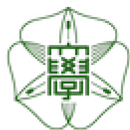

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INFORMATION

Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 40 graduates of the School of Veterinary Medicine on March 25, 2004.

The authors summaries of their theses are as follows :

Localization of 3-PGDH, enzyme for de novo L-serine biosynthesis, in the peripheral nervous system and non neuronal tissues of mice

Yoshiaki Oota

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To clarify which cells synthesize L-serine in tissues outside the central nervous system (CNS), the author examined the cellular localization of 3-phosphoglycerate dehydrogenase (3-PGDH), a rate-limiting enzyme for L-serine production, by immunohistochemistry and, in part, *in situ* hybridization (ISH).

In the peripheral nervous system (PNS) of adult mice, 3-PGDH immunoreactivity was found in satellite cells of dorsal root ganglion, and submucous nerve plexuses of the small intestine. Endocrine tissues contained 3-PGDH immunoreactive supporting cells, such as stellate cells of pituitary gland and sustentacular cells of adrenal medulla. In genital tissues, Sertoli cells and spermatogonia of the testis and oocytes in primary ovarian follicles were immunolabeled with the 3-PGDH antibody. The lens epithelium and retinal pigment epithelium were most intensely immunoreactive in sensory organs. The signals for 3-PGDH mRNA in the eyes and testis were detected on the same cells demonstrated by immunohistochemistry.

During developmental stages, the first 3-PGDH immunoreactivity was recognized in Sertoli cells and primordial germ cells of the primitive sex cord at embryonic day (E) 13, and in the lens placode and optic vesicles at E 10, when they were before completed organogenesis.

It is known that astrocytes in CNS synthesize L-serine, a neurotrophic factor, and provide it to surrounding neurons. The present study revealed that the major population of 3-PGDH-expressing cells out of CNS were supporting cells, suggesting that they, as astrocytes do, give L-serine to neurons in PNS and endocrine cells. Since 3-PGDH-deficient patients are affected with hypogonadism and congenital cataract, the author assumes that L-serine, namely 3-PGDH, has an important role in survival of genital primordial cells and homeostasis of lens.

Immunohistochemical localization of serotonin transporter in the non-neuronal cells of mice

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Since the source and disposition of serotonin are still unclear in non-neuronal cells, an author investigated the cellular expression of serotonin transporter (SERT) and vesicular monoamine transporter (VMAT) in mouse adrenal chromaffin cells, mast cells and enterochromaffin cells, all of which contain serotonin in their secretory granules. Immunohistochemistry using SERT antibody in the adrenal medulla demonstrated an intense immunoreactivity in the cell membrane of adrenalin (A) cells, but not in that of noradrenalin (NA) cells. On the other hands, using VMAT2 (neuron type) antibody demonstrated an intense immunoreactivity in the limiting membrane of granules of NA cell, but not in that of A cells.

Electron-microscopically, the expression of SERT appeared to occur mainly on the lateral cell membranes of A cells facing adjacent A cells. On the other hands, expression of VMAT 2 appeared to occur mainly on the membrane of granules of NA cells. Peritoneal mast cells also displayed a selective immunoreactivity for SERT along the plasma membrane, although the intensity varied cell to cell. In the tongue, stomach, nasal mucosa and trachea, connective tissue-type mast cells were immunoreactive, but not mucosal-type mast cells. On the other hands, Peritoneal mast cell displayed a selective immunoreactivity for VMAT 2 along the membrane of granules. In the alimentary canal, both connective tissue-type mast cell and mucosal-type mast cells were immunoreactive. Gastrointestinal epithelial cells including enterochromaffin cells were free of the immunoreactivity for SERT, although displayed a selective immunoreactivity for VMAT 2.
These findings suggest that SERT is involved in the uptake of serotonin in adrenal A cells and connective tissue-type mast cells, and VMAT is involved in the uptake of biogenic amines (serotonin and histamine) in granules.

Localization of neutral amino acid transporter ASCT 1 in the non-neuronal tissues of mice

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The author examined the expression of neutral amino acid transporter ASCT 1 in non-neuronal tissues of adult and developing mice by immunohistochemistry and immunoelectron microscopy.

ASCT1 was localized on the baso-lateral

membranes of basal cells in the stratified squamous epithelium from oral parietes to nonglandular region of the stomach, chief cells in gladular region, acinar cells of salivary gland and pancreas, and Paneth cells in the small intestine. In these cells, ASCT1 may act fundamental for uptake of extracellular neutral amino acids, which are materials for synthesizing their cytoskeletons, digestive enzymes and anti-microbial peptides. In the liver, ASCT1 was detected on the membranes of hepatocytes surrounding central veins, and a temporal increase of ASCT1 immunoreactivity was shown in the fetal or CCl4-treated adult livers. These findings suggest that ASCT1 may function to trasport plasma neutral amino acids alanine, which has been synthesized from ammonia, to be detoxicated in adult livers, and to transport alanine, the precursor of pyruvic acid which act as a material for energy production in growth and regeneration of ergastoplasms in hepatocytes of fetal or injuried adult livers.

ASCT1 was also expressed on the basal plasma membranes of proximal uriniferous tubular epithelial cells in the kidney of adult mice, and those of supporting cells in the medulla of adrenal gland. ASCT1 may function to reabsorb amino acids from proximal tubular epithelial cells into blood, and contribute to transport amino acids to medullary cells in the adrenal gland. As regards the ASCT1 expression in the pregnant luteal cells, ASCT1 would serve for amino acids uptake, which are used as materials of enzymes in the progesterone synthesis pathway.

These results suggest that ASCT1 is expressed in various non-neuronal tissues in mice, mainly such as epithelial cells, and ASCT1 functions as a fundamental molecule-contribute to the amino acids transportation in a variety of cells in non-neuronal tissues.

Regulatory mechanism of UCP2 gene expression in hepatocytes

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Uncoupling protein (UCP)2 is a member of the UCP family that diverts energy from ATP synthesis to thermogenesis by uncoupling mitochondrial oxidative phosphorylation. UCP2 is not expressed in normal hepatocytes, but it is incidentally induced under various unphysiological conditions, such as in primary cultured hepatocytes, some hepatoma cell lines, fatty liver, and regenerating liver after hepatectomy. In this study, I investigated the regulatory mechanisms of UCP2 expression in hepatocytes, using two hepatoma cell lines, HepG2 and Hep3B cells.

RT-PCR analysis revealed that UCP2 mRNA was abundantly expressed in HepG2 cells, whereas not at all in Hep3B cells. Since some genes are known to be silenced by DNA methylation, the methylation pattern of the UCP2 gene in the two cell lines were analyzed. Bisulfite genomic sequencing analysis showed that the DNA region including the transcription start site of the UCP2 gene was highly methylated in Hep3B cells but rarely in HepG 2 cells. Treatment of Hep3B cells with an inhibitor of DNA methylation, azacytidine, induced UCP2mRNA expression. These results

suggest that the UCP2 gene in Hep3B cells is silenced by DNA methylation. However, in mouse liver, similar DNA methylation of the UCP2 gene was not found, suggesting that some other mechanisms are involved in the control of UCP2 expression in normal hepatocytes. Then, I investigated possible roles of some transcription factors in UCP2 expression by promoter-reporter gene assay in the two cell lines. No silencer element was found in the UCP2 promoter, while the proximal promoter region of -100bp to -33bp from the transcription start site was found to be important in its activation. This region contained consensus sequences for sterol regulatory element binding protein (SREBP) and Sp 1 transcription factors. Indeed, transient expression of SREBP enhanced the promoter activity, but neither Sp 1 nor Sp 3 stimulated it. However gel shift assay indicated another protein bounds to the Sp 1 sites. Furthermore, the stimulatory effect of SREBP was partially suppressed by transient expression of another factor, inhibitor of DNA binding (Id), which is known to interact with SREBP. Considering abundant expression of SREBP and Id proteins in the liver, it seems likely that relative amounts of these two transcription factors may regulate UCP2 expression in hepatocytes.

Establishment of a regulated expression system for the uncoupling protein gene in Hep 3 B human hepatocellular carcinoma.

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Uncoupling protein (UCP) located in the inner mitochondrial membrane mediates proton leak into the mitochondrial matrix, resulting in dissipation of the mitochondrial membrane potential formed during respiration as heat rather than ATP synthesis. UCP1, a representative member of the UCP family, is known as a thermogenin in brown adipose tissue, and UCP1-dependent thermogenesis is controlled by the sympathetic nervous system. Fatty acids released by sympathetic activation of lipolysis not only are the substrate for thermogenesis but also act as a mediator of the protonophoric activity of UCP1. In addition to fatty acid metabolism, glucose utilization is also activated in parallel with UCP1-dependent thermogenesis. However, little is known about the possible role of UCP1 in the metabolic control. To this end, in the present study, I developed a new system for analyzing UCP 1 function in mammalian cells by using Stratagene's LacSwitch II Inducible Mammalian Expression System. In brief, Hep3B human hepatocellular carcinoma expressing Lac I but no endogenous UCP was transfected with Lac I-regulatable rat *UCP1* gene, and then stable transfectants were cloned. Of 92 clones selected, five clonal cells were capable to inducing UCP1 by treatment with isopropyl -β-D-thiogalactopyranoside (IPTG), an inhibitor of the Lac I. UCP1 was induced 4 ~ 72 hours after the addition of IPTG, and found in a mitochondrial fraction but not in a cytosolic or microsomal fraction. Measurement of mitochondrial membrane potential by rhodamine 123 revealed that mitochondria containing

UCP1 had decreased membrane potentials in the presence of succinate and rotenone compared to those without UCP1, and that the decrease was abolished by the addition of GDP, which is known as an inhibitor of the protonophoric activity of UCP1. In conclusion, the mammalian cells expressing functional UCP1 under the control of Lac I system were established. They would be useful for the studies on the cellular and molecular mechanisms of the UCP1-dependent metabolic control.

Neuropeptide Y activates protein kinases in vascular endothelial cells.

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Neuropeptide Y (NPY) is one of the most abundant peptides in both central and peripheral nerous systems. NPY is involved in various physiological functions, such as control of food intake, anxiety, seizures, circadian rhythm, memory, and pain. In the peripheries, NPY is co-stored and co-released with norepinephrine in sympathetic nerve fibers to induce a long-lasting vasoconstriction and to increase vascular permeability and revascularization of ischemic tissues. NPY mediates its effects through five receptors subtypes, all of which act through pertussis toxin-sensitive G protein to inhibit adenylate cyclase. In addition, NPY leads to mobilize Ca^{2+} from intracellular stores and to activate p44/p 42mitogen-activated protein kinase (p44/p42 MAPK) and other effectors. However, it is not clear whether NPY also activates another group of protein kinases, tyrosine kinase.

In the present study, I examined the effects of NPY on the activation of Janus kinase 2 (JAK2), a non-receptor tyrosine kinase in mouse lung microvascular endothelial cells (LE II), by measuring a site-specific phosphorylation of the enzyme. In LE II cells, NPY induced phosphorylation of not only p44/p42 MAPK but also JAK 2 in concentration-and time-dependent manners. However, the effective concentrations of NPY for JAK 2 phosphorylation were two-order lower than them for p44/p42MAPK. Furthermore, pretreatment of the cells with AG490, a JAK 2 inhibitor, blocked JAK 2 phosphorylation, but not p 44/p42MAPK phosphorylation, by NPY. My results do not support a reported idea of possible involvement of JAK 2 in the NPY-induced p44/p42MAPK activation, but rather suggest that NPY activates two protein kinase pathways independently, and that those may cooperatively mediate the NPY effects in vascular endothelial cells.

BIPHASIC EFFECT OF HYDROGEN PEROXIDE ON VASCULAR TONE IN ISOLATED RAT BASILAR ARTERY

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1. The effects of hydrogen peroxide (H_2O_2) on 5 HT - or high K^+-induced contraction and increase of intracellular Ca^{2+}concentration ($[Ca^{2+}]i$) were investigated in endothelial-denuded ring preparations isolated from rat basilar artery. In arteries precontracted with 5 HT or K^+, H_2O_2 caused a biphasic mechanical response : a transient potentiation of contraction followed by a persistent relaxation. The concentration of H_2O_2 required to cause the relaxation is lower in 5HT-precontracted preparation than in K^+-precontracted one.
2. In arteries precontracted with K^+, H_2O_2 caused a sustained increase of $[Ca^{2+}]i$ even though the biphasic mechanical response occurred. In arteries precontracted with 5 HT or phorbol ester, H_2O_2 increased $[Ca^{2+}]i$ with a transient contraction and decreased $[Ca^{2+}]i$ with a persistent relaxation, but increased $[Ca^{2+}]i$ did not return to the resting level when arteries relax to the basal tone.
3. Neither $\cdot O_2^-$ nor $\cdot OH$ scavenger affected the biphasic mechanical response to H_2O_2, while catalase eliminated the response.
4. FCCP (an uncoupler of oxidative phosphorylation in mitochondria) and SK&F 96365 (a non-selective cation channel blocker) significantly inhibited the potentiating effect of H_2O_2 on the 5 HT-induced contraction.
5. Chelerythrine (a PKC inhibitor) significantly shortened the time required to cause relaxation induced by H_2O_2, while MAPK inhibitors did not affect the biphasic mechanical response.
6. The biphasic mechanical response to H_2O_2 was not influenced by inhibitors of NO-cGMP pathway, a COX inhibitor, and a Na^+-K^+ ATPase inhibitor. K^+ channel blockers did not inhibit the H_2O_2-induced relaxation.
7. In organ-cultured rat basilar arteries in the presence of H_2O_2, 5HT - and K^+-induced contraction were attenuated. The inhibitory effect of H_2O_2 on 5 HT-induced contraction was greater than that on K^+-induced one.
8. These results indicate that H_2O_2 caused a biphasic mechanical response in 5HT-and K^+-precontracted basilar arteries. It is suggested that the transient potentiation of contraction is mediated by the Ca^{2+}release from mitochondria, the Ca^{2+}influx via non-selective cation channels, and the activation of PKC. The decrease of Ca^{2+} sensitivity to a contractile machinery may mediate the H_2O_2-induced persistent relaxation.

Development of new diagnostic methods of leptospirosis

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Leptospirosis is a zoonotic infection caused by pathogenic *Leptospira interrogans* and occurs worldwide. Isolation of leptospires from clinical samples such as blood or urine of animals is a standard diagnostic method of leptospirosis. It, however, takes 5 days to a few weeks to isolate leptospires by cultivation. In the present study, a sandwich ELISA which detects LipL 32, a common antigen shared by pathogenic leptospira species, has been developed for the rapid diagnosis of leptospirosis. A competitive ELISA to detect antibodies against LipL32 has been also developed as a new method for serodiagnosis of leptospirosis.

The sensitivity of sandwich-ELISA using anti-LipL32 monoclonal antibodies against 6 different serovars of *L. interrogans* was $10^{4.9}$-$10^{5.5}$ leptospires/50µl. A competitive ELISA using recombinant LipL 32 and anti-LipL 32 monoclonal antibody was investigated for the serodiagnosis of leptospirosis. In this assay, antibodies against LipL 32 were detected in rabbit hyperimmune sera against 6 different serovars of pathogenic *L. interrogans*, but not in that against non-pathogenic *L. biflexa*. Specific antibodies were also detected in serum samples of mice experimentally infected with pathogenic leptospira and its sensitivity was equivalent to that of MAT. This method also detected in the sera of sows with abortion, indicating that the assay should be useful for epidemiological surveillance of leptospirosis in animals.

Epitope mapping of the hemagglutinin and neuraminidase molecules of H9N2 influenza viruses

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Since the beginning of 1990s, H9N2 influenza viruses have been circulating in Asian countries and caused extensive economic damage in poultry industry. H9N2 viruses are isolated not only from poultry but also from pigs and humans. Since pandemic of H9N2 virus may occur in near future, it is important to analyze the antigenicity of H9N2 viruses and prepare proper vaccine strains for a possible pandemic. In this study, monoclonal antibodies against A/swine/ Hong Kong/10/98 (H 9N2) and A/duck/Hokkaido/13/00 (H9N2) viruses were generated and their characters were analyzed.

Fifty monoclonal antibodies have been obtained and divided into 7 groups of anti-HA antibodies and one group of anti-NA antibodies based on their different reactivity patterns

to escape mutants. Anti-HA antibodies recognized different 8 epitopes on the molecule, and 7 of them were located in 4 antigenic sites of the HA known with H1 and H3HA molecules. Anti-NA antibodies recognized two independent epitopes and they were located on the antigenic site known with N2NA molecule. With these 8 groups of anti-HA and anti-NA antibodies, antigenicity of 21 H9N2 virus isolates were analyzed. Antibodies to Sw/98 reacted with viruses of G9 lineage but not with other viruses. On the other hand, antibodies to Dk/00 reacted with most of the H9N2 viruses. These results indicate that Dk/00 should be a better candidate for vaccine strain.

Development of a novel diagnostic method and studies on the molecular epidemiology of bovine viral diarrhea-mucosal disease

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Bovine viral diarrhea-mucosal disease (BVD-MD) is caused by infection with bovine viral diarrhea virus (BVDV). Cattle infected with BVDV show symptoms of digestive and respiratory diseases, and the infection of pregnant cows may result in abortion, stillbirth and teratogenic damage for their fetuses. Cattle persistently infected with BVDV are immunologically tolerant and shed the viruses. For the control of BVD-MD, it is essential to detect and sacrifice persistently infected cattle, since they should become reservoirs for the virus, thus, lifelong carriers. It is, therfore, needed to develop novel diagnostic methods with rapidity and high sensitivity. In the present study, monoclonal antibodies to nonstructural protein NS3 that is conserved among BVDVs were established and evaluated for their usefulness for diagnosis. Genetic analysis of 127 BVDV strains isolated in Hokkaido were done to clarify the distribution of BVDV.

Ten monoclonal antibodies against recombinant NS3 protein expressed in *Escherichia coli* were obtained and checked their reactivity to BVDVs and other pestiviruses. Six clones of them were useful for diagnosis of BVD-MD, since they could detect all BVDV strains tested. One hundred and twenty-seven field strains of BVDV were identified from cattle in Hokkaido with the monoclonal antibodies established. Then, studies on the molecular epidemiology of these isolates were carried out. Phylogenetic analysis revealed that not only BVDV genotype 1 (BVDV-1) but also genotype 2 (BVDV-2) have spread widely in Hokkaido. In addition, the epidemiological studies and the genetic analysis demonstrated that BVDV-2 isolates in this study did not have higher virulence like those isolated in North America as hemorrhagic syndrome but same virulence as those of BVDV-1 strains.

Further epidemiological surveillance is ongoing for the control of BVD-MD in Japan. Development of rapid detection kit using established monoclonal antibodies is now in progress.

Identification and Characterization of Peptides Binding to Newcastle Disease Virus by Phage Display

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Phage display technology can be used to select for and produce novel peptides which bind to target molecules of interest. Newcastle disease (ND) is caused by the ND virus (NDV), and known as one of the most serious diseases in the poultry industry. Several live and inactivated vaccines are available to control the outbreak of ND. However, NDV can infect numerous kinds of unvaccinated fowls, including free-living birds, and such infections seem to play a significant role in the spread of ND. Thus, the development of new strategies is desired to control field ND problems.

In this study, 3 individual peptide sequences, EVSHPKVG, WVTTSNQW and SGGSNRSP, which have potentials to bind to NDV were identified by the biopanning method using phage display system. The binding specificities of these peptides presented on phages were confirmed by ELISA competition assay using chicken antiserum to NDV. The synthetic peptides designed based on the results did not inhibit the hemagglutination by inactivated NDV, but partially neutralized the infection of NDV *in vitro*.

In the future, it will be necessary to modify these peptides sequences, for example substitution of amino acids, to improve their abilities to bind to NDV and to inhibit NDV infection. It is also important to examine the protective efficacies of them against NDV *in vivo* for clinical application.

Attempt to Eradicate Bovine Viral Diarrhea Virus Infection in Cattle Herds Using Bulk Milk Samples

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Bovine viral diarrhea virus (BVDV) is a ubiquitous pathogen of cattle. Infection during the first gestation period can result in the birth of immunotolerant calves which are persistently infected (PI) with BVDV. Such calves are a major source of virus spread and thus, it is very important to detect and remove them from the cattle herd. In general, PI cattle may show varied clinical manifestations, but are often detected based on mucosal lesions. This work aims to detect PI cattle that do not show the mucosal disease using bulk milk samples. In cows with diarrhea, pneumonia, poor growth and those did not respond to treat-

ments, one out of six were PI cattle. However, some PI cattle did not show clinical manifestations, and it is practically not possible to detect all PI cattle basing only on clinical manifestations. It would, therefore, be more important to test the whole herd in order to determine the disease prevalence.

In this study, a survey was carried out by PCR using bulk milk samples on the prevalence of BVDV in a herd of cattle. This PCR was sensitive enough to detect one positive cow within150cows. We found6.6% of all the herds tested had positive PI cattle. And the number of herds with BVDV that previously had BVDV was 3.8times more than the number of herds that did not have BVDV in the past. In herds that had more cattle with diarrhea, pneumonia, or abortions, BVDV was detected2.4times more than in the herds without these diseases. These results demonstrate that PI cattle enhance the disease prevalence in their herds.

From detailed research on herds with BVDV, it can be ascertained that in herds with PI cattle, vertical and horizontal transmissions of the disease to healthy cattle can occur. BVDV infection in the herd can be eliminated by the removal of PI cattle, and the method based on BVDV detection by PCR using bulk milk samples can be very effective. This method can help in the eradication of BVDV from herds.

Characterization of a cell line persistently infected with Theileria orientalis

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Theileria orientalis is a tick-borne hemoprotozoan parasite of cattle and causes anaemia as intraerythrocytic piroplasms. The schizont stage in peripheral blood mononuclear cells (PBMC) is not clear in animals infected with *T. orientalis* as reported in other Theileria species. Preliminary, it was reported that cytopathic effect (CPE) was detected when the adherent cells were separated from PBMC of *T. orientalis*-infected cattle and cultured *in vitro*, though CPE did not appear in non-infected cell lines, suggesting the possibility that CPE is induced by *T. orientalis*. Thus, the characteristics of a cell line persistently infected with *T. orientalis* were analyzed in details in this research.

The adherent cells derived from the normal cattle did not show CPE until they overgrow. On the other hand, the adherent cells derived from the *T. orientalis*-infected cattle showed CPE. By nested-PCR, the major piroplasm surface protein (MPSP) gene was detected in the DNA samples extracted from those cells as well as the cells which detached as a result of CPE. By the immunofluorescence assays using a *T. orientalis*-specific monoclonal antibody, Rag 23, prepared by immunizing *T. orientalis*-persistently infected cells, the positive reaction was detected in accordance with the CPE parts in the persistently infected cell line. From these results, it was suggested that CPE observed in a cell line persistently infected with *T. orientalis* is indeed induced by the protozoan infection.

When a splenectomized calf was inoculated with the persistently infected cell line, this calf showed transient fever, and the MPSP gene was detected from its PBMC. However, this calf did not show any other clinical symptoms, and piroplasms were not found. These results showed a possibility that the persistent infection of *T. orientalis* was established in the calf by the inoculation of the persistently infected cell lines.

By this research, the properties of the cell line persistently infected with *T. orientalis* were clarified. In the near future, it is necessary to examine cell-mediated immune responses, which is considered to be important for prevention from protozoan infection, induced by the inoculation of this cell line to develop a live attenuated vaccine, as used to effectively control the *T. annulata* infection.

Development of a quick diagnostic method to detect Marek's disease virus (MDV) from feather tips and molecular epidemiology of MDV in wild birds.

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Marek's disease virus (MDV) is an avian oncogenic herpesvirus that disseminates through feather dust and chickens are infected by inhalation of the dust. Chicken is the most important natural host for MDV and cell free MDV is only produced in feather follicle epithelium of infected chickens. Serotype 1 strains of MDV (MDV1), except attenuated vaccine strains, are known to cause lymphomas in visceral organs of infected chickens, whereas little has been reported on the prevalence of MDV in other avian species. In 2001, a MD case was reported in a white-fronted goose (precious natural treasure) migrated from Russia in lake Miyajimanuma of Hokkaido. Though the roles and significance of wild geese on the epidemiology of MDV1 remains to be determined, they could play some important roles as reservoirs and carriers of MD to domestic poultry. Therefore, a reliable and easy-to-handle method for surveillance of the prevalence of MDV1 in the field would be desirable. In this study, a method for the detection of oncogenic MDV1 using feather tips has been developed because these sites are suitable for the collection of samples in the field.

To distinguish oncogenic from non-oncogenic MDV1, the *meq* gene, one of the candidate oncogenes in MDV1, was chosen as a region for the detection of viral DNA, since a difference in the *meq* open reading frame (ORF) between oncogenic and non-oncogenic MDV1 was reported: L-*meq*, in which a 180-bp sequence is inserted into the *meq* ORF, is detected in non-oncogenic MDV1. When chickens were infected with Md5, an oncogenic MDV1, the *meq* gene was detected in total DNA extracted from feather tips throughout the experimental period for 10 weeks post inoculation (pi) determined by nested polymerase chain reaction (PCR). In the case of chickens infected with CVI988, an attenuated MDV1, the L-*meq* gene was detected at 2 to 10 weeks pi. Furthermore, when the *meq* or L-*meq* gene was monitored using DNA samples

from feather tips in chickens co-infected with Md5 and CVI988, *meq* gene was detected in most of the chickens at 2 to10 weeks pi. These results suggest that the detection of the *meq* gene by nested PCR using feather tip-derived samples is an effective method for the diagnosis of oncogenic MDV1.

This method for the detection of oncogenic MDV1 using feather tips was applied in the field to survey the prevalence of MDV1 in wild geese. The *meq* gene was detected in white-fronted geese, canada geese and bean geese, suggesting that oncogenic MDV1 is widely spread among goose populations.

Species identification method for the eggs of taeniid cestodes -DNA extraction from eggs and PCR-amplified of CO I and ND-1 gene-

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Taeniid cestodes include important parasites in dogs and cats, such as *Echinococcus multilocularis, E. granulosus, Taenia taeniaeformis, T. hydatigena, T. pisiformis etc*. In Hokkaido, *E. multilocularis* is an important zoonotic parasite. In order to diagnose and determine the prevalence of the parasite in the definitive hosts such as foxes, dogs and cats, reliable diagnostic methods for the definitive hosts are required. The current standard laboratory procedures are microscopic examination for the detection of eggs and coproantigen detection test. To provide a more sensitive and specific diagnosis, PCR-amplified CO I and ND-1 genes were evaluated.

In the present study, the method of DNA extraction from taeniid eggs, and the sensitivity and specificity of the *E. multilocularis* specific primers E.mSP1-A and E.mSP1-B, and species specific identification method of taeniid cestodes were examined. Eggs were efficiently collected from feces by combination of flotation method with a sugar solution and sequential sieving with meshes sizes of 40μm and 20μm. DNA could be extracted from taeniid eggs using QIAamp DNA Mini Kit (Qiagen) without pre-treatment for disruption of the keratin embryophore. DNA extracted from eggs heated at 70℃ for 12 hours or frozen at -80℃, both of which methods are required for the parasite egg sterilization, were amplified by PCR similarly, but DNA from the eggs stored in 1% formalin was not amplified. DNA extracted from *E. multilocularis* eggs heated at 70℃ for 12 hours was amplified with primers of mitochondrial (mt) CO I gene, PR-A and PR-B, with *E. multilocularis* specific primers, E.mSP1-A and E.mSP 1-B. Amplified sequences were detected in electrophoresis from 10 and 1 egg(s) equivalent amount of DNA by PCR with 30 cycles and 50 cycles, respectively.

Finally, for CO I gene, sequences of 6 species (9 samples) of taeniid cestodes (*E. multilocularis, E. granulosus, E. vogeli, T. taeniaeformis, T. crassiceps and T. pisiformis*) were determined, and aligned with sequences of available references and GenBank data. Consequently, the specificity of the primers E.mSP1-A and E.mSP1-B was confirmed. Furthermore, the diagnostic value of some restriction enzymes were validated for CO I

gene of the cestodes. For possible usage of ND-1 gene for species specific identification, sequences of 6 species (12 samples) of taeniid cestodes (*E. multilocularis, E. vogeli, T. taeniaeformis, T. crassiceps, T. hydatigena and T. pisiformis*), were determined and aligned with the sequences of available references and GenBank data.

These results indicate that eggs of *E. multilocularis* can be detected by PCR with the primers E.mSP1-A and E.mSP1-B from a small number of eggs, and other taeniid cestodes can be identified the species by PCR-RFLP.

Epizootiology of *Echinococcus multilocularis* in the northeastern region of Sapporo

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Echinococcus multilocularis is a zoonotic parasite which causes the human alveolar echinococcosis. In Hokkaido the reservoir hosts are red foxes (*Vulpes vulpes schrencki*) as the definitive host and red-backed voles (*Clethrionomys rufocanus*) as the intermediate host. In order to obtain the basic data for countermeasures against the parasite, the epizootiology of echinococcosis in the northeast of Sapporo was investigated.

A total of 25 foxes were shot in Sapporo City (Kita-ku and Higashi-ku) and it's environs (Ebetsu City and Tobetsu-chou) between May and September in 2003, and were examined the parasite. The foxes were divided into 9 groups by their locality of captured places and den sites. Although 7 foxes (3 groups) were captured in Tobetsu-chou, infected foxes were not found. At the northeast of Sapporo City and Ebetsu City, 18 foxes (6 groups) were captured. Among them *E. multilocularis* was detected in 6 foxes belonging to 4 groups. In the study area, three fox dens were found under the floor of a warehouse at a pasture, at a deserted house in a farmland, and at a small wood between a small swamp and a river. In Sapporo City, fox captured places or den sites are in a wildland of farmlands or in riverside. The respective distances from captured places or den sites to the nearest private house are in 0.08-0.8km, and the respective distances from these sites to a residential section are in 0.7-2.3km. Fox feces (15pieces) were collected in habitats of the foxes, and 2 *E. multilocularis* antigen and egg positive feces were found from the feces. Furthermore, one of the antigen and egg positive feces was collected at one habitat of two groups in which infected fox was not detected. Sixteen voles were captured on the border of farmland with Sasa bamboo and wildland of riverside. Infected voles were not found among them.

In conclusion, foxes infected with *E. multilocularis* and potential intermediate hosts, red-backed voles were found in the northeastern region of Sapporo, suggesting that the life-cycle of *E. multilocularis* is maintained in this sub-urban area.

Detection of *Babesia gibsoni* gene from blood smear and tissue specimens by *in situ* hybridization

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Babesia gibsoni is a blood protozoan in dogs and a causative pathogen of severe hemolytic anemia in infected dogs. In general, Babesia parasites can be easily detected on a Romanowsky-stained blood smear. However, it is difficult to detect the parasites in the tissue of infected animals. In the present study, *In situ* hybridization (ISH) was tested for the specific detection of *B. gibsoni* in blood smear and tissues, because ISH could detect the parasites by the nucleotide sequences of its genes.

B. gibsoni heat shock protein 70 (BGHsp 70) was selected as a target gene for ISH, because it was thought to be an essential protein for the survival of *B. gibsoni*. The BGHsp 70 probe was prepared according to the nucleotide sequence of that gene. At first, slides prepared from *B. gibsoni* cultured in vitro were fixed with three kinds of fixative, methanol, acetone and paraformaldehyde, respectively, and used for ISH. *B. gibsoni* was well detected when the slide was fixed with paraformaldehyde. From the result, paraformaldehyde was seemed to be a suitable fixative for the detection of *B. gibsoni* on blood smear by ISH.

For the detection of *B. gibsoni* in the tissues, paraffin-embedded sections of liver, kidney, limph node and bone marrow from *B. gibsoni* infected dogs were examined by ISH. As the result, some BGHsp 70-positive cells were found only in the liver. These cells seemed to be the Kupffer cells or macrophages containing *B. gibsoni*-infected erythrocytes. Those results suggested that the detection of *B. gibsoni* in the tissue specimens by ISH might be possible.

Rapid mutation screening and molecular epidemiology of GM_1 gangliosidosis in Shiba dogs

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GM_1 gangliosidosis is a lysosomal disease which develops from a deficiency of β-galactosidase and is inherited as an autosomal recessive trait. GM_1 ganglioside, the major substrate of the enzyme, accumulates in the brain and other visceral organs resulting in progressive neurologic dysfunction. GM_1 gangliosidosis in Shiba dogs is due to the deletion of cytosine residue at nucleotide position 1647 of the putative coding region for canine β-galactosidase, which can be detected by a polymerase chain reaction (PCR)-restriction

fragment length polymorphism (RFLP) method. In the present study, we developed a rapid mutation screening method utilizing a novel PCR reagent cocktail, Ampdirect, and an epidemiologic survey was performed in northern Japan using this method.

The strategy of this mutation screening is based on the identification of the nucleotide deletion by the PCR-RFLP method, coupled with the direct PCR amplification from blood specimens utilizing Ampdirect. The target sequence of the canine β-galactosidase gene could be amplified directly from various forms of canine whole blood specimens, including anticoagulated blood, blood frozen for more than 1 year, dried blood held in filter paper for 1 year at room temperature and dry powder of blood stripped from Giemsa-stained blood films prepared approximately 10 years previous. Preliminarily, the epidemiologic survey of Shiba dogs was performed in northern Japan mainly using blood films. Sixty-eight Shiba dogs were examined and 2 heterozygous carrier dogs (2.9%) and 66 normal dogs were found.

The rapid mutation screening method developed in the present study was useful for the epidemiologic survey of GM_1 gangliosidosis in Shiba dogs. The result of the survey suggested that there may be many widespread homozygous carriers. It is desired that this canine disease is controlled and prevented based on the data obtained using the rapid mutation screening.

Diferrentiation and maturation of canine erythroid cells from peripheral blood by in a two phase liquid culture system

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For studying the differentiation and maturation of canine erythroid progenitors *in vitro*, mononuclear cells from the peripheral blood of dogs were cultured in a two-phase liquid culture.

In the two phase liquid culture (TPLC) system, hematopoietic stem cells increase in the first phase culture in the presence of some cytokines, and then differentiate and maturate to erythrocytes in the second phase in the presence of erythropoietin.

In the present study, phytohemagglutinin stimulated-leukocyte conditioned medium (PHA-LCM) or a combination of interleukin-3 (IL-3) and stem cell factor (SCF) were used as cytokines for the first phase of the TPLC system. PHA-LCM was a culture supernatant including cytokines which were released from peripheral blood mononuclear cells stimulated by phytohemagglutinin.

Canine erythroid progenitors in the peripheral blood differentiated and maturated to erythrocytes by the TPLC system in the presence of either cytokine. However, those erythrocytes showed many morphological abnormalities, such as crenated cells, and cells containing a degenerated nuculeus. These results suggested that the maturation of erythrocytes in the TPLC system might have been impaired by an unknown mechanism.

Next, the effects of cytokines, PHA-LCM and the combination of IL-3 and SCF on the

differentiation and maturation of canine erythroid cells were compared. In the presence of PHA-LCM in the first phase culture, the proliferation rate of nonphagocytic mononuclear cells was higher than the phase in the presence of IL-3 and SCF, but the ratio of proerythroblasts in the PHA-LCM culture was lower than that in the IL-3 and SCF culture at the beginning of the second phase. Moreover, the appearance of erythrocytes occurred earlier in the presence of IL-3 and SCF than that in the presence of PHA-LCM. These results indicated that the proliferation, differentiation and maturation of erythroid progenitors might depend on the kind of cytokines used in the first phase.

Establishment and Characterization of a New Cell Line from a Canine Transitional Cell Carcinoma

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Transitional cell carcinoma (TCC) is one of the most common malignancies of canine urinary bladder, with a very poor prognosis. For developing effective diagnoses and treatment of canine TCC, it is necessary to have relevant cell lines, but until now there are only few established.

In this study, we tried to establish a new cell line derived from metastatic regions of an internal iliac lymph node in a dog with TCC of bladder. Tumor characterization was done by inoculation TCC cell suspension subscutaneously on SCID mice. Additionally, we analyze anti-tumor activity of piroxicam on the TCC by using tumor bearing mouse model. Previously, it has been reported that piroxicam, which belongs to a non-steroidal anti-inflammatory drugs (NSAIDs), has antitumor -activity on TCC in dogs.

The TCC cell line was expanded in monolayer manner *in vitro*, with doubling time of approximately 30 hours. We estimated that cells continuously could be cultured over 60th passages without losing potential of proliferating.

The tumor tissue produced by the inoculation of the cells into SCID mice was histopathologically identical to the primary and the metastatic tissue. Using immunohistochemical method epithelial marker was detected in the cells of tumor. Additionally, on electron microscopic examination, cells from monolayer culture had similar microstructure characteristic to TCC.

Inoculated TCC cell suspension formed tumor in the subcutaneous of SCID mice. The tumors increased in size during10days. Afterwards the tumor tended to regress but still after 30days TCC cells histologyicaly could be detected.

Notable anti-tumoral effect of piroxicam on TCC bearing mice was not observed. Only a few number of cells in the transplanting tumor tissue and the cell lines from monolayer expressed intensive Cox 1 and Cox 2 immunoreactivity. This result may suggest that there are just few cells sensitive to piroxicam in the cell line.

In conclusion, we established a new canine TCC cell line, which further can be used

for *in vivo* and *in vitro* experiments. Cloning of Cox positive cells and Cox negative cells will be essential to study the anti-tumor mechanism of NSAIDs.

Analysis of calving and housing properties as causes of foot diseases of dairy cattle in Hokkaido.

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Foot disease of dairy cattle is one of major factors of economical loss of productivity in dairy farms. Subclinical laminitis is the most recent and ruinous foot disease in dairy cattle. Claw horn lesion is a suggestive finding of this type of laminitis. The cause of this laminitis is multi-factorial, including nutrition, genetics and hormonal change during calving. The purpose of this study was to analyze calving and housing properties as predisposing factors to cause subclinical laminitis in dairy cattle in Hokkaido, which would contribute to prevent this disease in Hokkaido.

In 9 dairy herds including 445 cows, the condition of housing of cows was recorded. At the time of a regular claw trimming, the claw horn shape was measured before claw trimming and the sole horn lesions were evaluated after that. Information of the respective lactation period of each cow was also inscribed.

The incidence rate of sole haemorrhage was increased in perinatal period and during the highest and the midterm lactation periods. In contrast, the incidence rate of white line lesions was stable regardless of lactation status. In animals, which were feed on the hard or slippery wet floor, or on the insufficient length of stole, sole haemorrhage was more frequently observed. Sole haemorrhage was usually observed on the hoof that was bearing body weight unequally through abnormal claw horn shape. White line was usually observed on the hoof that was bearing body weight unequally through abnormal claw horn shape. White line lesion was seen on the hoof of the animal that was fed on hard or slippery wet floor, or on the insufficient length of stole, while the risk of the lesion was decreased in freer stole. The incidence of sole haemorrhages was significantly correlated to the claw horn shape. The different feature of the incidence between sole haemorrhage and white line lesion suggested that risk factors of incidence and severity for respective lesions should be contrasting.

In conclusion, detected abnormal sole lesions, including sole haemorrhage and white line lesions, would indicate the risk of calving and housing properties as predisposing factors of hoof diseases, especially subclinica ll aminitis. Further investigation and analysis, using increased number of examined herds of cows, would contribute to meet more precise understanding of hoof diseases in dairy cattle.

Analysis of nutrition as predisposing factors of foot diseases in dairy cattle in Hokkaido

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Foot disease of dairy cattle is one of the major causes of economic loss in dairy farms. Within foot pathology, subclinical laminitis takes a remarkable part. The cause of subclinical laminitis is believed to be multifactorial, in which nutrition, housing, genetics, stress and changes of hormones during parturition are emphasized. The purpose of this study was to analyze effects of cow nutrition on claw horn lesions, as a suggestive reason of subclinical laminitis, by using results from metabolic profile test (MPT).

Investigation was done in 9 commercial dairy herds including 445 cows in Hokkaido, in which cows were kept in free- or tie-stall yard. MPT was performed one week before claw horn lesions scoring, which was done at the time of claw trimming. Finally, the incidence of deviation from the reference range of MPT parameters was calculated. Correlation coefficients between claw horn lesion scores and the incidence of the deviation were analyzed statistically.

In a free stall group, deficiency of proteins in feed might be responsible for the incidence of claw horn lesions. In a tie stall group, ruminal acidosis might be related to claw horn lesions. In a longitudinal investigation of the same dairy herd, decayed roughage feeding might cause mycotoxicosis, which might be also responsible to the incidence of claw horn lesions.

In the present study hypothesis, which indicates that insufficient nutrition affects the incidence of foot disease, was not completely investigated. Our data raised from a week advance MPT, which was used to analyze and compare with the incidence of hoof lesions. However, changes of blood components in MPT were affected by milk production in respective groups of animals. Therefore it was difficult to conclude that only nutritional factors were accountable for the incidence of foot diseases in dairy cattle.

In conclusion, ruminal acidosis, insufficient of protein in feeding and mycotoxicosis alterated by roughage might be responsible for the incidence of claw horn lesions. Further investigation focusing on more aspects apart from nutrition would be necessary to understand the pathophysiology of this critical disease in dairy cattle.

The efficacy of nutraceutical (Agaricus blazeii, Pleurotus cornucopiae, Hericium erinaceum, arabinoxylan and shark substances) on the tumor growth in tumor-bearing mice

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Many kinds of neutraceuticals seem to be frequently applied to cancer patients in both medical and veterinary practice. Many works have been investigated the usefulness and usage of nutraceuticals, however the detailed information of mechanism of action and clinical efficacy of these agents is still unavailable. In this study, effects of immunopotentic neutraceuticals, including *A. blazeii, P. cornucopiae, H. erinaceum,* arabinoxylan, and antiangiogenic shark cartilage and its extract, were examined in tumor-bearing mice.

The C3H/HeN mice in examination groups were fed by diet supplemented with one of the studyed nutraceuticals for 3 weeks when a murine osteosarcoma cells (LM 8) with high metastatic potential to the lung were inoculated subcutaneously in the back of the animals. Afterwords, mice from each group were fed by certain nutraceutical for additional 3 or 4 weeks. On the other hand, the mice in the control groups were fed without nutraceuticals. Study 1; In the groups of *A. blazeii, P. cornucopiae, H. erinaceum* and arabinoxylan (n= 6), there were not notable changes of tumor growth. Study 2; In the groups of shark cartilage and shark extract (n=10), the tendency of growth inhibition in inoculated and metastatic tumor cells and decreasing of the number of newly synthesized blood vessels in each tumor lesions were observed. Study 3; In the groups of shark extract (n= 7), the number of blood vessels in tumor lesion were significant decrease and the results of study 3 implied the tendency of more effective inhibition of tumor growth than study2. These results proved the antiangiogenic activities of shark cartilage and its extract, which were reported previously. In addition, shark extract has more antiangiogenic effects than shark cartilage, and best antitumor effects within nutraceuticals that were used in this study. In this study any side effect related to nutraceuticals was not observed.

In conclusion, shark extract has the best antitumor effects within nutraceuticals that were used in this study. This agent could be a substitution or an alternative agent in cancer therapy.

Can persistently infected genomes of influenza A virus be rescued by superinfection?

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Influenza virus has been reported as one of the possible causes of the central nervous system (CNS) deseases, such as Parkinson's disease, and the significance of the persistently infected influenza virus for the CNS diseases have been disputed.

Several *in vitro* studies have demonstrated a persistent infection of influenza A, B and C viruses in cultured cells, and Park et al. (2003) demonstrated the genomic and antigenic persistence in the brain of mice recovered from the clinical signs of acute influenza A virus infection. In their mice, the virus could not be recovered from the brain, thus the mouse model corresponded to" chronic defective" persistent infection.

In vitro studies have demonstrated that the persistently infected influenza virus RNA segments in cultured cells can be rescued by superinfection of another strain of influenza virus. If the *in vitro* studies are applicable to *in vivo*, the viral genomes persistently maintained in host cells may be reactivated and rescued into superinfected virus particles. In chapter I of the present thesis, the persistent infection of the neurotropic influenza virus in the mouse was established and further details of the persistent infection were studied. The results showed that viral genomes persisted till 60 post infection day (PID) and viral protein till 20 PID, and they gradually disappeared with day. On 20 PID, all RNA segments but PB1 were detected in full-length. These results suggested that the mice on 20 PID are suitable for demonstrating that the persisted viral genomes can be rescued by superinfected influenza virus, thus giving rise to novel reassortant virus in the CNS of mice.

In chapter II, a strain of influenza virus was intracerebrally inoculated in the mice persistently infected with influenza virus of another subtype. However, no virus particles were reisolated from the superinfected brain. This may be attributable to immune-mediated clearance of the superinfected virus from the brain.

As a conclusion, genomes of influenza virus can be maintained in naturally-infected host brain, however, it is improbable that the persisted genomes be rescued by the superinfected another subtypes of neurotropic influenza virus in the CNS.

Epidemiological study on so-called fowl glioma

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So-called fowl glioma is suggested a virus-induced neoplasm caused by an avian leukosis virus subgroup A (ALV-A). This virus is suspected to spread in the flock of Japanese fowl of a zoological garden in Toyama prefecture.

Nested polymerase chain reaction (PCR) and reverse transcriptase (RT) nested PCR methods were developed for the detection of the causal ALV of fowl glioma in various tissues. The established nested PCR amplified the products with expected molecular sizes from the DNA sample of infected CEF and did not from those of CEF infected with 10 standard strain viruses. Various tissues from the experimentally infected chickens were examined by these methods. The provirus of the causal virus was detected in most of the tissues examined. Feather pulps were the clinical sample suitable for the detection of the viral genome, since sample collection and preservation were convenient by using the pulp in the present field study. The results of RT-nested PCR in feather pulps were consistent with those of the nested PCR. Nested PCR and RT-nested PCR were valuable for the detection of the causal ALV of fowl glioma.

The prevalence of the disease was examined in 131 Japanese fowls of the zoological garden in Toyama prefecture by enzyme-linked immunosorbent assay (ELISA) as well as the nested PCR and RT-nested PCR established in this study. Fifty-two (39.7%) chickens were positive by nested PCR and seventy-five (57.3%) were positive by ELISA. The zoological garden in Toyama prefecture has played an important role as a major supplier of Japanese fowls for other zoological gardens in Japan. Therefore, these results indicated that the disease has already been spread over Japanese fowls kept in at least 10 zoological gardens in Japan by the transport of the affected chickens and fertile eggs.

Isolation of CHO-K1 subclones showing different susceptibility to equine herpesvirus-1 infection and analysis of their viral gene expression

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CHO-K1, a cell line established from Chinese hamster ovary cells, has been considered nonpermissive for the replication of alphaherpesviruses. However, Equine herpesvirus 1 (EHV-1) infected CHO-K1 expresses some viral genes and is partially positive for viral antigen. In this study, a low susceptible subclone (114) and a highly susceptible subclone

(314) to EHV-1 infection were isolated from the CHO-K1 cells by limiting dilution-culture method. The virus titer in 314 at 24hour after infection was 8.5-fold higher than that in 114 by plaque assay. The characteristic intranuclear inclusion bodies were frequently noted in 314 at 24hour after infection with hematoxylin and eosin stain, whereas 114 had a few inclusions. The level of transcription of IE, ICP0, gK gene in 314 was 10 - to 19 - fold higher than that in 114 at 12 or 24 hour after infection by the dot blot analysis. The difference in the transcription of all genes between 114 and 314 was considered to result from the difference in the transcription of IE gene, because IE gene product controls the transcription of early and late genes. The transcription of IE gene depends on the activity of the promoter which is controlled by some cellular transcriptional factors. However no difference was observed in IE promoter activity between uninfected 114 and 314 by CAT assay. Therefore, the difference in the transcription of IE gene between 114 and 314 was not related to the efficiency of their cellular transcriptional factors. These results suggest that the difference in the susceptibility to EHV-1 infection between 114 and 314 may result from the difference in the levels of viral RNA transcription that might be controlled by viral transactivator, rather than cellular transcriptional factors.

Effects of thawing procedures on the survival of *in vitro* produced bovine expanded blastocysts frozen with glycerol and sucrose

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The objective of this study is to determine the effects of thawing procedures of frozen bovine blastocysts on their survival. Bovine expanded blastocysts were produced *in vitro*, and frozen with 1.4 M glycerol and 0.25 M sucrose in plastic straws using a conventional slow freezing procedure. Frozen embryos were thawed by exposing the straws in air for various times, and they were then transferred into a water bath. After thawing, embryos were cultured *in vitro* for 36 hr and evaluated by their morphology, live cell numbers and mitotic cell numbers. In experiment 1, the effect of time of holding straw in air (0-30 sec) on the survival of thawed embryos (the rate of re-expanded, hatching and hatched blastocysts) was examined. The highest survival was observed when frozen embryos were thawed by holding in air for 15 sec (81.3%).

The rate of embryos with damaged zona pellucida thawed by holding in air for 0 or 5 sec was higher than that by holding for 10 to 30 sec (50% vs less than 5%). The survival of embryos with damaged zona pellucida was lower than that with intact zona pellucida (46.2 vs 75.6%). In experiment 2, the effect of time of shaking the straw in air (5-20 sec) on the survival of thawed embryos was examined. As the time of shaking the straw in air was increased, the survival of thawed embryos was decreased (from 92.5 to 36.0%). The rates of embryos with damaged zona pellucida were lower than 5%, regardless of the shaking periods. In experiment 3, the straw

was held in air for 15 sec, and then transferred into a water bath at 35 or 20℃. Neither the water temperature nor agitation of the straws in a water bath affected the survival of thawed embryos. Temperature changes in the straws during the thawing in each procedure suggested that rapid warming (transferring to a water bath) between -140 and -110℃ may cause fracture plane leading damages on the zona pellucida of embryos, and that slow warming (holding in air) between -40 and -30℃ may lead devitrification in the embryonic cells.

Changes in infiltrating cells and endometrial epidermal growth factor (EGF) positive cells associated with alteration in endometrial EGF concentrations in dairy cows

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Absence of the peaks on Days 2-4 and 13-14 in endometrial EGF concentrations has been reported as an abnormality in repeat breeder cows. Since measurement of uterine endometrial EGF concentration is, however, not common laboratory practice, an alternative diagnostic method to EGF assay is needed. Numbers of infiltrating cells, including lymphocytes, NK-like cells, plasma cells and mast cells, vary under the influence of ovarian steroid hormones, which also regulate EGF production in the endometrium. Therefore, alterations in EGF concentrations could be estimated by changes in infiltrating cell population. Further, there is little information on the relationship between type and numbers of EGF positive cells in the endometrium and the endometrial EGF concentrations. Thus, the present study examined 1) the numbers of infiltrating cells and 2) changes in the type and numbers of cells that are immunohistochemically positive for EGF in the cows with normal and altered endometrial EGF concentrations. Holstein cows were divided into two groups with an abnormal group (n=4) and a normal group (n=3) by EGF concentrations on Days 2-4 and 13-14 (Day 0 = estrus) of the estrous cycle. Ovarian steroid hormone concentrations, the number of infiltrating cells and that of EGF positive cells were compared between the two groups. Plasma progesterone concentrations showed normal cyclic change in the both groups; however, plasma estradiol concentrations in the abnormal group did not show a peak on the day of estrus. Biopsy on Days 2-4, 6-7 and 13-14 obtained Endometrial tissues. In the endometrial stroma, cyclic changes in the number of lymphocytes, NK-like cells and plasma cells were similar in both groups. Number of all infiltrating cells and that of mast cells in the abnormal group were fewer than those of the normal group throughout the estrous cycle. In both groups, only the glandular epithelial cells were immunologically stained positive for EGF. The number of EGF-positive cells increased during the estrous cycle as days after estrus increased, but no significant difference was noted in the cell numbers between the two groups. In summary, the present study revealed that cows with abnormal EGF concentrations could be diagnosed by ex-

amination of endometrial mast cell number on Days 13-14. Further, suppression of endometrial EGF concentrations on Days 2-4 and 13-14 may be caused by decreased synthetic activity rather than decreased number of EGF producing cells.

Relationship between standing estrus and the changes in the activity measured by using radio-telemetric pedometer in dairy heifers

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Estrus detection is one of the major factors to obtain a high pregnancy rate in dairy herds. The traditional form of estrus detection has been visual observation; however, efficiency of this method is low. Thus, development of efficient estrus detection aids including a pedometry system has been needed. To determine the efficiency of pedometer for the detection of estrus, the author examined the relationship between standing estrus and the changes in the activity measured by a telemetric pedometer system. Holstein heifers (n = 6) were kept in a paddock. The pedometer was attached to the left hind leg and read the counts hourly. The heifers were visually observed estrous behavior for 30 min every 2 hr throughout estrous periods (2 natural and 10 prostaglandin $F_{2\alpha}$-induced estrous periods). After confirming a high accuracy of the pedometer counts, the changes in the total pedometer counts for every 6 hr were examined throughout the experimental period. The 6-hr pedometer counts significantly increased between 6 hr before and 11 hr after the onset of standing estrus (0 hr). To determine the relationship between standing estrus and the changes in the pedometer counts, activity ratio (the count for the last 24 hr to the average count for corresponding 24 hr for the last 5 days) was calculated hourly. The activity ratio started to increase before the onset of standing estrus and was 1.4 ± 0.4 at the first observation of standing behavior. By the end of standing behavior, the activity ratio reached 2.2 ± 0.6. Relationship between the duration of standing estrus and that of pedometer-detected "estrus" was investigated using the 95% confidence upper bound (1.7) of the activity ratio at the onset of standing estrus (threshold). The estrous period determined by the threshold was 20.0 ± 6.0 hr (ranged from 9 to 26 hr). Estrus was first detected by the threshold of activity ratio between 3 hr before and 16 hr after the onset of standing estrus. When the animals showed estrous behavior simultaneously, estrus was detected within 5 hr from the initiation of standing estrus. The present study confirmed the temporal relationship between onset of standing estrus and the increase in the activity measured by a telemetric pedometer system. The results indicated that the telemetric pedometer system could be used for the detection of estrus in dairy cattle.

Developmental Changes of Tight Junction Proteins in Murine and Bovine Mammary Epithelial Cells

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Transmembrane proteins claudins and occludin are major constituents of the tight junction strand in epithelial cells, and play barrier functions in paracellular transport of solutes, ions, and water. The present study was conducted to investigate gene expression and localization of tight junction proteins including claudins, occludin, and ZO-1 and their changes along with development of murine and bovine mammary glands. RT-PCR analysis of 19 distinct claudin mRNAs showed that claudins 1, 3-8, 10-12, 15 and 19 as well as occludin and ZO-1 exhibited expression at various levels in murine mammary glands. Some of them involving claudin-4 showed developmental changes in the level of mRNA expression, whereas the rest including claudin-3, occludin, and ZO-1 appeared to be expressed constitutively at constant levels. Cellular localization was analyzed for claudins 1-5 in murine and bovine mammary glands using immunofluorescent microscopy. Claudin-3, claudin-5, occludin, and ZO-1 exclusively localized at the apical end of the cell membrane relevant to the tight junction, indicating that these are predominant components of the tight junction in mammary glands and principal determinants for paracellular pathways. Interestingly, claudin-4 showed apical and lateral distribution with a transition to the tight junction at lactating stage, suggesting a possible mechanism for developmental change in distribution with the help of function of some unknown molecules. Claudin-1 was not located to the tight junction but was found in basement or basolateral membranes. Different distribution in cellular membranes of these claudins all possessing PDZ domain-binding motives supposed alternative mechanisms by which claudin proteins were linked to the cytoskeleton, in addition to the one through association with ZO-1.

Pathobiology for Bovine Band 3 Deficiency: Mild Hemolysis and Tissue Damages in A Fetus Homozygous for R664X Mutation.

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Band 3 (anion exchanger 1, AE 1), the most abundant transmembrane protein in mammalian blood cells, functions as an anion transporter mediating Cl^- / HCO_3^- exchange across the plasma membrane and participates in maintaining mechanical properties of red cells by attaching the membrane skeletal network to the plasma membrane. Molecular ba-

sis for band 3 deficiency with inherited spherocytosis in Japanese black cattle is a nonsense mutation, R664X mutation. The animals with aberrant deficiency of band 3 exhibit sever hemolysis due to remarkable instability of the red cell membrane and show high mortality presumably because of serious damages in many organs. The present study was carried out to analyze tissue damages at fetal stage of this disorder and distinguish them from those in neonates. A fetus homozygous for R664X mutation was produced by embryo transfer of the fertilized egg which was determined to be homozygous and was obtained by Caesarean operation at 100 days of pregnancy. The fetus apparently had no jaundice. The genotype of the fetus and the total lack of band 3 protein was confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of red cell membrane proteins and immunofluorescent microscopy of red cells. Red cells from the fetus showed spherocytosis and anisocytosis. Histopathological analysis was performed on the kidney, liver, and spleen from the fetus in comparison with those of a neonate with band 3 deficiency. The kidney from the neonatal calf had severe disposition of hemosiderin, calcification, and lipofuscin deposition in renal tubules and glomerulus, suggesting that an oxidation-mediated tissue injury was involved in pathogenesis of band 3 deficiency, whereas renal hemosiderosis was mild and the tissue damage was obscure in the fetus. Severe deposition of hemosiderin was also observed in the liver and spleen from the neonate but not in those from the fetus. Fetal liver revealed active erythropoiesis and erythroid cells apparently showed normal configuration without detection of polynuclear cells that were reported to be characteristic to band 3 deficiency. These findings suggest that tissue lesion is not crucial to survival in the fetal stage of animals with band 3 deficiency.

Pathgenicity of tick-borne encephalitis virus far-eastern subtype analyzed by a series of infectious cDNA clones

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Tick-borne encephalitis (TBE) virus, which is a member of the genus flavivirus in the flaviviridae family, causes severe encephalitis in humans. TBE viruses are known to be composed of European, Siberian and Far-Eastern subtypes. Far-Eastern subtype shows a higher case fatality rate than others and it is known as a high virulent subtype with serious sequelae.

Because of flavivirus polyprotein with transmembrane domains could be toxic for bacteria, it is difficult to construct an infectious cDNA clone of flavivirus. Although there are two reports of the infectious cDNA clones of TBE virus European and Siberian subtypes, there is no report about Far-Eastern subtype. We have constructed an infectious cDNA clone (O-IC) of Far-Eastern subtype TBE virus Oshima 5-10 which was isolated in Hokkaido.

In this study, we determined the complete genome sequence of O-IC clone. O-IC clone

had 9 nucleotide changes in entire sequence ; one amino acid change in the E protein, two amino acid changes in NS 5 protein and two nucleotide changes in 3' UTR as compared with Oshima 5 -10 sequence. Focus formation of O-IC virus in BHK-21 cells was slower than that of parent virus Oshima 5 -10.

In order to determine which amino acid changes were responsible for a focus size reduction of O-IC, we constructed three infectious cDNA clones, including O-IC-pt which is replacing all amino acid changes to parent sequence. The viruses which were recovered from a series of infectious clones were compared each other for genetic and biological characteristics. O-IC-pt virus showed no differences of the focus formation, growth cycles in culture cells and neurovirulences in mice compared with parent virus. The 40th amino acid change in the envelope protein was responsible for focus size reduction, growth cycle reduction in culture cells and attenuation of neurovilurence in mice. The two amino acid changes in the NS 5 influenced attenuations of neurovilurence and neuroinvasiveness in mice.

Since the infectious cDNA clone enables to generate mutants with any kind of nucleotide replacement and is stable, it will be very useful for future studies on pathogenesis and vaccine development and of Far-Eastern subtype TBE virus

Analaysis of co-evolution between hantavirus and *Clethrionomys rufocanus* and development of simple ELISA for serodiagnosis

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It is thought that the hantavirus has been co-evolving with their rodent hosts and various serotypes or genotypes are distributed all over the world by specific natural rodent hosts. Hantavirus causes two form of severe human illnesses, which are hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. In our country, although no patients of hantavirus infection are reported in recent years, brown rats (*Ruttus norvegicus*) hold Seoul type hantavirus in main port areas all over the country, and gray red-backed vole {*Clethrionomys* (*C.*) *rufocanus*} which inhabit Hokkaido hold the hantavirus closely related with the Puumala type. In this paper, development of the simple serodiagnostic method of hantavirus infection and epidemiological investigation were conducted at Hokkaido, Sakhalin, and Far East Russia. Furthermore, the genetic analysis of the virus and also *C.rufocanus* cytochrome b gene were performed.

A total 321 *C.rufocanus* were captured at the survey point and anti-hantavirus antibodies were detected by indirect fluorescence antibody assay (IFA) with Puumala type Sotkamo antigen. Seroprevalences at each survey points and year were as follows ; 2.9% (3/104) at Tobetsu in 2000, 14.6% (15/103) at Tobetsu in 2003, 100.0% (1/1) at Kiritappu in 2000, 2.9% (2/69) at Sakhalin in 1998, 25.0% (1/4) at Vladivostok in 1999, and 17.5% (7/40) at Khekchir in 2002.

The hantavirus S gene was cloned from

an infected *C.rufocanus* and nucleocapsid proteins (NP) was expressed in *Escherichia coli* system. The recombinant NP was applied for ELISA antigens for antibody detection. Then, *C. rufocanus* sera were examined by the ELISA, and IFA positive sera showed high optical density (OD) values. Strong correlation was seen between IFA titer and ELISA OD value in *C.rufocanus* sera (r=0. 9349). Therefore, the ELISA is useful as a serodiagnostic method of hantavirus infection in *C.rufocanus*.

Full length of virus S gene derived from *C. rufocanus* was sequenced. The identities of the S genes among Hokkaido origin were within 95%. The identities of the Hokkaido S gene to Sakhalin, and the Far East Russia were 87% and 80%, respectively. Strong relationship between the geographical distance of the virus origin and the genetical distance of the gene was observed. It appeared that there is the selection pressure which keep amino acid sequence of NP because the mutation rates in the first and second nucleotides of the codon were significantly lower than that of the third nucleotide. Furthermore, since the phylogenetic tree shapes of the virus S genes and *C.rufocanus* cytochrome b genes were almost identical, co-evolution between hantavirus and *C.rufocanus* is strongly indicated.

Antigenic characterization of Sin Nombre virus and establishment of serodiagnosis of hantavirus pulmonary syndrome.

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Sin Nombre virus (SNV) and Puumala virus (PUUV) are different serotypes of genus *Hantavirus* in the family *Bunyaviridae*. SNV and PUUV are causative agents of HPS and HFRS, respectively. HPS is a severe human illness characterized by acute pulmonary failure, with a high mortality rate of about 40%. SNV has a single rodent host *Peromyscus maniculatus* belonging to the subfamily *Sigmodontinae*. Because the SNV-related rodents only inhabit the American continents, there is no report of HPS occurrence in other places. However, there is a possibility that a traveler is infected with SNV in the America and shows symptoms of HPS after getting into Japan. In addition, wild rodents which are possibly carrying SNV may be imported from the America. Therefore, establishment of a serodiagnostic method for SNV infection is strongly required. Serological surveillance of imported and indigenous rodents are also important. SNV and PUUV, however, have cross-reactivity to each other, so antigenic comparison between SNV and PUUV is necessary. In this study, recombinant nucleocapsid protein (rNP) of PUUV and SNV were prepared by baculovirus expression system. In antigenic profiling of SNVrNP by using monoclonal antibody (MAb) in indirect fluorescent antibody assay (IFA), SNVrNP had strong cross-reactivity to PUUV NP. In enzyme linked immunosorbent assay (ELISA), we examined the cross-reactivity between SNV and PUUV antigens in patient sera. Twelve out of fifteen SNV-infected patient sera had cross-reactivity against PUUVrNP. The OD value to

PUUVrNP was 50.8% of that to SNVrNP. Sixteen out of twenty six PUUV-infected patient sera had also cross-reactivity against SNVrNP. The OD value to SNVrNP was 42.8% of that to PUUVrNP. However, by comparing the OD values to SNVrNP and PUUVrNP in patient sera, differential serodiagnosis was possible.

In ELISA by using SNV155 and PUUV 155, which were rNPs lacking 1 to 154 amino acids of the N terminus, SNV155 had low reactivity to SNV patient sera and no cross-reactivity to PUUV patient sera. PUUV155 had specific reactivity to PUUV patient sera and no cross-reactivity to SNV patient sera. To know the information of rNP multimerization, competitive sandwich ELISA using E5G 6 monoclonal antibody as capture and detection antibody was carried out. The OD value to SNV155 was below that to SNVrNP in any antigen concentration while the OD value to PUUV155 increased in antigen-dose dependent manner. Therefore, SNV155 and PUUV155 may be monomer and multimer, respectively.

Mechanism of the induction of neurite-like outgrowth in PC 12 cells by nitrone spin trap reagents.

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A nitrone spin trap, α-phenyl-N-*tert*-butylnitrone (PBN), is widely used for studies of the biological effects of free radicals in vitro and in vivo. Recent studies in this laboratory showed that PBN had the unique NGF-like activity to induce neurite-like outgrowth in rat pheochromocytoma PC12 cells and that the Ras-ERK pathway played a critical role in this event. The NGF-like activity of various nitrone spin trapping reagents such as POBN, S-PBN and DMPO was first evaluated in the present study. The percentage of cells showing neurite-like outgrowth induced by PBN, POBN, S-PBN and DMPO was 59%, 37%, 10% and 2%, respectively. Since PBN is known to be a hydrophobic spin trap compared with other reagens, the hydrophobicity of nitrone spin trap might be an important factor in the induction of neurite-like outgrowth in PC12 cells. Subsequently, to examine whether cysteine residues of Ras were involved in PBN-induced neurite-like outgrowth in PC 12 cells, mammalian expression vectors having a mutation at cysteine residues of Ras, Ras (C51S), Ras (C80S), Ras (C118S), Ras (C181S) and Ras (C184S) were constructed and introduced into PC12 cells. The enhancement of neurite-like outgrowth by PBN was observed in PC12 cells overexpressed with Ras (WT) but not in those with Ras (C118S), Ras (C181S) and Ras (C184S), suggesting that the cysteine residues at cys-118, cys-181 and cys-184 were targets of PBN or its metabolites. From these results, it was concluded that the hydrophobicity of nitrone spin traps and cysteine residues of Ras in host cells were important in differentiation in PC12 cells.

Non-invasive assessment of the JNK inhibition against malonate-induced ischemic brain injury by using 7.05 T MRI

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There is increasing evidence that stress-activated protein kinase / c-Jun N-terminal kinase (SAPK/JNK) of mitogen-activated protein kinase (MAPK) family is closely associated with ischemia-induced neuronal death. Therefore, the development of compounds having an ability to inhibit SAPK/JNK as a protector against the ischemic brain injury is important. In this study, to confirm the role of SAPK/JNK pathway in ischemic brain injury, malonate, a reversible mitochondria-toxin, was injected into the left striatum of male Sprague-Dawley rat without or with the simultaneous injection of a peptidic JNK inhibitor, (L) -HIV-TAT$_{48-57}$-PP-JBD$_{20}$. The protective effect of this peptide on the ischemic brain injury was evaluated by non-invasive visualization technique with apparent water diffusion coefficient (ADC) mapping MRI method.

Malonate injection induced the hyperintense region at the left striatum in ADC mapping of the brain. The ADC value in the region was significantly high at 6 h, whereas the region surrounding the hyperintense region was imaged as a hypointense one in ADC mapping and the ADC value was low at 3 and 6 h, suggesting that the edema appeared in this region. In the JNK inhibitor-injected rat, changes in the ADC values of both regions were significantly suppressed, and the size of the edema also became smaller than that of no JNK inhibitor-injected one. Histological examination of the brain at 6 h and 2 days after malonate-injection proved that the JNK inhibitor protected the brain against not only neuronal cell death but also axonal swelling and intramyelinic edema. Immunoblot analysis revealed that the activation of JNK and the expression of c-Jun were observed in the edema region. This was not observed in the corresponding region of JNK inhibitor-injected group. Furthermore, malonate injection gave no influence on the activation of extracellular signal-regulated kinase (ERK) and p38-mitogen activated protein kinase (p38 MAPK). These data indicated that the induction of brain injury by ischemia is controlled by the JNK-pathway. In conclusion, the inhibition of JNK pathway may protect brain tissues against degenerated and edematous injuries in the brain. ADC mapping MRI method can be utilized for non-invasive visualization of brain injury at the early stage and for the pertinent evaluation of neuroprotective ability of compounds.

Suppression of cell proliferation in Colon 26 solid tumor by a treatment combining X irradiation and a novel anticancer drug, 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl) cytosine (ECyd)

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The combined treatment of radiation with anticancer drugs is often used to improve therapeutic efficiency for solid tumors. ECyd, 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl) cytosine, has been newly developed as an anticancer drug having the cytotoxicity by inhibiting RNA synthesis. Previous studies in this laboratory have demonstrated that a low dose of ECyd can amplify X-ray-induced apoptosis in a variety of tumor cells in vitro. The aim of this study was to investigate antitumor efficacy of radiation treatment combined with ECyd administration in vivo.

Colon adenocarcinoma Colon 26 cells were subcutaneously inoculated in the footpad of right hind limb of BALB/c mice. After 10 days, they received treatments of a low dose of ECyd (0.1mg/kg) and/or X irradiation (2 Gy), and tumor growth was monitored. When mice were treated with X-rays or ECyd alone, the inhibition rates of tumor growth were 10.6% and 0.8% 5 days after treatment, respectively. However, when mice were treated with the combination of X-rays and ECyd, the inhibition rate significantly increased (45.5%). The tumor growth suppression by this combined treatment was largely abrogated in the presence of 0.5mg/kg cytidine, indicating that phosphorylation of ECyd by uridine/cytidine kinase (UCK) was required for its ability to amplify the antitumor activity of X-rays. Furthermore, to examine whether repetetive treatments of X-rays and ECyd increase more efficiently the cure rate than those of X-rays or ECyd alone, mice were treated with combined treatments for three times in two-day interval. The complete remmission of tumors was observed in three among six mice. Histological examination by hematoxylin-eosin staining and TUNEL staining methods showed that no increase in the number of apoptotic cells in tumor was observed even in the case of combination of X-rays and ECyd. However, the induction of apoptosis in vascular endothelial cells in tumor was demonstrated. These results suggested that the induction of apoptosis in vascular endothelial cells in tumor was partly responsible for the inhibition of tumor growth by the combined treatment. These data might provide useful information about the clinical application of radiation combined with ECyd for treatments of solid tumors.

Biomonitoring of the Harbor Seawater Environment on the Hokkaido Coast with Induced Hepatic Cytochrome P4501 A of Minnow

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[Introduction] Harbors are the most polluted spots of the coastal water, but the means to assess levels of impact of pollution on biota has not been developed. We assessed environmental pollution of the seawater of three harbors in Hokkaido by monitoring the hepatic cytochrome P4501A (CYP1A) of the dace, *Leuciscus* (*Tribolodon*) hakonesis. CYP 1 A is known to be specifically induced with environmental pollutants as dioxins, PCBs and polycyclic aromatic hydrocarbons (PAHs), and has been used as one of the biomarkers to monitor environmental impact. The dace is an omnivorous minnow and inhabits broadly in fresh or brackish water in Japan, the Korea peninsula, Sakhalin and other places.

[Methods] Wild male and female daces were collected from three different harbors in Hokkaido, Otaru, Ishikari and Bikuni, respectively. Otaru and Ishikari harbor were assumed to be polluted heavily, and the Bikuni harbor less so. Hepatic microsomes were prepared to measure the catalytic activities and protein levels from the daces. The ethoxyresorufin-O-deethylase (EROD) activities and expression levels of CYP1A protein by western blotting method were measured using livers of daces from these harbors. PAHs levels of seawater absorbed into Blue Rayon, which was hanged underwater in each harbor for72hours, were analyzed using spectrofluorometry. The estradiol levels in blood were measured with ELISA kits.

[Result and discussion] 1. The daces caught in the Otaru harbor gave the highest EROD activities and protein levels of CYP1A among the daces caught in these three harbors, which are correlated with the finding that the highest concentration of PAHs was found in the seawater from the Otaru harbor. Thus, CYP1A in the liver of the dace was confirmed to be a good biomarker to monitor the environmental pollution with PAHs in the sea. 2. In the spawning season, June and July, EROD activities and CYP1A protein levels were found to rise in their ratio, although they were low during the post-spawning season, August to October. 3. Sex differences disappeared in these two parameters between male and female daces in the highly polluted arbor of Otaru and Ishikari, although clear differences were observed in the Bikuni harbor. Female dace may react more sensitively to pollutants, and such sex differences could be good indicators for environmental pollutants.

Sexual differentiation of the brain induced by steroid hormone ~The mechanism of amplification of testosterone in the neonatal brain~

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It has been suggested that sexual differentiation of the brain is caused by its exposure to testosterone or its metabolites at a critical period. At the neonatal period, the expression levels of each steroidgenic enzyme in glia is different from that in neurons. It is proposed that glia cells interact with neurons in steroid metabolizing pathways. The purpose of this study is to examine the hypothesis that neonatal exposure of the brain to testosterone released from testes shortly after birth initiates the amplification of testosterone production by means of increased metabolism of blood dehydroepiandrosterone (DHEA) in astrocytes constituting the blood-brain barrier. Administration of testosterone propionate (TP) to neonatal female rats exhibited dose dependent 3 β-hydroxysteroid dehydrogenase type 1 (3 β-HSD-1) mRNA induction in hypothalamus. TP also induced 3 β-HSD-1 mRNA induction in C 6 glioma cells and primary cultured astrocytes. In primary cultured astrocytes, 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD-1) was also induced by a high dose of TP exposure. The mRNA induction was confirmed by the metabolizing ability of DHEA. DNA microarray analysis indicated that the administration of TP and DHEA decreased $GABA_B$ receptor subunit mRNA. These results indicate that exposure of brain to testosterone shortly after birth enhanced biosynthesis of testosterone in astrocytes and repressed $GABA_B$ receptor expression. Amplification of testosterone synthesis induced by circulating testosterone in astrocytes may lead to the masculine nature of the brain.

The repression of uroporphyrin accumulation and uroporphyrinogen oxidase activity in iron-deficient mice

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It has been reported that the increased formation of uroporphyrin by increased activity of uroporphyrinogen oxidase is responsible for uroporphyria and it is believed that CYP1 A2 is responsible for uroporphyrinogen oxidation. In experimental uroporphyria, not only the induction of CYP1A2 caused by polyhalogenated aromatic compounds and polycyclic aromatic hydrocarbons, but also sufficient levels of iron are essential for accumulation of uroporphyrin. However, it is not clear why a sufficient level of iron is essential for uropor-

phyrin formation. We have measured uroporphyrinogen oxidation and uroporphyrin accumulation in 9000g supernatants (S 9) from the livers of iron-deficient mice (ID mice) treated with CYP1A2 inducer3-methylcholantherene (MC) and 5-aminolaevulinic acid. We found that the concentration of uroporphyrin and the level of uroporphyrinogen oxidation were not raised in ID mice. This phenomenon was not due to the depletion of CYP1A2, since CYP 1A2 levels and their activities in iron-deficient mice were increased to the same levels as in normal mice (N mice). In N mice, alteration of uroporphyrinogen oxidase activity and CYP1 A2 activity a 4-week period after an injection of MC were parallel to each other, while they were completely disassociated in iron-deficient mice. UROX activities were also tested in liver microsomes. The difference in uroporphyrinogen oxidation between mice fed a normal diet and an iron-deficient diet was not as great as those observed in liver S9. Addition of liver cytosol from iron-deficient mice repressed the uroporphyrinogen oxidation in microsomes. These results suggest that uroporphyrinogen oxidase activity was inhibited by some unknown factor in cytosol. And this may suppress accumulation of uroporphyrin in iron-deficient mice.