth factor and heregulin alpha are differentially regulated in 3T3-L1 adipocytes.

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Development of mammary gland occurs during embryonic stages and also in postnatal life. Recent evidence indicates that ovarian steroids such as 17β-estradiol (E2) and progesterone (P) influence both mammary epithelial and stromal mesenchymal cells, resulting in regulating mammary gland development through reciprocal epithelial-stromal interactions. This interaction occurs via several growth factors such as stroma-derived hepatocyte growth factor (HGF) and heregulin alpha (HRGα) that induce branching morphogenesis and lobulo-alveolar formation of epithelial cells, respectively. However, regulatory mechanisms of expression and production of HGF and HRGα in stromal cells were not fully elucidated. In the present study, I examined the effects of E2 and P, and also nutritional state of cells on the expression and production of HGF and HRGα in 3T3-L1 mouse preadipocytes as a model of mammary stromal cells.

HGF mRNA was abundantly expressed in preadipocytes and diminished after the adipogenic differentiation by treatment of cells with isobutylmethylxanthine, dexamethasone, insulin and

trogritazone. HGF protein was found in the conditioned medium from both preadipocytes and mature adipocytes, but the amounts of which were decreased after the differentiation. Treatment of preadipocytes with either E2 (1～10 nM) or P (1～10 nM) increased the expression of HGF mRNA, while those with P at higher concentrations (100～1,000 nM) did not. Treatment of preadipocytes with combination of E2 and P (10 nM each) resulted in significant enhancement of mRNA expression and production of HGF, compared to those of E2 or P alone. However, neither treatment of mature adipocytes with E2 nor P increased the expression of HGF mRNA.

HRGα mRNA was also abundantly expressed in preadipocytes and diminished after the adipogenic differentiation, while the amounts of HRGα protein in the conditioned medium were not altered before and after the differentiation. The reason of this contradictory protein expression were not clear, but the activity of cleavage enzyme for membrane-bound pro-HRGα such as a disintegrin and metalloproteinase 17 (ADAM17) might be altered, because mRNA expression of ADAM17 inhibitor, tissue inhibitors of metalloproteinase 3 (TIMP-3) being rich in preadipocytes markedly decreased after the differentiation, although mRNA expression of ADAM17 itself did not changed by the adipogenic differentiation. In addition, neither treatment of preadipocytes and also mature adipocytes with E2 nor P modified the mRNA expression and production of HRGα.

In summary, the present results indicate that production of HGF in 3T3-L1 cells as a model of mammary stromal cells is regulated at the transcriptional levels under the control of E2 and P. In contrast, production of HRGα in 3T3-L1 cells is unaffected by the sex steroids, and possibly controlled by a posttranslational modification such as protein cleavage.

Adiponectin inhibits nerve growth factor-induced neurite outgrowth of PC12 cells by direct protein-protein interaction

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Adiponectin is a 28-kDa protein consisting of a collagen-like domain and a globular domain similar to collagens VIII and X and compliment factor C1q, respectively. Adiponectin is secreted exclusively by adipose tissue, and plays a pivotal role in controlling whole-body metabolism, particularly by enhancing insulin sensitivity in muscle and liver through its specific receptors, termed as Adipo R1 in the skeletal muscle and Adipo R2 in the liver. In addition, recent studies show that a reduced adiponectin level in plasma is significantly correlated with the risk of various cancers, and that adiponectin inhibits the growth of mouse fibrosarcoma *in vivo*. Indeed, adiponectin binds directly with some growth factor such as platelet-derived growth factor (PDGF-BB) and fibroblast growth factor (FGF). However, molecular basis of interaction between adiponectin and a certain growth factor is currently unknown.

Recently, it is found that nerve growth factor-β (NGF), a neurotrophin, is secreted by adipocytes and that the secretion is enhanced by treatment of adipocytes with proinflammatory cytokines such as TNF-α. Interestingly tertiary structure of NGF resembles that of PDGF-BB forming β cystine-knot structure, although these primary structures are completely different from each other. To address the question whether adiponectin binds to growth factor(s) depending on their tertiary structure, I examined direct interaction between adi-

ponectin and NGF by surface plasmon resonance method using BIACORE X, and compared with those of PDGF-BB. Perfusion of increasing concentrations of NGF on the surface of full-length adiponectin-bound chip caused specific increase of mass bound, comparable to that of PDGF-BB. The dissociation constant (K_D) of the adiponectin and NGF interaction was 1.0×10^{-7} M and binding ratio to 1 mole adiponectin was estimated as 0.6 mole of NGF, while those of PDGF-BB was 0.25×10^{-7} M and 2.6 moles, respectively. Perfusion of either NGF or PDGF-BB on the surface of globular adiponectin-bound chip decreased specific mass bound with reduction of K_D and the binding ratio.

Next, to examine the interaction of adiponectin and NGF in a solution and its possible role in physiological condition, I assessed the biological activity of NGF using PC12 cells. Treatment of the cells with NGF, but not adiponectin, induced neurite outgrowth and cell swelling. However, treatment of the cells with NGF and adiponectin simultaneously decreased number of cells with neurite and cell size, compared with those of NGF alone. Treatment of the cells with adiponectin, but not NGF, induced activity-related site-specific phosphorylation of AMP-kinase α subunit (AMPKα), and the cells expressed Adipo R 1 and Adipo R2 mRNAs. Treatment of the cells with siRNA for Adipo R1 and Adipo R2 prevented adiponectin-induced phosphorylation of AMPKα but failed to affect adiponectin-caused inhibition of neurite outgrowth by NGF. These results clearly indicate that adiponectin interact with NGF in solution, thereby inhibits NGF function such as neurite outgrowth.

In conclusion, adiponectin binds to both NGF and PDGF-BB that has common β cystine-knot structure, and inhibits their biological activities. Physiological relevance of the present results is currently obscure, but the information gives a new insight on adipnectin-growth factor interaction and would be useful for therapeutic use of adiponectin for preventing cancer, atherosclerosis and inflammation.

Role of adiponectin and leptin in the control of hypothalamus sympathetic nervous system in rats

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Adiponectin is secreted exclusively from adipose tissue, and plays major roles in controlling whole-body metabolism, particularly by enhancing insulin sensitivity in muscle and liver, and by increasing fatty acid oxidation in muscle. In addition, recent studies suggests that adiponectin may control feeding behavior and energy metabolism through direct action on the central nervous systems, but the actual central function of adiponectin remains obscure.

On the other hand, leptin, the obese gene product, is produced from adipose tissue and acts centrally via its specific receptor in the hypothalamus to regulate feeding behavior, energy homeostasis, reproduction and immune system, and part of which are mediated through activation of the corticotropin-releasing hormone (CRH)-sympathetic nervous system (SNS).

In order to elucidate central functions of adiponectin, I examined effects of adiponectin and leptin as a reference on noradrenaline (NA) turnover, as a biochemical marker of the activity of SNS in peripheral organs and of adrenergic neurons in the brain.

The tissue NA content varied considerably among organs, roughly reflecting the density of

sympathetic innervation of each organ. NA content in the hypothalamus and frontal cortex were also varied, reflecting the projection of adrenergic neurons in the brain. NA turnover rate was assessed from a decrease in tissue NA concentration after the inhibition of catecholamine biosynthesis with α-methyl-*p*-tyrosine, and varied greatly among organs, indicating the difference in the tonus of sympathetic nerve activity in each organs.

To investigate the possible central effect of adiponectin, the tissue NA turnover in the brain and peripheral organs was assessed after intracerebroventricular injection of adiponectin (2 μg/rat). NA turnover rate in the retroperitoneal white adipose tissue was marginally but significantly accelerated, while those in the other regions of white adipose tissue, brown adipose tissue, skeletal muscles, heart, lung, pancreas, kidney, frontal cortex and hypothalamus were not. Thus, it is unlikely that adiponectin affects the tonus of hypothalamus-SNS axis and noradrenergic neurons in the brain

However, NA turnover rates after intracerebroventricular injections of leptin (2 μg or 10 μg/rat) and CRH (10 μg/rat) were not accelerated in any central and peripheral organs tested, except hypothalamus after CRH injection. This conflicts with previous findings, and might be attributed to the dose of these substances, because there are reports only selective dosage of leptin and CRH could activate SNS. Therefore, to conclude the effect of adiponectin on SNS, additional experiments may be requested.

Activation of transient recepter potential VI (TRPV1) by allylisothiocyanate-related compounds

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I examined the effect of allyl isothiocyanate (AITC) and related compounds on pig TRPV1 expressed heterologous in human embryonic kidney 293 cells (HEK293) by measuring intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) with fruoroscent Ca^{2+} indicater, fura-2.

AITC (3 mM or less) induced dose-dependent increases of $[Ca^{2+}]_i$ in cells responding to capsaicin. AITC did not evoke $[Ca^{2+}]_i$ increase in HEK293 cells untransfected with TRPV1 cDNA (naïve cells). The $[Ca^{2+}]_i$ increase induced by AITC (3 mM) was inhibited by capsazepine (10 μM). Allyl disulfide (AD) (1 mM or less) and allyl sulfide (AS) (10 mM or less), both of which contain allyl base in their structures, induced dose-dependent increases of $[Ca^{2+}]_i$ in cells responding to capsaicin. Neither AD nor AS caused $[Ca^{2+}]_i$ increase in naïve cells. The $[Ca^{2+}]_i$ increase induced by AD (1 mM) or AS (10 mM) was inhibited by capsazepine (10 μM). AD at 3 mM or more caused $[Ca^{2+}]_i$ increase even in cells unresponding to capsaicin and evoked cell swelling (toxic effect). A methylated allyl compound, allyl methyl disulfide (AMD) induced dose-dependent increases of $[Ca^{2+}]_i$ in cells responding to capsaicin. The $[Ca^{2+}]_i$ increase induced by AMD (10 mM) was inhibited by capsazepine (10 μM). The toxic effect did not occur even at a high concentration (30 mM) of AMD. Similar effects were obtained with allyl methyl sulfide (AMS). Phenylethyl isothiocyanate (PI) and benzyl isothiocyanate (BI), both of which contain isothiocyanate structure, induced dose-dependent increases of $[Ca^{2+}]_i$ in cells regardless of responsiveness to capsaicin. Moreover, the toxic effect was caused by all concentrations used. The $[Ca^{2+}]_i$ increase induced by PI or BI (3 mM) was not inhibited by capsazepine (10 μM). Methyl salicylate (MS) (3 mM or less), which is known to stimulate other TRP channel subtypes induced

dose-dependent increases of $[Ca^{2+}]_i$ in cells responding to capsaicin. The $[Ca^{2+}]_i$ increase induced by MS (3 mM) was inhibited by capsazepine (10 μM). A high concentration (30 mM) of MS induced $[Ca^{2+}]_i$ increase even in cells unresponding to capsaicin. Propyl disulfide (PD) induced dose-dependent increases of $[Ca^{2+}]_i$ in cells responding to capsaicin. PD did not evoke $[Ca^{2+}]_i$ increase in naïve cells. The $[Ca^{2+}]_i$ increase induced by PD (3 mM) was inhibited by capsazepine (10 μM). PD at 1 mM or more produced the toxic effect in cells regardless of the expression of TRPV1 or not.

These results indicate that allyl base-containing compounds related to AITC are capable of activating TRPV1 and that their methylated compounds have high specificity to TRPV1. Since TRPV1 plays a role in the pheripheral nociceptor and the desensitization of this receptor is related to anti-nociceptive action of vanilloid compounds, it is suggested that these compounds are important target for development of new analgesic.

Changes in transient receptor potential V1-mediating contractile responses of detrusor muscle in cyclophosphamide-induced cystitis of mice

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I compared the degree of inflammation between wild and *TRPV1*$^{-/-}$ mice with cyclophosphamide-induced cystitis. Cystometrogram was performed to examine the change of the voiding responses by cystitis. I also examined contractile responses of the detrusor muscle isolated from cyclophosphamide-treated (cystitis) and untreated (control) mice. The administration of cyclophosphamide induced cystitis with dilation and hemorrhage. The increase of the bladder weight by cystitis was more remarkable in wild than *TRPV1*$^{-/-}$ mice. Histological analysis indicated that the degree of inflammation was more serious in wild than *TRPV1*$^{-/-}$ mice, suggestive of the involvement of TRPV1 in inflammation. Cytometrogram revealed that intercontraction interval (ICI) was shortened by cystitis in wild mice, but not in *TRPV1*$^{-/-}$ mice. Cystitis enhanced spontaneous contractions of the detrusor muscle in both wild and *TRPV1*$^{-/-}$ mice. The extent of this enhancement was greater in wild than *TRPV1*$^{-/-}$ mice. The amplitude of spontaneous contractions of the detrusor muscle isolated from control and cystitis was not decreased by a TRPV1 antagonist. Contractile responses to carbachol and α, β-methylene ATP were not influenced by cystitis, and there were no difference between wild and *TRPV1*$^{-/-}$ mice. The electrical field stimuation-induced contraction was suppressed by atropine, a muscarinic receptor antagonist and desensitization of the purinergic receptor by α, β-methylene ATP. The degree of these suppressions was not changed by cystitis. Capsaicin, a TRPV1 agonist, evoked a sustained contraction of the detrusor muscle from wild but not *TRPV1*$^{-/-}$ mice. The capsaicin-induced contraction was significantly enhanced by cystitis and disappeared by a TRPV1 antagonist. Repetitive application of capsaicin produced marked desensitization, the extent of which was much less in cystitis. The capsaicin-induced contraction was not affected by atropine, but suppressed by the desensitization of purinergic receptors to the same extent in control and cystitis. The capsaicin-induced contraction was suppressed by each tackykinin receptor (NK1 and NK2) antagonists, and blocked by both of them. It is suggested that

substance P and neurokinin A released from the sensory neurons are involved in contractile response to capsaicin. In cystitis, the capsaicin-induced contraction was more sensitive to NK1 antagonist than NK2 antagonist. In control and cystitis, the removal of urothelium did not affect the capsaicin-induced contraction. Substance P-induced contraction was significantly enhanced by cystitis in both wild and *TRPV1*$^{-/-}$ mice. On the other hand, cystitis slightly enhanced neurokinin A-induced contraction in wild but not *TRPV1*$^{-/-}$ mice.

These results suggest that TRPV1 is involved in inflammation and the cause of the frequent urination in cyclophosphamide-induced cystiits. The results indicate that the capsaicin-induced detrusor muscle contraction occurs solely via the activation of TRPV1 and that is enhanced by cystitis. It is suggested that the enhancement of the capsaicin-induced contraction by cystitis is related to the increase of tackykinin (especially, substance P) responsiveness to the detrusor muscle.

The depression of spinal reflex potentials during hypoxia in isolated spinal cord of neonatal rat *in vitro*

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In order to investigate the mechanism of the hypoxia-induced inhibition of the neuronal activity, we studied the effect of hypoxia on spinal reflex potentials by exposing isolated spinal cords to hypoxic ACSF (5% CO_2+ 95% N_2) *in vitro*.

The monosynaptic reflex potentials (MSR) and slow ventral root potentials (sVRP) were recorded from a ventral root (L3-L5) with the electrical stimulation of the corresponding lumbar dorsal root in rat isolated spinal cords. The MSR and sVRP were reversibly depressed by a brief exposure (10-15 min) to hypoxia. The inhibitory effects of hypoxia on spinal reflex potentials were smaller in younger rats (Day 0-4) than older rats (Day 5-8). Application of adenosine (0.1 μM～1 mM) caused a concentration-dependent depression of MSR and sVRP. The sensitivity to adenosine was a little higher in MSR than sVRP. The depression of MSR and sVRP by adenosine was attenuated by a selective adenosine A_1 receptor antagonist, CPT. CPT (3 μM) potentiated MSR and sVRP by about 20%. In the presence of CPT, the hypoxia-induced depression of MSR was virtually abolished. The depression of sVRP during hypoxia was significantly attenuated, but not abolished by CPT. Inhibitors of equilibrative nucleoside transporter (ENT), NBTI (5 μM) and dipyridamole (10 μM), had no effect on MSR, but reduced sVRP by about 10%. Both NBTI and dipyridamole were ineffective in the depression of MSR and sVRP during hypoxia. A $GABA_A$ receptor antagonist, bicuculline (5 μM), did not affect MSR but markedly potentiated sVRP. In the presence of bicuculline, the hypoxia-induced depression of MSR was rather profound compared with that in the absence of bicuculline. The depression of sVRP with hypoxia was significantly attenuated, but not abolished by bicuculline. A glycine receptor antagonist, strychnine (1 μM) did not affect MSR but potentiated sVRP dramatically. There were no significant change in the hypoxia-induced depression of MSR and sVRP in the presence of strychnine, but the degree of the depression of sVRP tended to be smaller. An acetylcholine muscarinic receptor antagonist, atropine (10 μM), had no effect on MSR and sVRP, nor affected the depression of MSR and sVRP during hypoxia.The NO synthase inhibitor, L-NAME (10, 100 μM), slightly increased MSR and sVRP.

L-NAME were not effective in the depression of MSR and sVRP by hypoxia.

These results suggest that hypoxia depresses MSR through adenosine release and subsequent activation of A_1 receptors. ENTs seem to play a role in regulating extracellular adenosine concentration in rat spinal cords, but have little effect on the adenosine release by hypoxia. Hypoxia also reduced sVRP, but adenosine did not seem to be the main factor in this depression. $GABA_A$ receptors, glycine receptors, muscarinic receptors, and NO were not relevant in this depression of sVRP. It is concluded that the inhibitory effects of hypoxia on spinal reflex potentials are associated with several factors.

Basic study of pathogenicity and prophylaxis of pestivirus infection

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Classical swine fever viruses (CSFVs) that did not show the exaltation of Newcastle disease virus (END) phenomenon (END-CSFV) induced clear cytopathic effect (CPE) in porcine kidney serum-free cultured cell line, CPK-NS. The accumulation of the nonstructural protein NS3 was observed in the cells infected with END-CSFV. In this study, viral and cellular factors were identified and elucidated the mechanism for this cytopathogenicity in CPK-NS cells. In CPK-NS cells infected with END-CSFV, a large amount of type I interferon (IFN) was induced, resulting in manifestation of CPE. The involvement of nonstructural protein N^{pro} of CSFV in this phenomenon was revealed by experiments of CPK-NS cells infected with mutant viruses. CPE was observed in CPK-NS cells after addition of recombinant type I IFN into the culture media. Apoptosis were induced in CPK-NS cells that showed CPE by END-CSFV infection or IFN treatment. The amount of NS3 was greater than that of NS2-3 in the CPK-NS cells infected with CSFV that show the END phenomenon after the induction of apoptosis with either recombinant type I IFN or apoptosis inducer Camptothecin. These results suggest that the amount of induction of type I IFN in CPK-NS cells infected with END-CSFV concern the manifestation of CPE. Moreover, dominant expression of NS3 resulted from apoptosis caused by type I IFN.

To establish an effective vaccination program for bovine viral diarrhea (BVD) and infectious bovine rhinotracheitis, commercial polyvalent vaccines which contain inactivated BVD virus were injected in cattle. The single shot of vaccination induced low titer of antibody in cattle which were seronegative to BVD virus and bovine herpes virus 1. One month after the second vaccination, neutralizing antibodies were detected significantly in cattle and antibody titers decreased gradually. It was also demonstrated that an additional shot of vaccination at 12 months after the first vaccination boosted antibody response. On the basis of the present results, two shots of vaccination and an annual booster shot in seronegative cattle are recommended for the prevention of these diseases.

Studies on the development of novel influenza vaccine using baculovirus vector

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Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have occurred worldwide, and these HPAI viruses are lethal not only for domestic poultry but also for feral water birds. In the present study, a recombinant baculovirus expressing the hemaggulutinin gene of A/duck/Hokkaido/Vac-1/04 (H5N1) was constructed as an influenza vaccine. Chickens were inoculated intranasaly with the recombinant baculovirus and then challenged with HPAI virus, A/chicken/Yamaguchi/7/04 (H5N1).

Antibodies to the HA of the influenza virus were detected in the serum of chickens inoculated with the recombinant baculovirus. These chickens were challenged intranasaly with 100 CLD_{50} of an HPAI virus. Chickens that were not inoculated with baculovirus died on 2 days after the challenge. One out of 4 chickens of the group either given 1 or 3 shots of the baculovirus vaccine survived1day longer than the uninoculated chickens after the challenge. The present results indicate that the immune response elicited by the baculovirus is not enough to protect chickens from the infection of HPAI virus. Further research how to express the antigen in baculovirus, and volume of inoculation and route of vaccination should improve the efficacy, and hence enable to develop an efficient vaccine against influenza using baculovirus.

Analysis of pathogenicity of highly pathogenic avian influenza virus in ducks and establishment of a novel serodiagnostic method for avian influenza

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Ducks are natural reservoir of non-pathogenic influenza A viruses. However, it is known that some H5N1 highly pathogenic avian influenza viruses (HPAIVs) kill ducks since 2002. In order to define the key gene segments of pathogenicity of H5N1 HPAIV in ducks, genetic reassortant viruses were generated by reverse genetics between A/Hong Kong/483/97 (H5N1) which is low pathogenic in ducks and A/Whooper swan/Mongolia/3/05 (H5N1) which kill ducks, and examined their pathogenicity in ducks. The results indicate that the PB2, NP and NS genes are responsible for pathogenicity in ducks. To clarify the molecular basis of PB2, NP, NS1 and NS2 associated with the pathogenicity, investigations are underway.

To differentiate infected from vaccinated chickens, a novel diagnostic method, NS1-ELISA, using recombinant NS1 has been established in the present study. Allele A NS1s were found to react with antibodies to NS1 more broadly than allele B NS1 by antigenic analysis of NS1 derived from allele A and B. Antibodies to NS1 were not found in vaccinated chickens but found in chickens infected with influenza viruses. HPAI vaccine in-

hibited manifestation of disease signs but did not inhibit the infection, so that antibodies to the NS1 were detected in vaccinated chickens challenged by HPAIV. The present results indicate that NS1-ELISA is available for differentiating infected chickens from vaccinated ones.

Prevalence and source of trypanosome infections in field-captured vector fly *Glossina pallidipes* in Eastern Zambia

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The prevalence of trypanosome infections was assessed by PCR in tsetse flies (*Glossina pallidipes*) captured from two regions with endemic trypanosomiasis in Zambia. Out of the 550 *G. pallidipes* captured by baited biconical traps, 163 (29.6%), including 130 females and 33 males were found to harbor trypanosome DNA. In the Chiawa region, infection rates of tsetse with *T. brucei brucei, T. vivax* universal, *T. congolense* savannah, *T. congolense* forest and *T. congolense* kilifi were 18.2% (90/492), 2.8% (14/492), 4.2% (21/492), 1.2% (6/492) and 1.8% (9/492), respectively. Out of the 58 flies captured in the Chakwenga area, 1 (1.7%), 49 (84.4%), 9 (15.5%) and 5 (8.6%) were positive for *T. brucei rhodesiense, T. brucei brucei, T. vivax* universal and *T. congolense* savannah, respectively.

To determine the mammalian hosts of the *T. brucei, T. congolense* and *T. vivax* infections in the tsetse flies, mammalian mitochondrion DNAs in these flies were analyzed by PCR and subsequent sequence analysis of the amplicons. Forty-eight (31.1%) of the 154 infected flies examined were found to harbor mammalian mitochondrion DNA, suggesting that these tsetse flies recently obtained a mammalian blood meal. Sequence analysis of the DNA amplicons showed the presence of mitochondrion DNA from 9 different mammalian species. The *Caprine cytochrome b* gene was among the 13 identified mitochondrion DNA in the tsetse flies, and thus, the prevalence of trypanosome infections was examined in the native goat population in the Chiawa area. Of 86 goats sampled, 35 (40.6%), 4 (4.6%), 5 (5.8%), 4 (4.6%) and 4 (4.6%) were positive for *T. brucei brucei, T. vivax* universal, *T. congolense* savannah, *T. congolense* forest and *T. congolense* kilifi, respectively. These results showed that the host source of the trypanosome infections in the vector fly can be determined by the analysis of the fly extracts for the presence of both parasite and mammalian host DNA.

Identification of biologically significant factors derived from *Ixodes persulcatus* and characterization of antimicrobial peptide "defensin" of the tick

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Ticks transmit several pathogens which cause diseases, and cause great damages on livestock industry and human public health. Currently, the use of acaricides is the most effective method to control tick infestation, but it has many disadvantages such as the selection of acaricide-resistant ticks and environmental contamination. As an alternative strategy, many efforts have been made to develop anti-tick vaccines.

Ixodes persulcatus is a vector for *Borrelia afzelii* and *B. garinii* causing Lyme borreliosis in Japan. Though information on genes expressed during blood feeding of ticks is necessary to search for candidates for anti-tick vaccine or to understand the mechanisms of the transmission of tick-borne pathogens, little is known about that of *I. persulcatus*. Thus, the aim of this study was to identify a factor important for blood feeding of *I. persulcatus* and to apply it as a candidate for anti-tick vaccine.

A cDNA library was constructed from whole bodies of fed nymph of *I. pesulucatus*. From this library, one cDNA encoding defensin-like antimicrobial peptide was identified and named as *Ip-Def*. Defensins are found in other ticks and arthropods, and considered to be important for their innate immunity. The amino-acid sequence of *Ip-Def* showed high similarities to those of the defensins of other ticks and arthropods. RT-PCR analysis showed that the *Ip-Def* gene is expressed strongly in the midgut, and up-regulated after blood feeding. The synthetic Ip-Def peptide inhibited the growth of several kinds of bacteria at different concentrations. Ip-Def is a concealed antigen, but vaccination of hamsters with the recombinant Ip-Def resulted in 13.6% mortality of adult ticks after engorgement.

Ip-Def may be involved in the defense system against microbes in the midgut of the blood-fed tick. Additionally, Ip-Def could be a candidate antigen for anti-tick vaccine, though other antimicrobial molecules should be also characterized.

Molecular epidemiological analysis of sandfly species in Pakistan by PCR-RFLP method

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Sandfly belonging to Diptera, Psychodidae, Phlebotominae is a minute bloodsucking insect and includes two genera in the old world. One is the genus *Sergentomyia* which takes blood of cold-blooded animals and the other is the genus *Phlebotomus* which takes blood of warm-blooded animals. Since sandfly is known to be a vector of leishmaniasis, it is important to research the sandfly species and their habitats in the endemic areas for control of leishmaniasis. In Pakistan, although

it is suspected that *Leishmania (Leishmania) major* is transmitted by *P. papatasi* and *Leishmania (Leishmania) tropica* is transmitted by *P. sergenti,* the accurate distribution of leishmania protozoa and sandfly species is not fully investigated. The purpose of this study is to develop the PCR-RFLP (PCR-linked restriction fragment length polymorphism) method targeting 18S rRNA gene for identification of Pakistani sandflies and to determine the distribution of sandfly species in southern Pakistan.

From May 28 through June 2, 2007, a total of 507 sandflies were collected at four villages in Sindh Province, Pakistan, using two kinds of light trap named Shannon and CDC trap in the night time. The ratio of male and female sandflies collected was about 1 : 2. DNA samples extracted from 26 individual sandflies were subjected to PCR amplification of the 18S rRNA gene and to sequencing of the products. Based on DNA sequences of these Pakistani sandfly samples and those of *Phlebotomus* and *Sergentomyia* species registered in GenBank, restriction enzymes were selected for identification of sandfly species by PCR-RFLP. Using this method, a total of 289 samples (142 males and 147 females) were analyzed and we found that *Sergentomyia* and *Phlebotomus* species occupied 86% and 14% of the total, respectively. Among *Phlebotomus* species, 71% and 29% of the samples were most likely to be *P. papatasi* and *P. alexandri,* respectively. Additionally, *P. papatasi* species appeared to habit in an environment close to human residential places rather than animal sheds, suggesting that recent outbreak of cutaneous leishmaniasis in southern Pakistan was mediated by *P. papatasi.*

Efficacy of bruceine A against *Babesia gibsoni in vitro* and *in vivo:* Seeking novel antibabesial agents in traditional medicinal plant

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In the present study, we evaluated the efficacy of bruceine A *in vitro* and *in vivo* against *Babesia gibsoni.* Bruceine A exhibited better *in vitro* antibabesial activity than diminazene aceturate in terms of minimum dose required for 50% parasites growth inhibition and rapid deduction in parasites number within 24 hours. From the results obtained in *in vitro* antibabesial assay, it was suggested that bruceine A has the effects on parasite clearance and inhibition on multiplication.

Bruceine A was also preliminary tested as the chemotherapeutic agent for dogs experimentally infected with *B. gibsoni.* The dogs were orally administrated bruceine A daily for 6 days at a dose of 6.4mg/kg. No clinical sign was observed in dogs treated with bruceine A, whereas control dog developed acute babesiosis along with severe anemia and high fever. However, complete elimination of *Babesia* parasites from peripheral blood was failed in the treated dogs.

Additionally, cytotoxicity assays were conducted on human tumor cell lines. Bruceine A showed the potent growth inhibitory effects on human gliosarcoma cells (GI-1), human colon cancer cells (HCT-15) and human promyelocytic leukemia cells (HL60). This result will be utilized for further research on bruceine A as an antibabesial agent.

The first trial of RNA interference in protoscolex of *Echinococcus multilocularis* (order Cestoda)

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Echinococcus multilocularis causes a serious helminthic zoonosis. The parasite proliferates and produces numerous protoscoleces in intermediate hosts. This study was aimed to establish a suitable protocol to inhibit gene expression by RNA interference in protoscoleces of a cestode, *Echinococcus multilocularis* for gene function analysis.

For introducing siRNA, soaking and electroporation were applied because of their simplicity and ability to manipulate a large number of protoscoleces at once. Firstly, the suitable conditions were verified by evaluating the survival rates of the protoscoleces treated by those two methods. For soaking, the concentration of 10 μl *NeoFX* (reagent for RNA introduction) in 100 μl culture medium was determined optimum. For electroporation, 100 V-800 μF and 200 V-100 μF were optimum as the combinations of electrical pressure (V) and capacity of capacitor (F). In those conditions, survival rates of the protoscoleces were higher than 80%. Secondly, protoscoleces treated with 5 μM fluorescent labeled siRNA under the selected conditions were subjected to confocal microscopy. Strong fluorescence was observed in protoscoleces treated by electroporation, but not by soaking, indicating that siRNA was introduced to protoscoleces by electroporation, but less by soaking.

Finally, siRNA targeting mRNA of EmAgB8/1, which is one of the major antigens of protoscolex, was introduced to protoscoleces by soaking (*NeoFX* 10 μl) and electroporation (100 V-800 μF). The result showed that the expression of EmAgB8/1 mRNA was highly depressed by electroporation but moderately by soaking. However, it was also observed that the applied conditions of electroporation caused the formation of bubble-like structures on the surface and of vacuoles inside of the protoscoleces. Moreover, expression of EmAgB8/1 mRNA was moderately depressed by electroporation without siRNA. Those results indicated that electroporation in the selected conditions itself may have caused the damage and the depression of mRNA expression in the tegument (surface syntidial cells) of protoscoleces.

This study demonstrated for the first time that RNAi could be induced in *E. multilocularis*. Although the protocol has to be improved, it would be a powerful tool for gene function analysis of *E. multilocularis*.

Fine mapping of the *hooded* gene in the rat

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The analysis of genes deficient in the LEC rat

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The LEC rat was originally established from a closed colony of non-inbred Long-Evans parental rats. The LEC rat is a unique animal model which shows hepatitis and hepatoma (*hts*), immunodeficiency (*thid*) and high sensitivity to radiation (*xhs*). Previous reports have shown that a mutation of the *Atp7b* gene is responsible for the *hts* mutation. The mutation indicated a large deletion of carboxy-terminal region, however, a precise deletion breakpoint in the *Atp7b* gene was not known. In the present study, I identified a deletion breakpoint of the *Atp7b* gene in the LEC rat genome precisely. There was 13 kb-deletion from intron 15 to the 3'-flaking region of the gene. This region included three expressed sequence tags (ESTs), whose expression was not seen in the LEC rat. In addition, I developed genotyping method of the $Atp7b^{hts}$ allele. This technique will be useful for the manufacture and maintenance of the $Atp7b^{hts}$ congenic rats.

The LEC rat also shows immunodeficiency, which is called T-helper immunodeficiency (*thid*) mutation. The *thid* mutation shows a defect in maturation of CD4 single positive (SP) thymocytes and an abnormality of peripheral helper-T cells. I attempted to identify a gene responsible for the *thid* mutation. I first performed genetic linkage analysis and mapped *thid* locus between *Myb* and *D1Rat392* on Chromosome 1. In this region, I found a～380 kb deletion from intron 3 of the *Ptprk* gene, which encodes a receptor-like protein tyrosine phosphatase type κ (RPTPκ), to intron 1 of *RGD1560849* predicted gene in the LEC rat genome. Reconstitution with syngenic BM cells transduced *Ptprk* but not *RGD1560849* predicted gene rescued development of CD4 SP cells in the LEC rat thymus. It is confirmed by this result that the *Ptprk* gene is responsible for the *thid* mutation in the LEC rat. These results further suggest that RPTPκ plays a critical role in the development of CD4 SP cells in the thymus.

Analysis of the female infertility of DW/J-*grt* mouse

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Changes in the specific gravity of canine erythrocytes with inherited high glutathione concentration during their aging

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Normal canine erythrocytes do not have Na, K-ATPase on their membrane and contain low potassium (K^+) and high sodium (Na^+) concentrations (i.e., canine LK erythrocytes). In contrast, some canine erythrocytes contain high K^+ and low Na^+ concentrations as a result of an inherited high Na, K-ATPase activity (i.e., canine HK erythrocytes). It is known that canine HK erythrocytes have a lower specific gravity and a larger cellular volume than canine LK erythrocytes, that it is easy to fractionate canine HK erythrocytes according to their specific gravity, and that those with a much lower specific gravity have much higher amino acids and reduced glutathione (GSH) concentrations. The specific gravity of canine LK erythrocytes increases and their cellular volume decreases during their aging. Recently, however, it was suggested that canine HK erythrocytes with a lower specific gravity and larger cell volume might be older than those with a higher specific gravity and smaller cell volume. Accordingly, we employed proteins 4.1a and 4.1b as aging markers. The 4.1a/4.1b ratio is a general aging marker, increasing during erythrocyte aging. We investigated the relationship between the cellular volume and aging of canine HK erythrocytes using the 4.1a/4.1b ratio.

Canine HK erythrocytes were fractionated according to their specific gravity, and the 4.1a/4.1b ratio, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), the methemoglobin (metHb) concentration which is a general marker for oxidative damage, and osmotic and intracellular Na^+, K^+, and chloride (Cl^-) concentrations of each fraction of canine HK erythrocytes were determined. As a result, canine HK erythrocytes with a lower specific gravity and larger cell volume had a higher 4.1a/4.1b ratio and higher MetHb concentration than those with a higher specific gravity and smaller cell volume. Generally, it is known that aged erythrocytes undergo oxidative damage. Therefore, the increase in the metHb concentration is correlated with aging. From these results and previous reports, it is shown that the cell volume of canine HK erythrocytes increases during their aging. Moreover, in canine HK erythrocytes, the intracellular Na^+ concentration and MCHC gradually decreased and the intracellular K^+ concentration and osmotic fragility increased during aging. Also, it was previously reported that aged canine HK erythrocytes have higher amino acid and GSH concentrations than younger ones. Based on these results and previous reports, it was suggested that canine HK erythrocytes might accumulate K^+ and amino acids, resulting in elevating the intracellular osmotic pressure. This elevated pressure might increase the MCV of canine HK erythrocytes by inducing an increase in intracellular water.

In conclusion, the present study indicates that the cell volume of canine HK erythrocytes increases during aging. Since the mechanism of this phenomenon is unclear, further studies are necessary.

Effects of an ionophore, amphotericin B, on *Babesia gibsoni in vitro*

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The effects of ionophore compounds, amphotericin B (AMB) and liposomal amphotericin B (L-AMB), on *Babesia gibsoni* and canine erythrocytes were investigated *in vitro*.

B. gibsoni was cultured with normal canine erythrocytes. When AMB was added to the culture, the number of intracellular parasites was immediately decreased depending on both the AMB concentration and incubation time. Meanwhile, when L-AMB was added to the culture, the number of parasites was slightly decreased after 24-hr incubation, and was significantly decreased after more than 48-hr incubations. L-AMB is a liposomal formulation of AMB, developed to reduce the toxicity of AMB. Therefore, it was supposed that L-AMB could not directly attack *B. gibsoni* in canine erythrocytes. Consequently, the activity of L-AMB against *B. gibsoni* free from canine erythrocytes was examined. When free *B. gibsoni* and canine erythrocytes were cultured together with L-AMB, the parasites could not invade the erythrocytes. These results suggest that L-AMB might attack only extracellular *B. gibsoni,* resulting in much longer times to decrease the number of parasites.

Subsequently, the hemolytic activity of each drug on canine erythrocytes was examined. The normal canine erythrocyte exhibits almost no concentration gradient regarding sodium and potassium ions. Accordingly, it was considered to be unaffected by ionophore compounds. However, each drug could hemolyze normal canine erythrocytes. This result indicated that these drugs might affect canine erythrocytes not only as ionophores but also as chemical denaturants of the erythrocyte membrane. Further, it was suspected that they might affect *B. gibsoni* indirectly by modifying the host cell membrane and environment. Furthermore, each drug hemolyzed infected more than uninfected erythrocytes. This suggested that both drugs might selectively hemolyze erythrocytes infected with *B. gibsoni*. In addition, the hemolytic activity of AMB was stronger than that of L-AMB, indicating that the toxicity of AMB on erythrocytes could be weakened by binding together with liposome.

In the present study, it was apparent that AMB exhibited both a potent anti-babesial activity against *B. gibsoni* and a potent toxicity against canine erythrocytes. On the other hand, L-AMB, a liposomal formulation of AMB, was much less toxic to canine host erythrocytes, although its anti-babesial activity was also weakened. In the future, the improvement of ionophore compounds and liposome technology may lead to the development of excellent anti-babesial drugs.

Assessment of canine thyroid function by thyroid hormone measurement and ultrasonography

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In the present study, circadian variations in free thyroxine (FT4) and canine thyroid-stimulating hormone (cTSH) concentrations in healthy dogs were investigated. In addition, thyroid gland volumes were measured using ultrasonography.

Serum FT4 and cTSH concentrations in 13 healthy dogs, aged 3 to 16 years old, were measured every two hours for 12 or 24 hours. Serum FT4 concentrations fluctuated randomly throughout these two time periods, suggesting that there was no specific pattern in circadian variation of the serum FT4 concentration in the dogs. The minimum serum FT4 concentration in each dog did not correlate with the body weight (BW), age, or sex. Exercise was not suitable for stimulating the thyroid gland to elevate the serum FT4 concentration in the dogs. These results indicated that the thyroid function of dogs does not seem to be assessable on single measurement of the serum FT4 concentration.

The serum cTSH concentration also fluctuated randomly, and the ranges of its fluctuation were smaller than that of the serum FT4 concentration. This smaller fluctuation in the serum cTSH concentration was not related to the serum FT4 concentration. Transiently high concentrations of serum cTSH were observed in two dogs. In one of them, this transiently high concentration seemed to induce an increase in the serum FT4 concentration. These temporarily high concentrations of cTSH might constitute a cTSH-pulse. On overall consideration, this study showed that the physiological characteristics of cTSH have not been clarified sufficiently and that further investigations into cTSH are required to clarify the relation between FT4 and cTSH.

Ultrasonography of the thyroid gland revealed that the thyroid gland volume was positively correlated with the BW of dogs. Further, the volume/BW ratio was negatively correlated with the serum FT4 concentration in healthy dogs. These results suggest that the thyroid function might be depressed in dogs with both a low serum FT4 concentration and small thyroid gland.

In summary, single measurement of the serum FT4 concentration does not seem to be a reliable test for assessing the thyroid function of dogs. The physiological characteristics of cTSH have not been clarified sufficiently, and so further studies on cTSH are necessary. Additionally, ultrasonography of the thyroid gland will be a useful examination for evaluating thyroid function based on its morphology.

Basic study on clinical application of antitumor immunotherapy using dendritic cells in canine tumor patients

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Dendritic cells (DCs) are considered to be essential to induce tumor antigen-specific cytotoxic T lymphocytes (CTL) in antitumor immune response. The methodology of DC immunotherapy against human malignant tumor cases has been established, however, very little information regarding its therapeutic evidence and clinical approach is available in the veterinary oncology.

In this study, DCs were derived from canine peripheral blood mononuclear cells (PBMC) by treatment with recombinant human (rh) granulocyte-macrophage colony stimulating factor, recombinant canine (rc) interleukin (IL)-4 and rc tumor necrosis factor-α. These cells were fused with two kinds of tumor cells originated from different tumor cell lines, canine malignant melanoma (CMeC) and canine osteosarcoma (HMPOS). The fusion efficiency of cell treatment with polyethylene glycol was evaluated. Toxicity of repeated subcutaneous injection of rcIL-12 was clinically evaluated in 6 healthy dogs at the dosage of 1 μg/m^2. Clinical effect of immunotherapy with fused DCs was evaluated for 6 dogs with spontaneous tumors. In addition, tumor cells specific cytotoxic activity assay was performed in one of these dogs.

It was observed that as the DCs: tumor cells ratio is increased the more efficient is the cell fusion. Following first administration of rcIL-12 at the dosage of 1 μg/m^2, serum interferon-γ levels were raised and ratios of tumor-specific CD4+ and CD4+ CD8+ T cells in canine blood were increased, whereas toxic effect related to repeated administrations of rcIL-12 at this dosage was not clinically observed. No side effect was found following repeated administration of autologous DCs fused with autologous tumor cells and rhIL-12. PBMC obtained from one tumor dog after repeated administrations displayed autologous tumor cells specific cytotoxic activity. Furthermore, size reduction of a distant metastatic tissue could be identified in one patient.

These results suggested that autologous DCs fused with tumor cells might be efficient in order to promote necessary immunological reaction towards canine tumorous tissue without clinical recognition of side effects and to preserve general physical condition in tumor-bearing animals.

The utility of local photodynamic therapy by intratumor injection of benzoporphyrin derivative monoacid ring A (BPD-MA) in mice

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Photodynamic therapy (PDT), involving the use of photochemical reactions mediated through the interaction of an intravenously administrated photosensitizer, light and oxygen, makes it possible to achieve highly selective antitumor treatment. In recent years, topical applications of the

agent have been investigated and are expected to lead to less systemic adverse reactions performed by high local tissue concentration with lower dose of the drug and shorter treatment time. The purpose of the present study was to evaluate the pharmacokinetics and PDT effects following intratumor injection of BPD-MA in murine tumor models.

Cytotoxity of PDT depending on time exposed by BPD-MA was evaluated in cultured tumor cells *in vitro*. Phototoxicity was most effective in 3 hour-exposure of BPD-MA, and remained stable even in longer exposure. It was suggested the difference of cells influenced the sensitiveness of cytotoxity by PDT.

The pharmacokinetics and PDT effects following intratumor injection of BPD-MA was evaluated in murine tumor models. It was suggested the tumor selectivity of BPD-MA administered directly into tumor tissue resulted in reaccumulation of the agent in the tumor tissue after distribution to systemic circulation. Once light was given to the tumor, the region of tumor destruction tended to be relatively stable (approximately 65%). The length of time between administration of the agent and irradiation did not affect the phototoxicity.

In conclusion, the results suggested that local PDT with intratumor injection of BPD-MA is a promising antitumor treatment in respect of high local tissue concentration with lower dose of the drug administered and shorter treatment time, though it did not necessarily lead to highly destruction of neoplastic tissue.

Effect of cartilage extracellular matrix on canine cultured synovial cells and chondrocytes *in vitro*

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Since arthrosis is estimated to affect as much as 20% of canine population, a new therapeutic approach and understanding of it are expected to be necessarily improved. Hyaluronic acid (HA) and polysulphated glycosaminoglycan (PSGAG) are commonly used as a symptomatic treatment for joint disorders in small animal practice. However, it is still unclear how exactly these drugs produce clinically favorable effects. The purpose of this study was to elucidate mechanisms, through which HA and PSGAG contribute to alter progression of articular pathology using canine cultured synovial cells and chondrocytes.

In this study it was investigated the effect of HA and PSGAG on the proliferation of cultured synovial cells and chondrocytes by means of MTT-assay along with its proapototic status evaluated by nuclear staining with hoechst33342. In separate trials, the expression of the canine *CD44* gene and canine hyaluronic acid synthase (*HAS*) genes in cultured canine synovial cells and chondrocytes was examined by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, the effect of HA and PSGAG on the expression of canine *HAS* in these cells was investigated by semi-quantitative RT-PCR.

As a result, HA enhanced proliferation in cultured synovial cells and chondrocytes when stimulated with either recombinant human (rh) interleukin (IL)-1ß or prostaglandin (PG) E_2. PSGAG in high concentration induced proapoptotic effects to cultured synovial cells and chondrocytes. The expression of canine *CD44* gene was detectable in these cells, suggesting its role of a cell surface receptor for HA and PSGAG. HA, as well as rhIL-1ß and PGE_2, induced activation of canine *HAS-1, 2* and *3* genes in cultured synovial cells whereas the activation of canine *HAS-2* and *3* genes were

found in cultured chondrocytes. PSGAG did not affect the expression of canine *HAS* genes in these cells.

In conclusion, these results of this study suggested that exogenous HA induced activation of canine *HAS-2* gene in canine synovial cells and chondrocytes, leading to the synthesis of high molecular weight HA, might explain inhibitory effect of HA on inflammation.

Epidemiological Study on Avian Retroviruses with Unique Oncogenicity in Nervous System

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Avian leukosis virus (ALV) mainly causes hematopoietic neoplasms in chicken, whereas fowl glioma-inducing virus (FGV), which belongs to ALV subgroup A, induces astrocytoma in chickens. Fowls kept in a zoological garden in Toyama have been considered as source for the infection in other Japanese zoological gardens. However, the background and process how FGV acquired the unique oncogenicity in nervous system has not been clarified. Ancestors of Japanese fowl are thought to be brought to Japan in the Edo era from Southeast Asia. Along the propagation path, feather pulps and cloaca swab were collected from 264 chickens in Indonesia, Philippines, South Korea and Japan. These chickens were not related by birth to any fowls of the zoological gardens in Toyama. These materials were examined to detect FGV by nested polymerase chain reaction (PCR). Twenty nine chickens, including 9 birds in the foreign countries, were positive for nested PCR and the amplified products showed 97% nucleotide sequence homology to the corresponding region of FGV. Six strains of FGV were isolated from these chickens in Japan. On the other hand, two Japanese fowls of a flock in Japan showed perineurioma and neurofibroma, respectively. This was the first case of avian perineurioma in the field. An ALV strain was isolated from neurofibroma and its nucleotide sequence of corresponding to the *env*-3' LTR region showed 98% homology to that of the *ev-1* strain. This result suggests that the ALV isolated from neurofibroma is a mutant from the *ev-1* because *ev-1* is an non-infectious endogenous virus. From these findings, domestic and foreign chickens unrelated to the birds in the zoological garden in Toyama are suggested to be infected with FGV. It is speculated that FGV has appearred outside of the zoological garden and a part of FGV-infected chickens were brought to this garden and used for breeding.

Does the fever enhance the pathogenicity of influenza virus to mouse brain?

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Influenza encephalopathy is the central nerve system (CNS) disease induced by influenza virus infection. The disease has been characterized by peracute loss of conscious and coma following high fever, high mortality rate and poor prognosis. Influenza virus has not often demonstrated in the brain tissue and cerebrospinal fluid of many patients, thus the pathogenesis of the disease is unknown.

In this study, we investigated histologically and immunohistochemically the effect of the fever on the pathogenicity of influenza virus in mice. In experiment 1, we confirmed a pyrogenicity of intracerebroventrical injected prostaglandin E2 (PGE2), an endogenous pyrogen. In experiment 2, we infected mice with H3 subtype influenza A virus which has often been isolated from respiratory tract of patients affected with influenza encephalopathy. Infected mice showed severe pneumonia, but other organs including the CNS were unremarked. We could detect the viral antigen only in the pulmonary lesions.

PGE2 injected to the mice infected with influenza virus didn't show the pyrogenicity, and survival rate was higher than that of influenza virus infection plus saline injection group. In pulmonary lesions, the neutrophilic infiltration was more prominent in the former than the latter group.

We here demonstrated that, intracerebroventrical injection of PGE2 enhanced defense mechanism against influenza virus infection. Cytokines induced by PGE2 treatment and their effects on influenza virus infection should be examined to clarify the detail of the enhanced defense mechanism.

Evaluation of therapeutic potential of intracerebral immunization against rabies

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Rabies is one of the lethal viral zoonoses occurred in many mammals including humans. Once the clinical signs of rabies appeared in patients, almost all of them are invariably fatal. So, we definitely need to develop therapeutic measures for the disease. In the previous study of my laboratory, we could completely prevent rabies virus-inoculated mice from developing clinical signs by intracerebral (I.C.) immunization, inoculation of the antigens directly into the brain parenchyma or subarachnoid spaces to induce antibody response in the cerebrospinal fluid. In this study, I tried to examine the therapeutic values of I.C. immunization by using experimental animals, mice and hamsters.

In the first, to estimate therapeutic effect of I.C. immunization after the invasion of rabies viruses into brains, I prepared the I.C. immunized

mice and inoculated the rabies virus CVS strain into the brain. Clinical signs, body weight changes of treated mice were recorded every day and histopathology, immunohistochemistry and Western-blotting were performed. Virus antigens were detected in the brains of I.C. immunized, subcutaneously immunized and control mice in the early stages. But the incidence of the clinical signs and mortality was significantly improved in I.C. immunized group. These results indicated that I.C. immunization had a potential to rescue the rabid mice.

In the next experiments, the mice or hamsters were performed I.C. immunization after showing neurological signs. Unfortunately, I couldn't observe significant effect of I.C. immunization in these animals. Oral administration of diet and feeding water were failed to prolong the survival time of paralytic mice and hamsters.

Further studies are needed to establish the I.C. immunization-based treatment against rabies by using another rabid animal model which survives longer after showing clinical signs of rabies.

Vitrification of embryos for cryopreservation of primordial germ cells in zebrafish (*Danio rerio*)

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During the last decade, cryopreservation of oocytes and embryos has become an important technology in the field of animal reproduction and conservation. However, this technology has not been well developed in fishes. In cryopreservation of primordial germ cells (PGC), cryopreservation of intact whole embryos will reduce loss of PGC than cropreservation after isolation from embryo. Therefore, vitrification of embryos for cryopreservation of PGC in zebrafish was attempted.

First, the sensitivity of embryos of the 64-256 cell stage, 50% epiboly stage and the 14-20 somite stage for cryoprotectants were compared. Embryos of the 14-20 somite stage had the lowest sensitivity to cryoprotectans. Propylene glycol (PG) and 1,3-butylene glycol (1,3-BG) showed the lowest toxicity to embryos. Therefore, in the later experiments, vitrification of the 14-20 somite stage embryos with PG snd 1,3-BG were examined. The toxicity and glass forming property of PG and 1,3-BG at concentrations of 5 and 6 M, mixture of 2 M 1,3-BG and 3 M PG (BP23) and mixture of 3 M 1,3-BG+4 M PG (BP34) were investigated. 1,3-BG of 5 M and BP23 showed high embryos survival rate after exposure for longer periods. 1,3-BG of 6 M and BP34 showed high embryos survival rate after exposure for shorter periods and high glass forming property. Step-wise exposure of embryos to 1,3-BG at concentration of 5 to 6 M resulted in damage of yolk. Embryos vitrificating with BP23 and BP34, 32% of embryos remained morphologically intact after warming, but no live embryo was obtained. Histological investigation of these vitrificate-thawed embryos showed collapse of the layer covering yolk syncytial layer and degeneration in most embryos. Finally, when embryos in which PGC was visualized by microinjection of GFP-*nos1* 3' UTR mRNA, were vitrified and warmed, some PGC (about 13-14 cells/embryo) were found alive.

Examination of *in vitro* culture systems for growth of mouse secondary follicles and maturation of oocytes

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The final purpose of this experiment is to establish *in vitro* culture systems for growth of mouse secondary follicles and maturation of oocytes. At first, effects of different medium (αMEM and Waymouth) and oxygen tension in the gas atmosphere (5 and 20%) on maturation and developmental competence of oocytes from *in vivo* grown antral follicles of mouse were investigated. Maturation of oocytes in αMEM under 5% O_2 increased hatching and hatched blastocyst rate after *in vitro* culture for 120 h.

Then, secondary follicles mechanically isolated from ovaries were treated with collagenase and cultured in membrane insert for 10 days and effects of different basic medium (αMEM and Waymouth) and duration of collagenase treatment (5 and 10 min) on growth ability of follicles (survival rate) and maturation competence (Metaphase Ⅱ rate) after *in vitro* growth were investigated. *In vitro* growth in both medium after collagenase treatment for shorter time (5 min) increased survival rate of follicles and maturation rate of survived follicles.

Next, secondary follicles mechanically isolated from ovaries were cultured in droplets for 12 days and effects of the addition of FSH and LH to medium and difference of culture dish (Falcon1007 and 3004) on growth ability of follicles and maturation competence after *in vitro* growth were investigated. As a result, the addition of FSH increased survival rate and antral formation rate of follicles, and maturation rate of survived follicles. Oocytes of follicles formed antrum matured at higher rate.

The present results indicated that culture in αMEM under 5% O_2 is suitable for maturation of mouse oocytes, suitable collagenase treatment is necessary for *in vitro* growth of secondary follicles in membrane insert, and the supplement of FSH to medium is essential for *in vitro* culture of secondary follicles in droplets to obtain viable oocytes to maturation.

Handling and thawing procedures on the viability of *in-vitro* produced bovine blastocysts frozen with glycerol and sucrose

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This study was designed to study the effects of several handling and thawing procedures on the viability of *in-vitro* produced bovine blastocysts frozen with 1.4 M glycerol and 0.25 M sucrose in 0.25 plastic straws. The embryos were thawed by holding the straws in air for seconds prior to being plunged into water (35˚C) for 10 sec. Thawed embryos were examined whether zona pellucida was intact, and cultured *in-vitro*. Each embryo was assigned a developmental stage after 36 h, 48 h and 72 h. Experiment 1-1 was conducted to compare the effects of holding times (0-30 sec) in air on the

viability of thawed embryos. As a result, a lower survival rate was obtained by holding the straws in air for 30 sec, and holding the straws for 10 to 25 sec resulted in higher survival rates. In addition, when the embryos were thawed by holding straws in air for 0 and 5 sec, rates of embryos with damaged zona pellucida were 60% and survival rate was lower. Experiment 1-2 was conducted to compare the effects of holding straws in light breeze (wind scale: 2, wind speed: 1.6 to 3.3 m/sec) on the viability of thawed embryos. In light breeze, temperature changes in the straw during thawing procedures indicate the rapid warming, and holding straws for 10 sec in light breeze, holding 5 sec in light breeze prior to holding 5 sec in air resulted in lower survival rates. Experiment 2 was conducted to compare the effects of repeated holding of straws in light breeze or calm on the viability of thawed embryos. In calm, repeated exposure of straws to air for 15 sec resulted in a higher survival rate. But, repeated exposure of straws to air for 20 sec resulted in a lower survival rate. In light breeze, no embryo survived by repeated exposure of straws to air for 5 and 10 sec. Therefore, the embryos frozen in straws should be handled or thawed in air of perfect calm. These results suggest that intracellular devitrification occur above -70°C and intracellular ice crystals followed by devitrification are critical for the viability of embryos frozen in straws.

Effect of intra-vaginal seminal plasma injection on endometrial epidermal growth factor (EGF) concentrations in cattle

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Studies in rodents, pigs and horses implicate that the seminal plasma plays a role in enhancing fertility via not only the regulation of sperm metabolism and function (*e.g.,* capacitation) but also the regulation of endometrial function. In cattle, however, the effect of seminal plasma on uterine endometrium has not been examined. The present study examined the effect of intra-vaginal injection seminal plasma on endometrial epidermal growth factor (EGF) concentrations: an important regulator of uterine function.

First, the temporal change of endometrial EGF concentrations after intra-vaginal seminal plasma injection was examined using ovarectomized cows. Endometrial EGF concentrations increased, and peaked 4 h after the injection. Then, 14 seminal plasma samples (0.5 ml) obtained from 7 bulls were injected at estrous into the vagina of cyclic cows between 60 and 80 days postpartum. Concentrations of EGF were determined 4 h and 3 days after the injection. The effect of seminal plasma was different between samples. Endometrial EGF concentrations at 4 h and on days 3 with 8 and 2 samples after the injection, respectively, increased compared with the control cows given PBS ($P \leq 0.05$). Pooled seminal plasma obtained from 5 sires was also injected into vagina of repeat breeder cows, in which changes in endometrial EGF concentrations had altered. Intravaginal seminal plasma injection normalized endometrial EGF concentrations and increased the pregnancy rate compared with the controls. Next, to estimate molecular weight range of protein(s) responsible for increasing EGF concentrations in the uterine endometrium, seminal plasma samples were separated by the gel filtration chromatography and electrophoresis, then, injected into vagina. The activity to stimulate endometrial EGF was found in different fractions and highest in a fraction ranging in 16-29 kDa. Finally, the relation-

ship between the volume of seminal plasma and the effect to endometrial EGF concentrations was examined. Endometrial EGF concentrations were higher in 10 ml-injected group than in 0.5 ml-injected group, and EGF concentrations were comparable to 10 ml-injected group when 0.5 ml of seminal plasma was diluted×20 and injected. The results indicate that the maximum responses were not obtained by 0.5 ml of seminal plasma injection.

In conclusion, the present study showed that intra-vaginal seminal plasma injection enhanced endometrial EGF concentrations in cattle. This may suggest the possibility of bovine seminal plasma taking part in regulation of fertility through the control of uterine function in cattle.

Analysis of seminal plasma proteins of bull by two dimensional electrophoresis

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Reports for bovine seminal plasma have been suggested that proteins in seminal plasma have influence on sperm metabolism, capacitation and female fertility. In recent years, a lot of proteins in bovine seminal plasma were identified. In 1998, Mortarino *et al.* visualized about 250 protein spots of bull seminal plasma by 2 dimensional (2D)-polyacrylamide gel electrophoresis (PAGE), and in 2006, Kelly *et al.* identified 99 proteins in bull seminal plasma. But there are few reports for variety of protein components among neither different bulls nor their semen. The objective of this report was to compare the protein components among different bulls and seminal plasma using 1 D-PAGE and2D-PAGE.

First I analyzed 13 seminal plasma samples (60 μg as a protein) that obtained from 7 bulls using 1D-PAGE. Each protein samples were separated 21-29 bands and I recognized total 30 bands. Four major bands (12, 15, 16 and 28 kDa) were expressed in all lanes on 1D-PAGE gel and their relatively quantity was about 95% of all bands. Then I analyzed 11 seminal plasma samples (120 μg as a protein) that obtained from 6 bulls using 2 D-PAGE. Each protein samples were separated 37-43 protein spots and I recognized total 51 spots.

Four bulls provided two or three samples that were from different ejaculations. These bulls' seminal plasma samples have some characteristic bands or spots on both electrophoresis. This result suggested that the protein components of seminal plasma were different from bulls.

Four major bands (12, 15, 16 and 28 kDa) on each 1D-PAGE lanes were separated into plural spots (max 8, 6, 6 and 4) on each 2D-PAGE gels. The total relatively quantity of these spots was about 90% of all estimated spots. This result was most equivalent to the four major bands' quantity on 1D-PAGE lanes. However, the presumed quantities of high molecular weight proteins on 2D-PAGE gels were likely to be higher than those of 1 D-PAGE lanes. And the presumed quantities of low molecular weigh proteins on 2D-PAGE gels were likely to be lower than those of 1D-PAGE lanes.

In conclusion, it was verified that 2D-PAGE has higher separation ability for seminal plasma proteins that 1D-PAGE. In addition, it was suggested that there were differences of the individual bulls for protein expression of seminal plasma.

Molecular basis for renal tubular dysplasia due to claudin-16 deficiency in Japanese black cattle: studies on the paracellular ion transport through the tight junction

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Claudin-16 is a member of claudin family and distributes specifically to the thick ascending limb (TAL) of Henle's loop in the kidney. Inherited deficiency of claudin-16 causes renal tubular dysplasia in cattle, although several other claudins are also present at the same tubular segment. Moreover, recent histological studies on *CLDN16*$^{-/-}$ bovine fetuses demonstrated that development of renal tubules was nearly normal even in the absence of claudin-16. Therefore, it is suggested that tissue lesions in bovine tubular dysplasia was caused by the loss of claudin-16 functions in paracellular transport in the TAL epithelia. The purpose of the present study is to determine the physiological function of bovine claudin-16, especially in its effect on ion selectivity of the tight junctions in paracellular transport. Several MDCK II cell clones stably expressing claudin-16 at the tight junction have been established and were investigated for the selectivity and permeability of Na^+, Cl^-, Ca^{2+}, and Mg^{2+} through paracellular pathway. MDCK II-CL16.5 cells, one of the isolated clones, showed remarkably increased magnitude of dilution potential, indicating that the ratio between the permeability of Na^+ and that of Cl^- was increased. MDCK II-CL16.5 cells also showed an increased permeability for Ca^{2+} and Mg^{2+} under the presence of concentration gradient of Na^+ toward the basal side. These findings indicate that bovine claudin-16 is more permeable to Na^+ than Cl^- and that it plays a key role in generating transepithelial voltage as the primary driving force for Mg^{2+} and Ca^{2+} reabsorption in TAL epithelia. The present findings also suggest a possibility that the deletion of claudin-16 would cause an imbalance of ion concentration across the TAL epithelia, leading to disruption of the epithelia and renal tubular dysplasia in cattle.

Potential roles of a chaperone protein calnexin in the quality control and intracellular transport of anion exchanger 1, AE1

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The endoplasmic reticulum (ER) has a quality control system for newly synthesized proteins. The quality control followed by proteasomal ER-associated degradation (ERAD) of the target protein is involved in pathogenesis of various membrane protein disorders as demonstrated for cystic fibrosis in humans and AE1 (anion exchanger 1, red cell band 3) deficiency in cattle. The previous study indicated that mechanisms underlying proteasomal ERAD of bovine AE1 significantly differ from those demonstrated for CFTR and its mutants. The purpose of the present study is to determine if a major chaperone protein calnexin plays a role in the quality control of bovine AE1 in the ER.

Immunoisolation studies showed that endogenous human calnexin (hCNX) specifically associated with WT and R664X bovine AE1 and their *N*-glycosylated mutants, P661S and P661S/R664X AE1, in transfected HEK293 cells. Co-transfection of wild-type hCNX epitoped with Myc tag (hCNX/WT-Myc) with AE1 proteins showed the signals for Myc epitope with high intensity in the immunoprecipitates of P661S and P661/R664X AE1. When hCNX-Myc lacking lectin site (hCNX/E425A-Myc) was transfected, contents of this hCNX mutant in the immunoprecpitates of P661S and P661S/R664 X were remarkably reduced to approximately 50-60% that was consistent with the levels observed in the immunoprecipitates of WT and R664X AE1. The presence of hCNX/E425A-Myc also resulted in subsequent increase in contents of endogenous hCNX in the immunoprecipitate. Immunofluorescence microscopy also demonstrated that hCNX/WT-Myc and hCNX/E425A-Myc were distributed to the ER, being consistent with that R664X and P661S/R664X AE1were retained in the ER. These findings indicate that hCNX interacts with AE1 and its structural mutants through binding *N*-glycan and by some unlabeled contacts.

Epidemiological study of hantavirus infection in Volga-side federal region in European Russia.

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Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne zoonosis, which is caused by viruses of the genus *Hantavirus*. At least six serotypes of hantaviruses, which are Hantaan, Seoul, Dobrava, Saaremaa, Amur, and Puumala viruses, are known to be the causative agents of HFRS. 100,000 hospitalized patients of HFRS are reported annually in the world. Since about 10,000 HFRS patients are reported annually in Russian Federation (Russia), this disease is considered to be an important problem of public health. The highest endemic area in Russia is the Volga-side federal region, which is eastern part of European Russia. In the Samara area, a part of the Volga-side federal region, there are some species of rodents, which are possible reservoir animals of hantavirues. To know the virus (viruses) causing HFRS and their reservoir animals in Samara area, epidemiological survey of hantavirus infection in rodents was carried out. Sera of HFRS patients of the Samara area were also analyzed for anti-hantavirus antibodies.

In 145 sera of captured rodents in the Samara area, anti-hantavirus antibodies were detected by immnofluorescence antibody assay (IFA) and enzyme-linked immunosorbent assay (ELISA). Rodent species which had anti-hantavirus antibodies were *Myodes glareolus* (6/68) and *Apodemus flavicollis* (1/19). No anti-hantavirus antibodies were detected in *A. agrarius* (0/21) and *A. uralensis* (0/37). Hantaviral genomes were detected from four seropositive and three seronegative *M. glareolus* by reverse transcriptase-polymerase chain reaction (RT-PCR). These results indicate that hantaviruses are circulating in *M. glareolus* in the Samara area.

Lung homogenates of three seronegative and hantaviral genome-positive *M. glareolus* were injected to Syrian hamsters to isolate the viruses. Two strains of hantaviruses were isolated through Vero E6 cells from lung homogenates of inoculated Syrian hamsters and named as Sam49 and Sam94.

Open reading flames (ORFs) of hantavirus S, M and L genome segments were sequenced from

two isolated hantaviruses, and a partial S and M segments were sequenced from 5 hantaviral genome-positive *M. glareolus*. Comparison of S segments between Sam49 and F-s808, which was detected from a fatal HFRS patient in Samara area, showed 99.3% and 100.0% identities in nucleotide and amino acid levels, respectively. This result indicates that strain Sam49 has pathogenicity to human. Results of phylogenetic analysis indicate that hantaviruses circulating *M. glareolus* in the Samara area belong to Puumala virus and similar to other Puumala viruses identified in other areas of the Volga-side federal region. In Addition, Puumala virus groups between Volga-side federal region (strain Sam49, Sam94, Kazan, Ufa 97 or so) and the other Europe region (strain Sotkamo, Umea/hu or so) occupied in apparently different clades. These results indicate Puumala viruses of the Volga-side federal region diverged from the ancestral Puumala virus long years ago.

To know the antigenecity of Puumala virus strain Sam49 and Sam94, neutralization test and IFA using a panel of monoclonal antibodies were carried out. Sam49 and Sam94 had similar neutralization titers to the other Puumala virus strains and the same reactivity against all monoclonal antibodies used this study. These results indicate that strain Sam49, Sam94 and other Puumala virus strains have similar antigenicity.

Serological specificity in sera from13 HFRS patients was analyzed by IFA and ELISA. All of the patient sera with anti-hantavirus antibodies had the highest reactivity against Puumala virus antigen. This result indicates that Puumala virus is the major pathogen of HFRS in the Samara area.

These results strongly indicate that Puumala virus is the major causative agent of HFRS in the Samara area and that *M. glareolus* plays an important role as the reservoir animal of Puumala virus in this area.

Evaluation of serological diagnoses of West Nile fever and the epidemiological study in Far Eastern region of Russia

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West Nile virus (WNV) is a member of genus *Flavivirus,* family *Flaviviridae*. In nature, virus transmission occurs between avian hosts and mosquito vectors. Humans and horses get infected with virus by infected mosquito bite, and some of the cases result in serious conditions such as fatal encephalitis. To prevent the epidemic of WNV in Japan, it is very important to develop highly specific diagnosis for the detection of viral infection in humans or wild birds.

Neutralization test (NT) is the most specific method among several serological diagnoses. But it is difficult to test many samples by standard NT. In this study, a small quantity neutralization test (SQNT) was established using 96-well micro plates. SQNT made it possible to test many serum samples with small quantity. SQNT could discriminate between WNV and JEV infection in chickens infected with each virus.

Ninety one sera of wild birds were collected in Far Eastern region of Russia and 15 samples were positive for WNV-specific antibodies by SQNT. This indicates that WNV was transmitted among wild birds in this region. Virus was not isolated, and viral RNA was not detected in any organ of these birds.

Because NT is required to use infectious live virus, it must be carried out under biosafety level 3 conditions. Therefore, I tried to establish the epitope-blocking enzyme-linked immunosorbent

assay (ELISA) that can be manipulated in safe conditions to detect specific antibodies to WNV rapidly. Antibodies to WNV in immune ascitic fluids of mice were specifically detected by the epitope-blocking ELISA using WNV-infected C6/36 cells and WNV specific MAb as an antigen and detector, respectively. With several modifications, this ELISA can be useful to detect antibodies to WNV in sera of virus infected animals of multiple species.

A role of severe acute respiratory syndrome (SARS) Coronavirus membrane M protein in virions formation

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An international outbreak of severe acute respiratory syndrome (SARS), an atypical pneumonia, spread to more than 30 countries and caused about 8,422 cases and 916 deaths worldwide from its emergence in November 2002 until August 2003. SARS-Coronavirus (SARS-CoV) is the etiologic agent of SARS.

Efforts to develop animal models of SARS are now underway and no effective vaccine and antiviral agent have been developed. Local SARS epidemics will most likely occur in near future, and there remains the possibility of a global outbreak of SARS. Therefore, development of animal models of SARS as well as basic information about assembly of SARS-CoV are necessary to discover and develop anti SARS-CoV drugs. Therefore, in this study I analyzed the function of M protein which plays an important role in virion formation.

Plasmids expressing SARS-CoV M protein in full length and a truncated form were constructed. The plasmids were transfected with cells and localization of M protein was analyzed by a confocal microscope. SARS-CoV M protein expressed in mammalian cells was accumulated in the Golgi apparatus and was not localized at the plasma membrane. On the contrary, a mutant M protein, named M196 protein, lacking the carboxyl-terminal 25 amino acids was not efficiently retained in the Golgi apparatus and resulted in the transportation on the surface of plasma membrane. Culture medium from cells which express M protein or M196 protein with N protein and S protein was harvested and was ultra-centrifuged in a 20% sucrose buffer and the precipitated protein was analyzed by Western Blotting. M and N proteins were detected in the M protein expressing cell culture supernatant, but none of the protein was detected in the M196 protein expressing cell culture supernatant. This result may be due to the lack of formation of virus like particles with M196 protein.

The results indicate that amino acids of M protein from 197 to 221 play a predominant role for the retention of M protein in Golgi apparatus as well as for intracellular formation of virus particles.

The role of hypoxia inducible factor-1α induced by hypoxia condition and its expression mechanism in human lung epithelial A549 cells.

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In solid tumor cells, hypoxic regions are known to exist as a result of excessive tumor growth with a deficiency of blood vessels. It is well -known that the low oxygen concentrations in the regions reduce the cell killing efficacy induced by ionizing radiation. In this hypoxic region, hypoxia inducible factor-1α (HIF-1α) is reported to be highly expressed. However, the role of HIF-1α is still unknown in cancer cells under hypoxic condition. To clarify the role of HIF-1α in tumor cells, the effect of down-regulation of HIF-1α on hypoxia-induced cell death was examined in human lung epithelial cancer A549 cell. Hypoxia-induced cell death was demonstrated to be induced when hypoxia-induced HIF-1α was down-regulated by antisense oligonucleotide for HIF-1α, whereas any hypoxia-induced cell death was not observed in the cells with scramble oligonucleotide. This fact indicated that hypoxia-induced HIF-1α played as a protective role in hypoxia-induced cell injury. Furthermore, antioxidant regents, N-acetyl cysteine (NAC), Trolox and N-*tert*-butyl-α-(2-sulfophenyl)-nitrone (S-PBN), and flavoprotein inhibitor, diphenylene iodonium (DPI), significantly abrogated hypoxia-induced accumulation of HIF-1α, suggesting that reactive oxygen species (ROS) produced by NADPH oxidase (Nox1) in tumor cells is associated to the hypoxia-induced HIF-1α. Moreover, the expression of Noxo1 and Noxa1, cytosolic regulators for Nox1, was confirmed in A549 cells by RT-PCR, and hypoxia treatment induced the expression of Noxa1 but not Noxo1. This hypoxia-induced expression of Noxa1 was significantly inhibited by cPKC inhibitor (GF109203X), p 38 MAPK inhibitor (SB203580) and ERK inhibitor (PD98059). These results indicated that the hypoxia-induced activation of cPKC, p38 MAPK and ERK is involved in mRNA expression of Noxa1. In conclusion, present data showed that hypoxia-induced HIF-1α protective molecule against hypoxia-induced cell death through redox regulation linked with ROS produced by Nox1. This information seems to be important in gene-targeting for radio-resistant/anti-cancer-drug-resistant hypoxic tumor cells.

Enhancement of radiation-induced apoptosis in MCF-7 cells by overexpression of caspase-3

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Since the human breast carcinoma MCF-7 cells lack caspase-3 to play as an important factor in apoptosis, the loss of this protein is considered to be involved in the radioresistance in MCF-7 cells. In this study, I constructed caspase-3 expression vector (pCI-casp3) and transiently transfected into MCF-7 cells. The effect of this overexpression of caspase-3 on radiation-induced apoptosis was

estimated. In addition, involvement of apoptosis-related proteins such as Bcl-2 and IAP families in this radiation-induced apoptosis were also examined. The expression vector was transfected into MCF-7 cells by using Lipofectamine 2000. After irradiation, the apoptotic cells were evaluated by morphological change by using propidium iodide (PI) staining. The overexpression of caspase-3 did not increase apoptotic cells without irradiation. However, X-irradiation significantly induced apoptosis in the caspase-3-overexpressed cells in comparison with that in the nontransfected cells. These results indicates that caspase-3 acts as an important role for the radioresistance of radiation-induced apoptosis in the MCF-7 cells. Next, I measured caspase-3 active fragment and cleavage activity in pCI-casp3-transfected cells without and with X-irradiation. The activation of caspase-3 was observed in pCI-casp3-trasfected cells without irradiation and the radiation-induced enhancement of caspase-3 activity was small. Furthermore, the effects of irradiation on the expression of antiapoptotic proteins such as Bcl-2 and IAP families were examined. X irradiation and pCI-casp3 transfection induced suppression of the expression of XIAP and Survivin but not Bcl-2 and Bax. These results suggested that the radiation-induced apoptosis in MCF-7 cells overexpressed with caspase-3 was associated with reduction of IAP family proteins such as XIAP and Survivin.

The mechanisms of warfarin resistance in *Rattus norvegicus*

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Warfarin is a commonly used rodenticide worldwide. It inhibits coagulation of blood by inhibiting vitamin K epoxide reductase (VKOR) activity. An inadequate supply of vitamin K blocks the production of prothrombin and cause hemorrhage.

Recently, warfarin resistant brown rats (*Rattus norvegicus*) were found around Aomori areas in Japan. There is no significant difference in warfarin metabolic activity in sensitive and resistant brown rats. To clarify the warfarin resistant mechanism, I cloned VKORC1 gene from rats and found novel substitution of arginine to proline at 33 position of VKORC1 amino acid sequence.

Then, I determined the differences in kinetics of VKOR activities between warfarin resistant and sensitive rats. The hepatic microsomal VKOR-dependent activities were measured over a range of vitamin K epoxide concentrations from 6.25 to 150 μM. The values of V_{max} in resistant rats (0.044, 0.092 nmol/min/mg) were about one fourth of those of sensitive rats (0.29 ± 0.12 nmol/min/mg). The values of K_m in resistant rats (81, 229 μM) is larger than those of sensitive rats (58 ± 18 μM). There is ten times difference (sensitive>resistant) in enzymatic efficiency (V_{max}/K_m) between resistant rats and sensitive rats.

The phenomenon of small enzymatic efficiencies in warfarin resistant Aomori rats at low substrate concentrations even without warfarin may mean that VKOR activity is almost lost. Further study is needed to clarify how these rats survive with a markedly reduced VKOR activity and acquired warfarin resistance at the same time.

Characterization of cytochrome P450 1A in Japanese amphibians, especially Japanese fire-bellied newt *Cynops pyrrhogaster*

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It was reported that, since 1980, up to 120 amphibian species have almost or completely disappeared from the earth. Numerous environmental factors have the potential to cause declines of amphibian population, e.g., habitat loss, UV light, disease, mineral depletion and chemicals. In particular, amphibians are known to be more sensitive to chemicals than other species because of their high permeability of skin. However the characteristics of cytochromes P450 (CYP) which metabolize xenobiotics are still unclear in amphibian species. The aim of this study is to characterize amphibian CYP 1As that might play important role in metabolism of numerous environmental chemicals.

Novel CYP1A cDNA fragments were isolated from the liver of 9 Japanese amphibian species using the method of reverse transcription/polymerase chain reaction. Degenerate PCR primers were used to amplify 122-bp fragments of CYP1A cDNAs. The cDNA nucleotide sequence of these CYP1A fragments showed identities ranging from 72 to 98% (whole), 72 to 78% (anura vs. urodela), 75 to 98% (anura), 81% (urodela), 74 to 80% (*Xenopus laevis* vs. 9 Japanese amphibians). In phylogenetic tree, two branches of urodela and anura amphibians were formed. Within anura, three branches were formed as follows; 1) Ranidae and Rhacophoridae, 2) *Bufo japonicus formosus* and *Hyla japonica,* 3) *Xenopus laevis*. From the sequence analysis, it may be suggested that there are differences in CYP1A dependent metabolism among these species.

To clarify the CYP1A mRNA induction ability in urodela, Japanese fire-bellied newts were exposed to 3-methylcholanthrene (3MC), which is a potent inducer of CYP1As. Induction of newt CYP 1A mRNA was performed with two different administration routes, aqueous exposure (0.4 mmol/L, 3 days) and intraperitoneal injection (0.2 mmol/kg, 3 days). Tissue distribution (liver, stomach, small intestine, skin, muscles and brain) of CYP1A mRNA was measured by the real-time PCR technique. As a result, the liver showed the highest expression level of CYP1A mRNA in control and 3MC exposed newt. Therefore, it seemed that newt liver might be major organ for the metabolism of xenobiotics. After the intraperitoneal injection of 3MC, mRNA expression level was elevated significantly only in stomach. In the group of aqueous exposure of 3MC, elevation of CYP1A expression levels were observed in skin and brain, in addition to that in the stomach. The aqueous route of 3MC administration showed the tendency of induction of CYP1A mRNA in all newt organ, indicating their high chemical permeability of skin.

The prevalence and characteristics of *Escherichia coli,* antibiotic-resistant bacteria and food-borne pathogens isolated from feces of Japanese macaques (*Macaca fuscata*) in Shimokita Peninsula

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The epidemiological study of wildlife is important because wildlife can act as reservoirs of zoonoses or indicators of pathogenic contamination in the environment. Especially, non-human primates attract interest because of their similarity to humans. In this study, fecal bacteria isolated from Japanese macaques (*Macaca fuscata*) in Shimokita Peninsula were investigated to determine the influence of macaques' ecological characteristics on fecal *Escherichia coli* and the prevalence of antibiotic-resistant *E. coli* and typical food-borne pathogens.

In 2005 and 2006, fecal samples were collected from wild macaques and their captive cousins. *E. coli* were isolated and identified by serotyping, pulsed-field gel electrophoresis and plasmid profiling. The susceptibility of these isolates to antibiotics was also examined. *Campylobacter* spp., *Listeria* spp. and *Salmonella* spp. were also examined.

A total of 290 *E. coli* isolates were isolated and genotyping was performed to 109 isolates (9 serotypes) out of them. The phenotypes and genotypes of *E. coli* strains isolated from wild macaques were different from those of the strains from their captive cousins. The divergence was observed at the level of regions or even troops among wild macaque' strains. These results suggest that the macaques' diet and the infection from humans, contaminated foods, or the environment may affect the divergence of *E. coli* strains.

Antibiotic-resistant *E. coli* was isolated from 49 out of 62 wild macaques' fecal samples (79.0%) examined over 2 years. This rate was higher than that of other wild animals in Japan reported by previous studies. This might be resulted from the fact that most of the wild macaques investigated in this study utilized areas inhabited by people. The resistance patterns of macaque' strains indicate that they were not originated from the strains of domestic animals.

As a result of the examination of typical food-borne pathogens, *Campylobacter jejuni, Listeria monocytogenes* and *L. ivanovii* were isolated. The prevalence of these pathogens was very low. This result suggests that the importance of wild macaques as a reservoir of infection is limited, and further study on the transmission between macaques and humans or domestic animals is required.

Examination of methods for evaluating body fat percentage as nutritional condition of the Hokkaido brown bear (*Ursus arctos yesoensis*)

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To investigate the cause of the frequent appearance of brown bears, *Ursus arctos* in human settlements of Hokkaido, we performed the following evaluations. To assess the usefulness of the following 4 parameters for evaluating the nutritional state, their relationships with the nutritional state were analyzed by chemical methods: perirenal fat mass (KFM), perirenal fat index (KFI), gluteal subcutaneous fat thickness (RFT), and abdominal wall fat thickness (ASF). In addition, the effectiveness as a nutritional state parameter was compared between KFI and KFM. For femoral bone marrow fat (FMF), the age at the completion of the fatty change of the bone marrow was estimated, and the age range allowing evaluation of the nutritional state based on FMF was estimated. Finally, using these parameters, the accumulation order of visceral and bone marrow fat and seasonal changes in the nutritional state were evaluated.

1. Analysis of 18 carcasses of brown bears in Hokkaido between April and September, 2006 and 2007 revealed the following results:

1) Though there were some exceptions, KFI was well-correlated with the body fat as well as visceral fat ratio, suggesting its usefulness for the evaluation of the nutritional state.

2) In young females, KFI was not associated with the body fat or visceral fat ratio. Therefore, KFI is not an appropriate parameter, and KFM should be used.

3) RFT was correlated with the body fat as well as subcutaneous fat ratio, and, therefore, allows the evaluation of the nutritional state.

2. Analysis of 2,246 carcasses of brown bears in Hokkaido between 1988 and 1994 and between 1998 and 2005 generated the following results (seasonal changes were analyzed by the multiple comparison test between months of being shot ($p < 0.05$)) :

1) The kidney weight was correlated with the body size with the following exceptions: In adults and bears at ages showing slowing of the increase in the kidney weight, the correlation was weak or absent. There was no correlation in young females, and the correlation was weak in female sub-adults. No seasonal changes in the kidney weight were observed in brown bears except for female adults that showed a low kidney weight in April.

2) KFI was correlated with KFM. However, seasonal changes were more pronounced in KFI in the analysis of bears at all ages and in KFM in the analysis of adult bears.

3) KFI in April in females did not facilitate the accurate evaluation of the nutritional state.

4) Multiple comparison tests according to age ($p < 0.05$) identified no significant differences in fatty changes in the femoral bone marrow at the age of $\geq$ 2 years in males and at the age of $\geq$ 4 years in females. Therefore, this parameter can be used for the evaluation of the nutritional state after this age.

5) The relationship between KFI and KFM as well as FMF showed that bone marrow fat is metabolized after visceral fat to a certain degree.

6) Seasonal changes in KFI showed that the nutritional state of males is aggravated after copulation and does not recover until autumn.

7) Seasonal changes in KFI and KFM showed aggravation of the nutritional state of females after hibernation and fat storage in the fall in both males and females.

These results suggested the usefulness of KFI, KFM, RFT, and FMF as parameters of the accumulation of visceral, subcutaneous, and bone marrow fat. To evaluate visceral fat mass, the combination of KFI and KFM is appropriate in the analysis of females and adult males, and the use of KFI is appropriate in the analysis of all males, young males, and sub-adult males. To evaluate the subcutaneous fat mass, the use of RFT (MAX) and RFT45 (degree of cutting angle) is appropriate. The bone marrow fat ratio can be employed as a parameter of the nutritional state in males aged $\geq$ 2 years and females aged $\geq$ 4 years. Using these parameters, information on the nutritional state can be readily obtained and used for applied studies on the nutritional state of brown bears such as for the clarification of the cause of their appearance in human settlements.

Biological importance of trophy score for Hokkaido sika deer (*Cervus nippon yesoensis*)

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In recent years, the number of sika deer, *Cervus nippon* has markedly increased in various districts of Japan. In Hokkaido, forage-related damage to farmlands and natural environments caused by *C. n. yesoensis* as a subspecies of *Cervus nippon* is a problem, and an increase in hunting pressure is necessary. The deer horn reflects the quality of each individual, but its measurement method varies among researchers or institutions at present. To standardize the horn measurement method, and thereby promote hunting, we measured horns of *C. n. yesoensis* populations in Hidaka, Daisetsu, and Akan and *C. n. centralis* in Hyogo Prefecture using the measurement criteria of the Conseil International de la Chasse et de la Conservation du Gibier (CIC). For *Cervus nippon* in Japan, trophy scores of 225.00-239.99 are classified as bronze, 240.00-254.99 as silver, and $\geq$ 255.00 as gold. Five (50.0%) of 10 *C. n. centralis* from Hyogo Prefecture were classified as bronze. All *C. n. yesoensis* were classified as bronze or higher, and 86.2% (25/29) in the Hidaka population, 97.2% (35/36) in the Daisetsu population, and 100% (16/16) in the Akan population were classified as gold. For larger *Cervus nippon* from the continent, higher-level criteria have been established. According to these criteria, only 13.8% (4/29) of the Hidaka population, 36.1% (13/36) of the Daisetsu population, and 25.0% (4/16) of the Akan population were classified as silver (350.00-399.99). Since *C. n. yesoensis* markedly exceeds the criteria of *Cervus nippon* in Japan, the establishment of criteria specific to *Cervus nippon* from the continent or *C. n. yesoensis* is desirable. When mutations in genes related to *C. n. yesoensis* horns in Hokkaido were evaluated, the Hidaka population showed significantly thinner horns than other populations and significant differences between the left and right in the first and second branches. These differences may be due to genetic predispositions and the nutritional state, requiring further surveys.

Studies on the conformational stability and infectivity of abnormal prion protein derived from the Chandler strain

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There are prion strains that are distinguishable by incubation periods and distribution of neuropathological lesions after inoculation to experimental animals. Mechanisms that determine the prion strain remain to be elucidated; however, in some cases, biochemical properties of abnormal isoform of prion protein (PrP^{Sc}) also differ among prion strains. Thus, it may be possible that prion strains can be classified by biochemical properties of PrP^{Sc}. In this study, an extensive discrimination of prion strains was performed by the combination of three biochemical properties of PrP^{Sc}, *i.e.,* molecular weight, glycoform, and conformational stability against the GdnHCl treatment. A total of 7 prion strains used in this study was classified into 3, 3, or 4 groups by molecular weight, glycoform, or conformational stability of PrP^{Sc}, respectively. The combination of the three biochemical properties could distinguish 7 prion strains into 5 groups, suggesting that the profile of biochemical properties of PrP^{Sc} is useful for the discrimination of prion strains. During the analysis of conformational stability of PrP^{Sc}, it was found that the N-terminal region of PrP^{Sc} derived from the Chandler strain is gradually denatured with the increase of GdnHCl concentration. The region from around amino acid (aa) 80 to around aa 140 began to be denatured by the treatment with 1.5 M or higher GdnHCl. Within this, the region between around aa 80 and aa 90 was denatured almost completely by the treatment with 2 M or higher GdnHCl. Furthermore, the region from around aa 90 to around aa 140 denatured completely by the treatment with 3 M or higher GdnHCl. However, compared with PrP^{Sc} derived from other prion strains used in this study, the C-terminal region of PrP^{Sc} derived from the Chandler strain from around aa 140 showed extremely high resistance to the GdnHCl treatment. This property was conserved in PrP^{Sc} obtained from cells persistently infected with the Chandler strain and PrP^{Sc} derived from the Chandler strain propagated in mouse with different PrP allotype. In addition, this property was not observed in PrP^{Sc} from other prion strains, suggesting that this property is specific to PrP^{Sc} of the Chandler strain. Furthermore, the denaturation and removal of the region from around aa 80 to around aa 140 resulted in the significant reduction of infectivity of the Chandler strain. This suggests that the conformation of this region in PrP^{Sc} of the Chandler strain is tightly associated with the prion infectivity.

The construction of subgenomic replicon of the West Nile virus and the application as a reporter gene

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West Nile Virus (WNV) belongs to the genus *Flavivirus,* the family of *Flaviviridae.* In nature, the transmission of the virus occurs between avian hosts and mosquito vectors. Humans get infected by mosquito bites caused from infected mosquitoes and in some cases, fatal encephalitis occurs by the

infection. An outbreak of WNV occurred in 1999 at New York City. Since then, WNV has been noticed as an emerging disease. Though WNV has never occurred in Japan, an imported infection has been reported in 2005, so there are some matters of concerns to Japan for the invasion.

To prevent the epidemic of the WNV in Japan, a rapid and specific detection system that is quick and safe to diagnose the WNV infection is needed. The most specific way to diagnose the WNV that has been developed so far is the neutralizing test (NT) in serological diagnosis. However, the NT requires to be handled in a biosafety level 3 conditions due to the use of WNV. For the substitution of the WNV, the use of virus like particles (VLP) is effective since VLP is usable safely in a biosafety level 2 conditions. VLP is a particle that packages a subgenomic replicon which contains large in-frame deletions of the structural region. Because of the deletion, subgenomic replicon transfected cells are unable to be packaged unless the structural region is expressed in the same cell. So VLP could be made by transfecting the subgenomic replicon into the cell that expresses the structural protein complementary and is infectious for only once.

In this study, we constructed a cDNA clone of the WNV subgenomic replicon by making a large in-frame deletion of the structural region from the cDNA of the WNV genome RNA. The subgenomic replicons were *in vitro* transcribed and were transfected to cells. The expression of the reporter protein that was coded into the subgenomic replicon and the replication in cells were also confirmed. The construct that contains the DsRed gene inserted in the deleted structural region functioned most sufficiently as a reporter gene.

Further research may lead us to establish a new detection system using the VLP with this subgenomic replicon. Also, using this subgenomic replicon may expand to promote basic studies such as the mechanism of the particle formational or the replication of the virus genome.