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INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on March 22, 2012 to 18 recipients.
The titles of theses and other information are as follows:

Characterization of influenza viruses isolated from domestic ducks in Vietnam and evaluation of H9 influenza virus vaccine

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In the surveillance of avian influenza in Vietnam, 26 H9N2, 1 H3N2, 1 H3N8, 7 H4N6, 3 H11N3, and 1 H11N9 viruses were isolated from tracheal and cloacal swab samples of 300 domestic ducks in April 2009, and 1 H9N6 virus from 300 bird samples in March 2010. Out of the 27 H9 virus isolates, the HAs of 18 strains were genetically classified into G1 and the other 9 into Korean sublineages. Phylogenetic analysis revealed that one of the 27 H9 viruses was a reassortant, in which the PB2 gene belonged to Korean sublineage and the other 7 genes to G1 sublineage. Three representative H9N2 viruses were intranasally inoculated into ducks, chickens, pigs, and mice. On the basis of experimental infection studies, it was found that each of the 3 viruses readily infected pigs and replicated in their upper respiratory tracts, and infected chickens with slight replication. Viruses were recovered from the lungs of mice inoculated with 2 of the 3 isolates. The present results revealed that H9 avian influenza viruses are prevailing and genetic reassortment occurs among domestic ducks in Vietnam. Thus, it is recommended that careful surveillance of swine influenza with H9 viruses should be performed to prepare for pandemic influenza.

It is postulated that H9N2 virus may cause pandemic influenza in humans. In the present study, as the preparedness for pandemic influenza, H9 virus strains stocked in the influenza virus library in our laboratory were analyzed antigenically and phylogenetically to select H9N2 virus proper for a vaccine strain. On the basis of antigenic and genetic analyses, H9N2 viruses isolated from birds, pigs, and humans were classified into three sublineages. The chicken antisera to H9N2 viruses of Korean sublineage reacted with those of different sublineages by HI test. A test vaccine prepared from a non-pathogenic Dk/Hok/49/98 (H9N2) strain of Korean sublineage, that has been stocked in the influenza virus library, induced immunity in mice to decrease the impact of disease caused by the challenge with HK/1073/99 (H9N2) of a different sublineage. The present results indicate that the inactivated whole virus vaccine prepared from a non-pathogenic influenza virus from the library could be used as an emergency vaccine at the early stage of pandemic caused by H9N2 infection.

The original papers of this thesis appeared in *Arch. Virology*., **157**: 247–257 (2012) and *J. Vet. Med. Sci*., **74**: 441–447 (2012).

Studies on the molecular epidemiology and pathogenesis of *Trypanosoma evansi*

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Trypanosoma evansi (*T. evansi*), belonging to the subgenus *Trypanozoon*, often causes a severe wasting disease, called Surra, of livestock and wild animals. This disease is endemic in Southeast Asia, Africa and South America, where thousands of animals die annually due to the disease. In this study, the molecular epidemiological survey and gene polymorphism analysis of *T. evansi* in Philippines and South America were performed. Then, the virulence analysis of *T. evansi* isolated in Philippines was conducted using experimentally infected animals. In the last part, the functional analysis of regulatory dendritic cells (DCs) in *T. evansi* infection was carried out.

In South American and Southeast Asian countries, animal trypanosomiases as a result of mainly *T. evansi* infection cause significant economic losses in livestock industry. Therefore, in the first part, the epidemiological survey of animal trypanosomiases in Peru, Bolivia and Philippines was performed by using conventional polymerase chain reaction (PCR).

Depending on the locations, the epidemics of *T. evansi* and *T. vivax* infection was found in those countries. Then, the *T. evansi expression-site-associated gene* (*ESAG*) *6*, which encoded the transferrin receptors of trypanosome, were cloned and sequenced. Previously, it was shown that ESAG6 depicts genetic diversity among different isolates of *T. evansi* in Asia. In addition to some of the previously observed variants, 20 novel variants of ESAG6, which could be categorized into three new clades among the various isolates, were found. These results show that the ESAG6 sequence represents a useful genetic marker for the classification within the groups of *T. evansi* in different areas.

In Philippines, *T. evansi* is present in all 13 regions, and Surra is the second most important disease of livestock after fasciolosis. In recent years, highly virulent *T. evansi* has appeared and caused a severe disease with high mortality in livestock. For this reason, in the second part, several *T. evansi* strains were obtained from infected water buffaloes in Philippines, and their pathogenicities were compared to one another. When mice were inoculated with those isolates, the duration of prepatent period and survival time varied significantly amongst isolates. Then, the highest and least virulent *T. evansi* were inoculated into cattle. In these cattle, the highest virulent one grew faster than the least virulent one. The highest virulent *T. evansi*-infected cattle showed rapid leucopenia and anemia. The expression of tumor necrosis factor α (TNF-α) was increased in the cattle which developed anemia. These results indicate the presence of highly virulent *T. evansi* which can develop leucopenia or anemia in cattle in Philippines.

T. evansi infection triggers uncontrolled proinflammatory responses which contribute to the development of inflammation-associated tissue injury. To determine what kinds of inflammatory molecule plays a crucial role in the pathogenicity of *T. evansi* infection, PCR array analysis was performed on samples from the infected mice. The inflammatory cytokine and chemokine storm, caused mainly by macrophages, was observed. On the other hand, the expression of the *Ccl8* and *Il10* transcripts in splenocytes was also significantly increased. These results suggested an augmentation in the number and activity of

regulatory DCs. Therefore, the kinetics of regulatory DCs in *T. evansi*-infected mice was investigated. During *T. evansi* infection, the regulatory DCs became prevalent, with reduced amount of the inflammatory DCs. Interestingly, the survival rate of *T. evansi*-infected mice was increased when the regulatory DCs were implanted into these mice. Taken together, these results showed that a subset of regulatory DCs act as a potential regulator of the inflammatory responses.

In conclusion, this study provides novel information on the pathogenesis and host immune responses during the *T. evansi* infection. In future, it will be necessary to continue the monitoring of the *T. evansi* infection and to develop the new strategy for the treatment of the disease.

The original papers of this thesis appeared in *Infect. Genet. Evol.* **9**: 1301–1305 (2009) and *Parasite Immunol.*, in press.

Studies on transmission of leishmaniasis and mechanisms of parasite persistence in the liver

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Leishmaniasis is a zoonotic protozoan disease caused by the genus *Leishmania* species. Parasites are transmitted by the bite of female phlebotomine sand flies. The increase in risk factors for leishmaniasis is worldwide. Some risk factors are due to natural environmental changes, which may cause changes in the distribution and feeding behaviour of sand fly vectors. Others are the man-made changes such as migration and deforestation, or changes in the human host's susceptibility to infection such as immunosuppression.

In chapter I, the identification of phlebotomine sand fly species and their blood meals in the endemic areas of cutaneous leishmaniasis in Pakistan was studied. PCR-restriction fragment length polymorphism and sequence analysis of the insect 18S ribosomal RNA gene was developed to distinguish the *Phlebotomus* and *Sergentomyia* species. The female sand flies identified were *P. papatasi* (7.4%), *P. alexandri*-like sandflies (3.4%), *S. clydei*/*S. ghesquierei*/*S. magna* (68.6%), *S. dubia* (17.1%) and *S. dentata* (3.4%). Amplification and sequencing of the vertebrate cytochrome *b* gene in blood-fed sand flies revealed that *P. papatasi* fed on cattle and wild rat whereas *P. alexandri*-like specimens fed on human, cattle, goat and dog. Although *Sergentomyia* species are generally known to feed on cold-blooded animals, *S. clydei*, *S. dubia*, and *S. ghesquierei* preferred humans, cattle, goat, sheep, buffalo, dog, donkey, wild rat and Indian gerbil. The epidemiological significance of the zoophilic feeding of various host species by phlebotomine sand flies in Pakistan is further required to study for better understanding the zoonotic transmission of sand fly-borne pathogens and for appropriate management of the vectors.

In chapter II, the study is aimed to improve the understanding of mechanisms of hepatic *L. donovani* persistence in an immunocompromised condition using alymphoplastic *aly*/*aly* mice. Hepatic parasite burden, granuloma formation, expression of cytokine/chemokine mRNA and induction of regulatory T cells (Tregs) for up to 7 months after intravenous inoculation with *L.*

donovani promastigotes were determined. While control *aly*/+ mice resolved the infection by 8 weeks post infection (WPI), *aly*/*aly* mice showed long-term hepatic parasite persistence in the chronic phase of infection, which was associated with the delayed and impaired granuloma maturation. Although hepatic $CD4^+Foxp3^+$ but not $CD8^+Foxp3^+$ T cells were detected in both strains of mice, the number of $CD4^+Foxp3^+$ T cells and expression of *Foxp3* mRNA were significantly increased in *aly*/*aly* mice. Immunohistochemical analysis demonstrated the presence of $Foxp3^+$ T cells in *L. donovani*-induced hepatic granulomas and perivascular neo-lymphoid aggregates. Laser microdissection and quantitative RT-PCR of mature granulomas revealed that the increase in the *Foxp3* mRNA level at 12 WPI was correlated to the increased mRNA level of *IL-10* more than *TGF*-β, suggesting that the impairment of granuloma maturation was mediated by the immunosuppressive IL-10. Furthermore, the treatment of infected *aly*/*aly* mice with anti-CD25 or anti-FR4 mAb resulted in significant reductions in both hepatic $Foxp3^+$ cells and parasite burden. This study offered a novel insight into the involvement of Tregs in hepatic *L. donovani* persistence in an immunodeficient condition. The manipulation of Tregs may provide a promising immunotherapeutic strategy for visceral leishmaniasis.

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The development of animal models for Hirschsprung disease carrying mutations of the endothelin receptor type B gene

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Hirschsprung's disease (HSCR) is a developmental disorder characterized by the absence of ganglion cells in the lower digestive tract. Aganglionosis is attributed to a disorder of the enteric nervous system whereby ganglion cells fail to innervate the lower gastrointestinal tract during embryonic development. HSCR is a complex disease with low, sex-dependent penetrance and variability in the length of the aganglionic segment, thought as a result of the interaction of several genes. The genetic complexity observed in HSCR can be conceptually understood in light of the molecular and cellular events that take place during the enteric neural crest (ENS) development. DNA alterations in any of the genes involved in the ENS development may interfere with the colonization process, and represent a primary etiology for HSCR.

Animal models, in which genetic background and input alleles can be controlled in genome-wide and candidate gene approaches, are a strong tool to identify the novel genetic factors or modifiers that influence the variable penetrance and inheritance patterns of complex diseases like HSCR. In Part 1, an AGH-*Ednrb*^{sl} inbred strain and two congenic strains (LEH-*Ednrb*^{sl} and F344-*Ednrb*^{sl}) with the same mutation were produced. By investigating tone burst-evoked auditory brainstem response (ABR), it was found that this mutation resulted in seriously congenital sensorineural deafness in AGH-*Ednrb*$^{sl/sl}$ rats. A histological examination of the cochleae showed that the stria vascularis was thinner in AGH-*Ednrb*$^{sl/sl}$ rats compared to heterozygous

rats, with none of the other abnormalities. Using these strains, the impact of genetic background was evaluated with respect to the three features caused by the *Ednrb*^{sl} mutation. It was found that the different genetic background markedly changed the aganglionosis, but resulted in only slight changes in hearing loss and pigment disorder. This provided the important evidence, in support of previous studies, that different lineages of neural crest-derived cells migrating along with various pathways are regulated by different signal molecules. In addition, sex bias was found in the penetrance of aganglionosis in the short segment aganglionosis of F344-*Ednrb*$^{sl/sl}$ rats. This is consistent with observation in HSCR patients.

AGH-*Ednrb*$^{sl/sl}$ and F344-*Ednrb*$^{sl/sl}$ rats show large difference in aganglionosis extent. It was concluded that resistant genes in the genetic background of F344 significantly modulated the severity of the aganglionosis phenotype. In Part 2, it is focused on the variation in aganglionosis between individual *Ednrb*^{sl}-mutated rats and QTL analysis was used to identify modifiers that are influencing the aganglionosis aspect of the phenotype. Genome linkage analysis identified one significant QTL on chromosome 2 for the severity of aganglionosis. Moreover, a known HSCR susceptibility gene, *Gdnf*, was found in the QTL interval that suggested a novel non-coding sequence mutation in the *Gdnf* modified the penetrance and severity of the aganglionosis phenotype in *Ednrb*-deficient rats. In Part 3, it was investigated whether the intestine of JF1 mice, which have decreased *Ednrb* expression levels due to the *s* mutation, show neuronal and intestinal malformations such as hypoganglionosis by histochemical staining. It was found that the enteric innervation and neuronal density was impaired along the whole colon in JF1 mice, indicating that the JF1 mouse is also useful animal model for HSCR. In conclusion, three strain rats (AGH-*Ednrb*^{sl}, LEH-*Ednrb*^{sl} and F344-*Ednrb*^{sl}) with the *Ednrb*^{sl} mutation produced in this study and the JF1 mouse are useful animal models providing new knowledge for HSCR.

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Phenotypic analysis of mice with congenital hypothyroidism caused by the *grt* mutation in the tyrosylprotein sulfotransferase 2 (*Tpst2*) gene

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Congenital hypothyroidism (CH) is caused by the insufficient activity of thyroid hormone. If untreated, CH induces irreversible growth delay and mental retardation, and sometimes, accompanies reproductive disorders in both sexes. About 1:3,000–4,000 newborns are affected by CH and it is one of the most popular endocrinological disorders in the world. CH is caused by thyroid dysgenesis (TD) or defects in biosynthesis and secretion of thyroid hormone. About 80–90% of the CH is caused by TD. Both environmental and genetic factors affect the development of TD. To

date, six genes (*TSHR*, *TTF1*, *TTF2*, *PAX8*, *NKX2.5* and *HHEX*) are identified to be TD-associated genes in humans. However, the incomplete penetrance and the variable expression observed in familial cases of CH suggest that TD-induced CH is a genetically heterogeneous disease and there is little information available on the genetic factors involved in thyroid disease.

DW/J-*grt* is a mouse model for TD-associated CH characterized by fetal onset growth delay, lowered tri-iodothyronine (T3) and thyroxine (T4) levels, and thyroid hypoplasia with an autosomal recessive manner. DW/J-*grt* mice have a missense mutation in *Tpst2* gene, which leads to decrease in the activity of tyrosine O-sulfation. TPST2 is one of the enzymes for tyrosine O-sulfation and catalyzes the sulfation of tyrosine 385 of thyroid stimulating hormone receptor (TSHR). Since this modification is indispensable for the activation of TSH signaling, and furthermore, since signal transduction *via* TSHR is a prerequisite for the development and function of thyroid gland, DW-*grt* mice develop CH with TD. In this study, I conducted phenotypic analysis of DW/J-*grt* mice.

In Part 1, by using congenic mice for $Tpst2^{grt}$, effects of the genetic background on TD-associated CH phenotypes were evaluated. The congenic mice were generated on the most standard strain; C57BL/6J (B6) and 129^{+Ter}/SvJcl (129). In contrast to B6-*grt* mice, which showed severe dwarfism similar to DW-*grt* mice, 129-*grt* mice showed no growth retardation. To validate the correlation between the severity of growth delay and thyroidal function, serological and histological analyses were conducted. Although B6-*grt* mice showed severe hypothyroid phenotypes characterized by lowered total T4 levels and thyroid hypoplasia, 129-*grt* mice did not show the hypothyroid phenotypes described above. This evidence demonstrates that 129 strain has tolenrace to CH.

In Part 2, to identify the resistant gene(s) to CH in 129 strain suggested in Part 1, quantitative trait locus (QTL) analysis was performed using backcross progenies between susceptible DW and resistant 129. As a result, QTL analysis identified a major QTL with a highly significant linkage in the distal portion of chromosome (Chr) 2; between microsatellite markers *D2Mit62* and *D2Mit304*, particularly close to *D2Mit255* in both sexes. Furthermore, DW congenic strain mice carrying both a $Tpst2^{grt}$ mutation and the 129-derived *Lrch* (locus for resistance to CH on Chr 2) showed recovery from both growth retardation and thyroid hypoplasia. These results confirmed that the resistant gene to CH in 129-*grt* is involved in thyroid development and locating between 104.0–154.3 Mb on Chr 2.

Besides TD-induced CH, DW-*grt* mice demonstrate decreased fertility in male and lifelong infertility in female. Accumulating evidence demonstrates that hypothyroidism is associated with a broad spectrum of reproductive disorders in many animals including human. On the other hand, follicle stimulating hormone receptor and luteinizing hormone receptor both of which have important roles in female reproduction, are reported to be tyrosine-sulfated, and furthermore, their tyrosine sulfation is suggested to be important for its agonist recognition. In Part 3, to determine whether female infertility in DW-*grt* mice is caused by hypothyroidism or decreased activity in TPST2, reproductive analysis of DW/J-*grt* female mice with thyroid powder treatment was conducted. Thyroid powder feeding not only ameliorated their growth retardation, but also the low weights of reproductive organs. In addition, the recovery from sterility was observed in the thyroid powder treated females. These results indicate that the female infertility in DW-*grt* is caused by CH, not by disorders caused by decreased TPST2 activity.

Through this study, I clarified some phenotypic characteristics of DW/J-*grt* mice. These results not only enhanced the usefulness of DW/J-*grt* as a mouse model for TD-associated CH, but also exploited the way to identify the resistant gene(s) for CH. The identification of tolerant gene(s) against TD-induced CH would lead to more detailed understanding about thyroid organogenesis and thyroid hormone biosynthesis. Moreover, the

discovery of novel modifier gene(s) for CH should lead to the development of treatment strategies for thyroid cell disorders including thyroid tumors and hyperthyroidism, as well as hypothyroidism.

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Development of diagnostic ultrasound and ultrasound contrast agent microbubbles mediated cisplatin delivery in cancer therapy

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Cancer is the leading cause of morbidity and mortality, both in humans and dogs. Cancer therapy still has many problems such as cancer recurrence and metastasis, toxicity and invasiveness of therapy itself. Ultrasound (US) is a popular modality for cancer diagnosis because of its non-invasiveness, convenience, and safety. In addition to cancer diagnosis, US is promising as a "fourth" major therapeutic tool following surgery, chemo- and radio-therapy. The combination of diagnostic US and microbubbles (MBs) has been studied as a drug and gene delivery tool for cancer treatment, although delivery efficiency and the tissue specificity of drugs and genes using diagnostic US is still unsatisfactory. Hence, the principle of this thesis is to establish anticancer drug delivery using diagnostic US as one of the US-mediated cancer therapy.

In Chapter 1, *in vitro* characteristics of cisplatin delivery using diagnostic US were investigated. Canine thyroid adenocarcinoma cells were exposed to diagnostic US in the presence of cisplatin and US contrast agent Sonazoid® MBs. The cytotoxic effect of cisplatin was significantly enhanced by diagnostic US and MBs. However, both high MB and cisplatin concentraion caused undesirable side effects. Further investigation revealed that only a few MB adjacent or attached to cells with short time US exposure efficiently enhanced cytotoxic effect of cisplatin with minimum side effects. Therefore, it may be preferable to bring only a few MBs near every targeted cells and to expose US in a short period.

In Chapter 2, feasibility of an intratumor injection of MBs and cisplatin, concurrent with diagnostic US exposure, was investigated in a xenograft mouse model. Based on the results of Chapter 1, the interaction of MBs with cells was created by an intratumor injection of MBs. Canine adenocarcinoma cells were implanted into the left back of nude mice. Diagnostic US was exposed for 15 sec to the tumor concurrently with an intratumor injection of MBs and cisplatin. The combination of diagnostic US exposure and the intratumor injection of MBs and cisplatin significantly delayed tumor growth, compared with tumors without any treatments; however, cisplatin alone and the combination of cisplatin and US did not delay tumor growth. No treatments reduced the body weight of mice, compared to without any treatment. In addition, the treatment did not influence the appearance of the skin.

Anticancer drug delivery using diagnostic US and an intratumor injection of MBs is promising

for noninvasive and local cancer therapy with minimum side effects. In the future, highly efficient and minimally invasive cancer therapy using diagnostic US may be applied not only in animals but also in human medicine.

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Study on the mechanism of disease modifying osteoarthritis steoarthritis drugs (DMOADs) for canine osteoarthritis

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Osteoarthritis (OA) in dogs is often managed by combinations of therapy, which is mostly symptomatic to relieve pains and is unlikely to affect the progression of the disease. Some of pathological processes of OA could be mediated by novel therapeutic agents to slow or improve the progression of structural changes in OA. These agents, termed disease modifying osteoarthritis drugs (DMOADs), are however under investigated evidence in scientific basis. The objective of this study was to prove some of the agents as new therapeutic ones for canine OA.

In the first part, tepoxalin, which could inhibit both cyclooxygenase (COX) and lipoxygenase (LOX) was examined. Cytotoxic effects of tepoxalin, meloxicam, carprofen and a 5-LOX specific inhibitor, AA-861, on cultured canine synoviocytes were evaluated by MTT colorimetric assay. Induction of apoptosis was detected by morphological observations with Giemsa and annexin-V staining, and by the inhibition of cellular caspase-3 activity with N-Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO). Cytotoxic effects of tepoxalin were evident in comparison with COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs), carprofen or meloxicam. Same tendency of the cytotoxicity of tepoxalin was observed when cells were treated by AA-861. Morphological findings and contradictory effects of Ac-DEVD-CHO to the cytotoxicity proved pro-apoptotic effects of tepoxalin. Results obtained in this part of the study suggested that tepoxalin could control osteoarthritic synovitis by inducing apoptosis on proliferated synoviocytes, while most NSAIDs with selective inhibition of cyclooxygenase-2 would unlikely suppress synovial proliferation.

In the second part of the study, chondroprotective effects of pentosan polysulfate sodium (PPS) were examined. This material has a heparin-like structure and is purified from the plant of European beech wood. Recent years, it was newly recognized that PPS reduce pain and inflammation of OA. The objective of this part was to investigate a mechanism of action of PPS on IL-1β-induced inflammatory reaction of canine cultured articular chondrocytes *in vitro*. By means of western blotting and real-time PCR, it was investigated that inhibitory effects of PPS on matrix metalloproteinase (MMP)-3 gene and protein expression on induced by IL-1β stimulation. As a result, PPS significantly suppressed MMP-3 expression by concentration-dependent manner. Then it was evaluated that effects of PPS on IL-1β-induced phosphorylation of mitogen-actiated protein kinases (MAPKs), such as p38, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) in chondrocytes by western blotting. As a result, in the presence

of PPS, IL-1β-induced phosphorylation of p38 and ERK were certainly inhibited, while JNK phosphorylation was not affected. In chondrocytes, p38 and ERK transmit its information to nuclear factor (NF)-κB, whereas JNK passes to activator protein-1. Then, intracellular localization of NF-κB was investigated by immunofluorescence staining. As a result, PPS suppressed IL-1β-induced nuclear translocation of NF-κB. All the result of this part of the study strongly suggested that PPS treatment can prevent inflammatory intracellular responses induced by IL-1β through inhibition of phosphorylation of certain MAPKs, p38 and ERK and then nuclear translocation of NF-κB in cultured chondrocytes. These PPS properties may contribute to suppressive consequence of catabolic MMP-3 synthesis.

In the third part of the study, it was examined that effects of exogenous hyaluronic acid (HA) on extracellular matrix (ECM) of articular cartilage. On the occasion of OA, ECM tends to lose its quantity, leading degradation of articular cartilage. Moreover, it is suggested that quality of ECM could depend on the molecular weight of endogenous HA from chondrocytes. Thus, promoting endogenous HA synthesis or supplementation of exogenous HA could prevent cartilage degradation. The quality of endogenous HA from chondrocytes could be estimated by investigating transition of 3 types of hyaluronan synthases (HAS), such as HAS1, HAS2 and HAS3. It is known that each HAS generates different molecular weight of HA. It was investigated that effects of exogenous HA on gene expression of three HASs in IL-1β stimulated chondrocytes. Canine cultured articular chondrocytes were incubated with or without different types of HA (500–1,200 or 6,000 kDa). Then cells were stimulated with IL-1β. Thereafter, gene expression of HAS1, HAS2, HAS3, collagen type II, and aggrecan were quantified by real-time PCR. As a result, stimulation of IL-1β reduced HAS2 expression and induced HAS3 expression, and it was suggested that IL-1β stimulation could adjust HAS expression and promote low molecular weight HA synthesis. Gene expressions of collagen type II and aggrecan were decreased by IL-1β stimulation. Pretreatment of exogenous HA (500–1,200 kDa) significantly reduced inflammatory effects of IL-1β, whereas other type of HA (6,000 kDa) showed no effects on IL-1β. From all the results obtained in this part of the study, it was suggested that stimulation of IL-1β on cultured canine chondrocytes may contribute to worsen the quality of endogenous HA generated by HAS through adjustment of HAS gene expression. Exogenous HA, its molecular size 500–1,200 kDa could inhibit IL-1β stimulation and improve quality of endogenous HA.

In conclusion, experimented agents, tepoxalin, PPS, and exogenous HA showed disease modifying effects, such as, reducing synovial proliferation, inhibiting cartilage enzyme production, improving the quality of ECM, respectively. These results could be related to a better understanding of the mechanisms of action of each agent as DMOADs and provide information to clarify the position of the drugs in the multimodal treatment of OA.

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Experimental studies for attracting antibody-secreting cells into the CNS to control neurotropic viral infections

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The induction of an effective immune response, especially antibodies (Abs) production within the central nervous system (CNS) is regarded to be critical to prevent the transneural spread of viruses. Previous studies have been shown that antigen-specific Abs could be induced in cerebrospinal fluid by experimental intrathecal (e.g., intracerebral or subarachnoid) immunization. However, the mechanism of Abs induction has not been well understood. Therefore, CNS humoral immune response, especially antibody secreting cells (ASCs) known as plasma cells were focused in this study.

In the first experiment, the immune responses in both the CNS and lymphoid organs were investigated following intracerebral (IC) immunization against pseudorabies virus (PRV) in mice. IC immunized mice had significantly higher PRV-specific serum Abs and neutralizing Abs titers than subcutaneously (SC) immunized mice. The spleen and cervical lymph nodes of IC immunized mice produced significantly more PRV-specific Abs than that of SC immunized mice. The mRNAs of some chemokines and cytokines were predominantly detected in the brain of IC immunized mice. Further, ASCs were infiltrated in intrathecal region of IC immunized mice, and these mice (86%) survived more than SC immunized mice (33%) by suppression of virus propagation, when PRV was inoculated directly into the brain.

In the second experiment, IC chemokine injection was tried to investigate the utility of the chemokine as an inducer of peripheral ASCs into the CNS. CXCL12 and cocktail chemokine (mixture of CXCL9, 10, 12 and 13) attracted antigen-specific ASCs most strongly, compared to CXCL9, 10 and 13 in *in vitro* chemotaxis assay and *in vivo* IC chemokines injection experiment. CXCL12 and cocktail chemokine injections after intraperitoneal immunization increased the survival rate against IC rabies virus challenge.

In summary, IC immunization induced more effective immune responses against a neurotropic viral infection by attracting ASCs into the CNS that is partly undertaken by chemokine expression, suggesting the potentials of IC immunization and chemokine treatment as preventive or therapeutic tools against the diseases caused by transneural infection of neurotropic viruses.

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The genomic diversity and emergence of neuropathologic avian retrovirus

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Fowl glioma is histologically characterized by multiple nodular gliomatous growths with disseminated nonsuppurative encephalitis. The first case in the world was described in 1935 in Spain and thereafter this neurological disorder sporadically occurred in Europe, South Africa, the United States and Australia. The pathogenesis had long been controversial. In 1995, the first case of fowl glioma in Japan was found in a Japanese native chicken, Japanese bantam (Chabo; *Gallus gallus domesticus*), kept in a zoological garden. A strain of avian leukosis virues, fowl glioma-inducing virus (FGV), belonging to avian leukosis virus subgroup A (ALV-A), was isolated from the affected chickens and this strain has been suggested to be a causal virus of fowl glioma. FGV is a replication-competent and nonacute transforming ALV and is suggested to be a recombinant virus composed of various avian sarcoma/leukosis virus (ASLV) genomes. FGV also induces cerebellar hypoplasia and is suggested as a causal candidate of avian multiple perineurioma. Moreover, FGV has already spread in the flocks of Japanese native chickens in the zoological gardens in Japan. However, it is unclear how and where FGV emerged and whether FGV is currently prevalent in other countries. Additionally, another strain of ALV, TymS_90, has also been reported as a causal ALV of fowl glioma. By the nucleotide sequencing of its genome, TymS_90 is suggested to be a recombinant virus between endogenous vurus (*ev*)-1 and other ASLVs. Thus, to clarify the genome diversities of neuropathogenic ALVs, and how and where FGV appeared in chcikens, histopathological and molecular biological analyses of peripheral nerve sheath tumors (PNSTs) were perfomed and the prevalence of FGV in native chickens in Germany and several Asian countiries were examined.

PNSTs are rare in animals, including avian species, and their etiology remains to be elucidated. In Chapter I, naturally occurring intraneural perineuriomas were pathogenically examined in a Japanese native fowl. A 2-year-old male Japanese native fowl (*Gallus gallus domesticus*) was identified with an inability to feed and torticollis. At a necropsy, there were cylindrical enlargements and yellow discoloration of multiple peripheral nerves, including nerves of the lumbosacral plexus, brachial plexus, and spinal ganglia. On histological examination, these lesions consisted of diffuse proliferations of spindle cells with characteristic onion-bulb structures around residual axons. The spindle cells were immunohistochemically positive for glucose transporter 1 (GLUT1) and negative for S-100 α/β. On the basis of microscopic and immunohistochemical findings, the tumors were diagnosed as multiple perineuriomas. Although no ALV strain was isolated from the neoplasm, a 2.3 kbp fragment equivalent to the *env*-3' long terminal repeat (LTR) region was amplified from the RNA of the affected peripheral nerves. This fragment had 79.8% similarity with that of FGV and 99.9% similarity with *ev*-1. These results suggest that ALVs that are different from FGV may be associated with naturally occurring perineuriomas in the chicken.

In Chapter II, a naturally occurring PNST in a Japanese native fowl was pathologically examined and the strain of ALV isolated from the neoplasm was characterized by molecular biological analysis. A 2-year-old, male, Japanese

native chicken, Japanese tail dragger (Ohiki; *Gallus gallus domesticus*) identified with a 6-months history of lameness. There was a firm subcutaneous mass, 5 × 4 × 3 cm in diameter, in the caudal cervical region. The mass, connected to the adjacent spinal cord (C9–14), was microscopically composed of highly cellular tissue of spindle cells arranged in interlacing bundles, streams, and palisading patterns with Verocay bodies and less cellular tissue with abundant collagen. Immunohistochemically, neoplastic cells were divided into two types: perineurial cells positive for vimentin, GLUT1, and claudin1; and Schwann cells positive for vimentin, occasionally positive for S-100 α/β but negative for GLUT1. Based on these findings, a diagnosis of neurofibrosarcoma was made. The complete nucleotide sequence of an ALV strain, CTS_5371, isolated from the neoplasm was determined and phylogenetic analysis indicated that this strain was a novel recombinant virus from avian sarcoma/leukosis viruses previously reported. Additionally, experimental infection revealed that CTS_5371 induces the proliferation of Schwann cells and perineurial cells in peripheral nerves. These results suggest that this ALV strain has the ability to induce PNSTs in chickens.

FGV and FGV variants have spread to ornamental Japanese fowl, including Japanese bantams in Japan. However, it is unclear how and where FGV emerged and whether FGV is related to the past fowl glioma in foreign countries. In Chapter III, the prevalence of FGV in European, Asian and Japanese native chickens were examined. FGV was not isolated from any chickens in Germany and Asian countries other than Japan. Eighty (26%) out of 307 chickens reared in Japan were positive by FGV-specific nested PCR and 11 FGV variants with an FGV-specific sequence in their 3' untranslated region (3'UTR) were isolated. In addition, 4 other ALVs lacking the FGV-specific sequence were isolated from Japanese bantams affected with fowl glioma and/or cerebellar hypoplasia. These isolates were considered to be distinct recombinant viruses between FGV variants and endogenous/exogenous avian retroviruses. These results suggest that the variants as well as distinct recombinant ALVs are prevalent among Japanese native chickens in Japan and that FGV may have emerged by the recombination among avian retroviruses, including unidentified endogenous viruses, in the chickens of this country.

The present studies demonstrated that several ALVs, which are different from FGV and TymS_90, are associated with the occurrence of PNSTs and a strain of ALV, CTS_5371 could induce neurofibrosarcoma. In addition, these studies suggest that FGV is likely to have appeared in Japanese fowls in Japan without a relationship to the past fowl glioma found in Western countries, and that FGV and FGV vatriants still spread in Japanese folws, showing genome diversity mainly based on the recombination between these strains and avian endogenous viruses.

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Changes in apoptosis-related genes during proliferation and maturation of late-stage erythroblasts from rat bone marrow

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To evaluate the effects of a variety of chemical and physiological stimuli on erythropoiesis, erythroid progenitor cells or pro- and basophilic erythroblasts, which are at the early stage of erythroid development, are commonly used in *in vitro* assays. Meanwhile, polychromatic erythroblast, which is at the late stage of erythroid development, is more susceptible to some chemicals. Therefore, studies using late-stage erythroblasts as well as progenitor cells and early-stage erythroblasts are required to better understand the effects of various stimuli on erythropoiesis. Moreover, Bcl-2 family proteins play important roles in the induction and suppression of apoptosis in the early-stage erythroid cells. The purpose of the present study was to establish procedures for isolation and culture of late-stage erythroblasts from rats, popular experimental animals in toxicological investigations, for the evaluation of the effects of various stimuli, and to investigate the roles of anti-apoptotic Bcl-2 subfamily members in rat late-stage erythroblasts.

Bone marrow cells from 3- and 10-week-old rats were separated by discontinuous density gradient centrifugation. Polychromatic erythroblasts were most highly enriched in the fraction from 3-week-old rats at the density interface between 1.040 g/ml and 1.058 g/ml. When incubated in the presence of 2 U/ml erythropoietin (EPO), polychromatic erythroblasts thus obtained exhibited proliferation and maturation into orthochromatic erythroblasts, whereas incubation without EPO showed maturation with no apparent cell proliferation. The addition of EPO resulted in increased expression levels of Bcl-xL and Mcl-1 mRNAs and concomitant reduction in apoptosis of late-stage erythroblasts, suggesting that Bcl-xL and Mcl-1 are important anti-apoptotic factors in rat late-stage erythroblasts. The cells incubated with succinylacetone, an inhibitor of heme synthesis, showed apoptosis with increased activities of caspase-3 and caspase-9 and reduced expression of Bcl-xL and Mcl-1 mRNAs, indicating that reduced cellular heme contents caused mitochondrial apoptosis via caspase-dependent pathway through reduction in expression of Bcl-xL and Mcl-1. These findings demonstrate that Bcl-xL and Mcl-1 play key roles in survival of rat late-stage erythroblasts through preventing apoptosis, possibly in an EPO- and/or heme-dependent manner.

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Development of an efficient method for the isolation of hantavirus by using Syrian hamsters and establishment of a mouse model for human hantavirus infection

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Puumala virus (PUUV) and other Arvicolinae-borne hantaviruses are difficult to cultivate in cell culture. To isolate these hantaviruses efficiently, hantavirus nucleocapsid protein (NP)-positive but seronegative wild rodents were selected by NP-detection ELISA. Three of 68 *Myodes glareolus* captured in Samara, Russia, were NP-positive and seronegative. Syrian hamsters were inoculated with lung homogenates from NP-positive rodents for virus propagation. Virus isolation *in vitro* was carried out by the inoculation of lung homogenates of NP-positive hamsters to Vero E6 cell monolayers. Two PUUV strains (Samara49/CG/2005 and Samara94/CG/2005) from *M. glareolus* were isolated in Vero E6 cells. Nucleotide and amino acid sequence identities of the S segment of these isolates to those of PUUV F-s808 from a fatal HFRS patient in the Samara region were 96.7–99.3% and 99.3–100.0%, respectively. Morphologic features of Vero E6 cells infected with PUUV strain Samara49/CG/2005 were quite similar to those of Hantaan virus-infected cells. Isolation of Hokkaido virus from *Myodes rufocanus* captured in Hokkaido, Japan, was also performed. Hokkaido virus NP and RNA were recovered and maintained in hamsters. These results suggest that the inoculation of Syrian hamsters with rodent samples is an efficient method for the isolation and maintenance of PUUV and other Arvicolinae-borne hantaviruses.

Hantaan virus (HTNV) is a causative agent of hemorrhagic fever with renal syndrome (HFRS). The pathogenesis of HFRS has not been fully elucidated, mainly due to the lack of a suitable animal model. In laboratory mice, HTNV causes encephalitis. However, this symptom is dissimilar to that of human hantavirus infections. HTNV strain AA57 (isolated from *Apodemus agrarius* in Far East Russia) was found to cause pulmonary disease in 2-week-old ICR mice. The clinical signs of the infected mice were piloerection, trembling, hunching, labored breathing, and body-weight loss. A large volume of pleural effusion was collected from thoracic cavities of the dead mice. Overall, 45% of the mice inoculated with 3,000 focus forming units (FFU) of the virus began to show clinical symptoms at 8 days post-inoculation, and 25% of the inoculated mice died within 3 days of the onset of the disease. The morbidity and mortality rates of the mice inoculated with 30–30,000 FFU of HTNV strain AA57 were roughly equivalent. The highest rates of virus positivity (11/12) and the highest titers of HTNV strain AA57 were detected in the lungs of the dead mice, while lower detection rates and viral titers were found in the heart, kidneys, spleen, and brain. Interstitial pneumonia, perivascular edema, hemorrhage, inflammatory infiltration and vascular failure were observed in the lungs of the sick mice. Hantaviral antigens were detected in the lung endothelial cells of the sick mice. The symptoms and pathology of this mouse model resemble those of hantavirus pulmonary syndrome (HPS) and, to a certain extent, those of HFRS. This is the first report that, in laboratory mice, the HFRS-related hantavirus causes a

HPS-like disease and shares some symptom similarities with HFRS.

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Enhancement of Radiation-induced cell death by nucleoside anticancer drugs

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The combination treatment with radiation and anticancer drug has been widely applied to cancer therapy for the enhancement of cell death. For this combination treatment, DNA damaging agents such as cisplatin and gemcitabine are generally used. Since ionizing radiation was also known to induce DNA damage, radiosensitization in this combination treatment is sometimes an additive but not synergistic effect. On the other hand, the suppression of radioresistance-related proteins by the gene targeting has a synergistic radiosensitizing effect. However, it is difficult to apply this approach in clinical setting because of difficulties in gene transfer method. Previous studies in our laboratory have suggested that down-regulation of radioresistance-related proteins by an inhibitor against RNA synthesis, 1-[3-C-ethynyl-β-D-ribo-pentofuranosyl]cytosine (ECyd), was a potent strategy for enhancement of radiation-induced cell death. However, the precise mechanism underlying radiosensitization induced by RNA synthesis inhibition remains elusive. In this study, I employed two nucleoside anticancer drugs, 8-aminoadenosine (8-NH_2-Ado) and ECyd, which have an ability to inhibit RNA synthesis, and examined whether these drugs could enhance radiation-induced cell death.

In the first experiment, I assessed the effect of the long-term post-treatment with 8-NH_2-Ado after X-irradiation on radiation-induced cell death in human lung carcinoma A549 cells. The combination of 8-NH_2-Ado and X-irradiation significantly induced reproductive cell death as well as apoptosis compared to X-irradiation alone. Furthermore, 8-NH_2-Ado suppressed expression of survivin and XIAP. These results suggested that this suppression of anti-apoptotic proteins by 8-NH_2-Ado contributed to the enhancement of radiation-induced apoptosis. In addition, when peptide inhibitors against caspase-3, -8, -9 were tested to evaluate the involvement of caspases in 8-NH_2-Ado-induced radiosensitization, all inhibitors suppressed the enhancement of radiation-induced apoptosis. This result suggested that 8-NH_2-Ado enhanced radiation-induced apoptosis through not only mitochondria-mediated apoptotic signal pathway but also death receptor-mediated pathway.

In the second experiment, I examined whether the long-term pre-treatment with ECyd before X-irradiation could enhance radiation-induced cell death in A549 cells, human larynx squamous carcinoma HEp-2 cells and Chinese hamster lung fibroblast V79 cells, since radiosensitizing effects by ECyd in previous reports had been observed in the long-term post-treatment after X-irradiation. The long-term pre-treatment of ECyd before X-irradiation also showed significant radiosensitizing effect in these solid tumor cell lines. By using sub-lethal damage repair assay and γ-H2AX foci formation assay, I found that

ECyd suppressed the repair capacity for radiation-induced DNA double-strand breaks. Furthermore, ECyd suppressed the expression of BRCA2 and Rad51, which are known as keys among DNA repair proteins in the homologous recombination (HR) pathway. Therefore, to investigate whether the radiosensitizing effect of ECyd was due to suppression of BRCA2 expression, BRCA2- deficient V-C8 cells was utilized. ECyd did not change radiosensitivity in V-C8 cells, whereas it sensitized parental V79 cells to X-irradiation. These results suggested that ECyd induced down-regulation of BRCA2 and subsequent impairment of HR pathway, resulting in the radiosensitizing effect.

In conclusion, two nucleoside anticancer drugs, 8-NH_2-Ado and ECyd showed significant radiosensitizing effect, indicating that these drugs were effective radiosensitizer in solid tumor cells. Furthermore, these data suggested that modification of radiation-induced signaling pathway and suppression of DNA repair capacity through RNA synthesis inhibition could be a potent approach to improve the therapeutic efficiency of radiotherapy for solid.

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Environmental monitoring of metal pollution in aquatic and terrestrial environment in the Republic of Zambia

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The African continent has experienced rapid economic development during the last decade. Unfortunately, this has also led to an increase in environmental pollution. Among the chemical pollutants, metal contaminants are now a major health hazard in many African countries. Humans and wildlife are exposed to metals in drinking water, air and soil through contamination from anthropogenic activities such as mining and metal smelting.

The Republic of Zambia is rich in mineral resources such as copper (Cu), cobalt (Co), zinc (Zn) and lead (Pb). Mining and smelting of these mineral resources are one of the most important economic activities in Zambia. Unfortunately, this has lead environmental metal contamination in Zambia. Consequently, levels of metal contamination by these activities should be monitored in order to prevent health hazard on humans, aquatic and terrestrial animals since metals are not bio-degraded and long-lasting in the environment. However, ecotoxicological studies on metal contamination in Zambia are scarce. In this thesis, I investigated and clarified metal pollution in both aquatic and terrestrial environment in Zambia as below.

In chapter 1, I measured metal levels in soil and sediment from broad areas of Zambia and clarified the source and characteristics of metal pollution. In Zambia, the major sources of heavy metals are the mining areas, Kabwe and the Copperbelt, and heavy metals are then transported within each area by rivers. Kabwe is highly polluted by Pb, Zn, Cu, Cd and As. The Copperbelt area is highly polluted by Cu and Co. Other sampling sites showed relatively low concentrations

of heavy metals. Furthermore, sources in each area were not only mining but other human activities such as metal industries and combustion of fossil fuels. High concentrations of heavy metals, especially Cu, were found in the aquatic environment in the Copperbelt area. This also affected concentrations in Lake ITT, located 450 km downstream on the Kafue River, and in the Kafue National Park.

In chapter 2, I studied metal pollution in aquatic environment, especially lake sediment, fish and crayfish from two economically important man-made lakes, Lake ITT and Lake Kariba. I observed spatial variation in the accumulation of heavy metals between Lake ITT and Lake Kariba. The sediments and the herbivorous teleost *O. niloticus* accumulated very high concentrations of Cu in Lake ITT, located 450 km downstream of a mining area. This is most likely due to the discharge of Cu waste from the Copperbelt region. Conversely, the aquatic species that I sampled from Lake Kariba had higher concentrations of Cr, Ni, and Pb. I believe that the contaminants in this lake were derived from the use of leaded petrol and Cr-Ni compounds in anti-fouling marine paints. I also noted significant differences in the accumulation of heavy metals among the three species (*O. niloticus*, *S. thumbergi* and *C. quadricarinatus*) that are likely related to their feeding ecology, physiology, or metal sensitivity. The coefficient of condition (*K*) was negatively correlated with the Ni concentration in the hepatopancreas of the crayfish, suggesting that this species is sensitive to Ni toxicity. Interestingly, differences in BSAF rankings (Cu, Zn, and Cd) relative to the general order were observed in both *O. niloticus* and *C. quadricarinatus*.

In chapter 3, I extended my study to reveal source of metal pollution in Lake ITT and Cu accumulation profiles in fish species, especially in *Oreochromis spp.* Results of GIS analysis in the lake sediment suggests that the northern part of the lake, probably the Copperbelt mining area, could be the source of Cu pollution in Lake ITT. Diet may not be the reason for high Cu accumulation in *Oreochromis spp*. Results from both field and laboratory studies imply that *Oreochromis spp*. contain high concentrations of Cu under normal physiological conditions.

In chapter 4, I investigated metal pollution in terrestrial environment using roadside soil and wild rat from extensive Pb-Zn mine, Kabwe. High concentrations of Pb, Zn, Cu, Cd and As were found in soil samples taken near Kabwe, Zambia. The source of this metal pollution was historical mining activity at the now abandoned Kabwe Pb-Zn mine. The south area of the mine had particularly high contamination levels when compared with other areas around the mine. Wild rats from Kabwe had significantly higher tissue concentrations of Pb than those from Lusaka. Interestingly, body weight and renal Pb level were negatively correlated. These results suggest that pollution due to historical mining might affect terrestrial animals in Kabwe. Further investigation of metal pollution in animals and humans in Kabwe is required.

In chapter 5, I extended the study of chapter 4 and also focused on another extensive mine in Chingola. I found that wild black rats (*R. rattus* and *R. tanezumi*) from two mining areas in Zambia, Kabwe, and Chingola accumulated metals that had likely originated from mining activities. Although concentrations of metals were lower than the levels that cause toxicological effects on rats, the accumulated metal levels caused biological responses such as MT induction in the livers and kidneys. I also observed that HO-1 mRNA expression levels could be a marker for Cr exposure.

In chapter 6, I studied metal accumulation profiles in wild hippopotami because of their unique lifestyle that use both terrestrial and aquatic environment. Metal concentrations in liver from hippopotami from the South Luangwa National Park in Zambia were generally low and similar to an earlier study. These results may provide standard physiological metal levels in hippopotami inhabiting non-contaminated areas. Higher concentrations of Cd and Hg in hippopotami

than in the surrounding environment suggests that these metals can bio-accumulate in hippopotami.

In conclusion, I clarified the current circumstances of metal pollution in both the aquatic and terrestrial environment in Zambia. My work will strongly support the countermeasure for metal pollution in Zambia and provide the basic data in African countries where metal pollution has been recently expanding.

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Studies on the metabolic mechanisms of fat accumulation before hibernation in the Japanese black bear (*Ursus thibetanus japonicus*)

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The Japanese black bear (*Ursus thibetanus japonicus*) undergoes cycles in body mass, food consumption, and reproduction. Although the accumulation of body fat is important for survival and reproduction, the metabolic mechanisms regulating circannual changes in body mass of bears have yet to be elucidated. The purpose of this study is to elucidate the fat storing mechanism in the pre-hibernation period.

First, we focused on the morphological changes, with special emphasis on lipid and glycogen accumulation in the liver of bears during the active season. Histologically, swollen hepatocytes with pale cytoplasm were observed. A periodic acid Schiff stain with and without amylase digestion showed extensive glycogen deposition. In wild bears, various degrees of glycogen accumulation were observed, suggesting variation in feeding. In captive bears, moderate to marked accumulation of glycogen in the liver was observed throughout the active season, suggesting good nutrition. The amount of glycogen in the liver in wild and captive bears tended to increase in November. In contrast, lipid accumulation in the liver was not observed in all bears, suggesting that fattening in bears is entirely different from morbid obesity in humans and other mammals.

Second, to establish an anesthetic protocol which is safe and effective and has minimal effects on blood biochemical values and intravenous glucose tolerance tests (IVGTTs) in bears, we evaluated the safety and efficacy of four combinations of anesthetics: TZN (Zoletil: TZ with no premedication), TZA (TZ and acepromazine), TZB (TZ and butorphanol), and TZM (TZ and medetomidine). As a result, induction and maintenance of anesthetic state were safely achieved with little adverse effect on cardiopulmonary function for each of the four combinations of anesthetics. However, proper care of hypothermia induced by anesthesia is necessary in cold environments. The results in this study indicated that administration of TZN, TZA, and TZB have little effect on basal blood biochemical values, and the results of the IVGTT. Thus, we conclude that these three combinations of anesthetics can be used as methods of

chemical immobilization to perform the IVGTT in Japanese black bears. In contrast, medetomidine, which has hyperglycemic and hypoinsulinemic effects, is inappropriate as a premedication in bears when investigating glucose metabolism controlled by insulin.

Finally, we hypothesized that bears have a metabolic mechanism that efficiently converts carbohydrates into body fat by altering insulin sensitivity during the hyperphagic stage before hibernation. To test this hypothesis, we investigated the changes in blood biochemical values and glucose and insulin responses to IVGTT during the active season. Basal triglyceride concentrations were noticed to decrease significantly with increase in body mass. The IVGTT demonstrated increased peripheral insulin sensitivity and glucose tolerance in early November. In contrast, peripheral insulin resistance was indicated, in late Novemver, by an exaggerated insulin response. Our findings suggest that bears have a lipogenic-predominant metabolism and accelerated glucose uptake from increasing peripheral insulin sensitivity during the hyperphagic stage. We also investigated the changes in peripheral insulin sensitivity by the insulin tolerance test. Although there were no significant changes in the glucose disappearance rate among months, the insulin sensitivity tended to increase in early November.

In summary, in this study, we established an anesthetic protocol which has minimal effects on glucose and lipid metabolism in bears, and we suggested that bears have a unique metabolic mechanism of fat accumulation before hibernation as follows. Bears have a lipogenic-predominant metabolism during the hyperphagic stage. It is thought that bears accelerate glucose uptake in peripheral tissues as well as glycogen and lipid syntheses in the liver by increasing peripheral insulin sensitivity during the hyperphagic stage. In addition, the significant decrease in plasma TG concentration with increase in body mass suggested the facilitation of lipids uptake and deposition in the white adipose tissue in bears. We speculate that bears accumulate much body fat by controlling a series of carbohydrate and lipid metabolism in the whole body, without presenting abnormalities. Our findings will contribute to further development of the research on glucose and lipid metabolism in bears.

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Studies on the mutations causing fluoroquinolone resistance in *Mycobacterium tuberculosis*

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Tuberculosis (TB) is a chronic human infectious disease, which remains a major public-health problem. TB is estimated to affect approximately one-third of the world's population, and 95% of cases occur in developing countries. Current estimates show that approximately ten million new cases and nearly 1.4 million deaths from TB occur each year, and TB remains a major cause of premature death. TB control in some regions is jeopardized by the human immunodeficiency

virus (HIV)/ acquired immune deficiency syndrome (AIDS). TB typically affects the lungs (pulmonary TB), but can affect other sites as well (extra pulmonary TB). The causative agent of TB, *Mycobacterium tuberculosis*, is spread usually from one person to another by breathing infected air by coughs, laughs, sings, or sneezes during close contact. In general, a relatively small proportion (5 to 10 %) of people infected with *M. tuberculosis* will go on to develop TB; however, the probability of developing TB is much higher among people infected with HIV/AIDS. TB can remain in an inactive (dormant) state for years without causing symptoms or spreading to other people. When the immune system of a patient with dormant TB becomes weakened, the TB can become active (reactivated) and cause infection in the lungs or other parts of the body.

Without treatment, mortality rates from TB are high (about 40 % deaths). Thus, active TB is treated with isoniazide (INH) in combination with one or more of first line anti-TB drugs, including rifampin (RIF), ethambutol (EMB), pyrazinamide (PZA), and streptomycin. This drastically reduced the mortality rates. Indeed, the advent of RIF in the early 1970s permitted a drastic reduction in the duration of therapy to six months while the efficacy of treatment improved. However, those familiar with drug resistance in general have predicted the emergence of resistant *M. tuberculosis* to first line anti-TB drugs and the most countries participating in a global survey of anti-TB drug resistance registered cases of multidrug-resistant (MDR)-TB (TB resistant to more than two anti-TB drugs, including RIF and INH) in the mid-1990s. A recently published World Health Organization (WHO) report reviewing the global status of tuberculosis has pointed to an increasing incidence of drug-resistant tuberculosis.

The increased incidence of MDR-TB has hampered the treatment and control of TB and is associated with an increase in mortality rates in people with TB. Consequently, the required drug dosage and new drugs for the treatment of TB have dramatically increased, and fluoroquinolones (FQs) are now considered to be important second-line anti-TB agents.

FQs are a large and widely used class of synthetic antibacterial agents, which are frequently used in treating patients infected with MDR-TB. The target of the FQs is type II Topoisomerases that transiently cleaves and unwinds double-stranded DNA to catalyze the negative supercoiling of DNA and is thus essential for efficient DNA replication, transcription, and recombination. Most of eubacteria, such as *Escherichia coli*, have two type II DNA topoisomerases, DNA gyrase and topoisomerase IV. In contrast, a few bacteria such as *M. tuberculosis* have only DNA gyrase, which is therefore the sole target of fluoroquinolones. DNA gyrase consists of two subunits, GyrA and GyrB, which form the catalytically active GyrA2GyrB2 heterotetrameric structures. Amino acid substitutions in DNA gyrase subunits have been reported in FQ resistant *M. tuberculosis* clinical isolates; however, acquisition mechanism of FQ resistance in these bacteria is not fully understood.

This present thesis consists of two chapters; in the chapter I, I have elucidated the contribution of an amino acid substitution located at position 540 in GyrB by in vitro DNA supercoiling and cleavage assays in the presence or absence of FQs. I have proposed the model of interaction between substituents of FQs and amino acid residues in the GyrB. In chapter II, in order to elucidate the influence of a lineage specific amino acid dimorphism in GyrA, threonine or serine, at position 95 of GyrA on the acquisition of FQ resistance, I conducted in vitro DNA supercoiling inhibition assay utilizing recombinant DNA gyrases with GyrA-Ser95 and its derivatives.

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West Nile virus causes accumulation of ubiquitinated proteins in neuronal cells

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West Nile virus (WNV) belongs to the *Flaviviridae* family of viruses and has emerged as a significant cause of viral encephalitis in humans, animals, and birds. It has been reported that WNV replication directly induces neuronal injury, followed by neuronal cell death proven as apoptosis. Therefore, it is important to understand the mechanism of neuronal apoptosis caused by this virus to develop strategies to control its pathogenicity. Accumulation of ubiquitinated proteins has been reported to be associated with neuronal apoptosis in some pathological conditions. A lot of cellular stresses prevent cellular protein quality control mechanisms, resulting in the accumulation of ubiquitinated proteins. To obtain a better understanding of the mechanisms of WNV-induced neuronal apoptosis, I evaluated the accumulation of ubiquitinated proteins in the WNV-infected neuronal cells. I have observed that WNV infection caused massive neuronal injury in the brain of mice. Viral antigen was detected in the neuronal cytoplasm of the cells exhibiting neuronal apoptosis. Notably, ubiquitinated proteins were detected in WNV-infected neuronal cells. In addition, accumulation of ubiquitinated proteins was markedly enhanced in mouse neuroblastoma, Neuro-2a cells after WNV infection. My histopathological and *in vitro* studies suggest that accumulation of ubiquitinated proteins in neuronal cells might be associated with the neuronal apoptosis caused by WNV infection.

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Effects of the antitumor nucleobase analogue trifluorothymidine on fragmentation, replication and repair of cellular DNA

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Fluorouracil (5FU) is an antitumor nucleobase analogue used widely around the world, that acts by inhibiting thymidylate synthase (TS). However, one of the problems associated with the clinical application of this drug is that it does not work effectively in some patients because of high TS activity in the tumor. Recently, the novel antitumor drug TAS-102 was developed, that has been shown

to exhibit antitumor activity on 5FU-resistant tumors. TAS-102 consists of two components: trifluorothymidine (TFT), a nucleobase analogue, and an inhibitor of thymidine phosphorylase that catalyzes the biodegradation of TFT. The strong antitumor effect of TAS-102 has been suggested to be due to the incorporation of TFT into DNA resulting in single-strand breaks of DNA, rather than as being due to TS inhibition, although the precise mechanism remains poorly elucidated. To clarify the mechanism of action of TFT, I examined the effects of TFT on the fragmentation, replication and repair of cellular DNA.

The antitumor effect of TAS-102 could be reproduced when it was given orally for 14 days to tumor-bearing mice implanted with CO-3 colon cancer cells; similar antitumor effect was observed following oral administration of the 5FU analogue, Capecitabine. Although both the drugs induced DNA damage in the cancer xenograft, as judged by the increased number of ring-open aldehyde forms at apurinic/apyrimidinic (AP) sites, TAS-102, but not Capecitabine, caused double-strand breaks of the DNA. Treatment of HeLa cells derived from human cervical cancer with TFT was found to have induced DNA damage-dependent phosphorylation of ataxia-telangiectasia and Rad3-related protein (ATR) and its down-stream cell-cycle controlling checkpoint kinase 1 (chk1) by 24 h after the treatment. Treatment with TFT was also found to have induced DNA double-strand break-dependent phosphorylation of ataxia-telangiectasia mutated (ATM) and its down-stream effector, breast cancer susceptibility gene 2 (BRCA2), known to be involved in homologous recombination, by 48 h after the treatment. In addition, treatment with TFT was found to have doubled the number of AP sites by 72 h after the treatment. These results suggest that TFT exerts beneficial antitumor effects by mechanisms different from those of 5FU or its analogues, and that TFT causes double-strand breaks of DNA.

Treatment of HeLa cells with TFT also induced its incorporation into the DNA, and the rate of incorporation was 62.22 pmol/1 $\times$ 106 cells/ 4 h. This rate was about the half of that of thymidine incorporation, but 300-fold faster than that of 5FU incorporation. In vitro DNA extension assay with DNA polymerase α revealed that TFT tri-phosphate was also incorporated into DNA like thymidine-5'-triphosphate (dTTP), but hardly competed with dTTP. Moreover, after development of the method for synthesis of single-strand oligonucleotides containing TFT-monophosphate with combination of DNA polymerase and ligase reactions, the DNA extension assay showed that the DNA was synthesized against DNA containing TFT-monophosphate. These results suggest that TFT metabolites are effectively incorporated into DNA at the T-site by DNA polymerase α, and that the synthesized DNA containing TFT-monophosphate could be a template DNA.

Next, a DNA glycosylase assay was performed to evaluate the role of the incision repair pathway in the antitumor action of TFT. As expected, uracil-DNA glycosylase (UDG) and single-stranded selective monofunctional uracil-DNA glycosylase 1 (SMUG1) excised DNA containing 5FU, paired to either adenine or guanine. In addition, thymine-DNA glycosylase (TDG) and methyl CpG-binding domain (MBD4) excised DNA containing 5FU paired to guanine. However, none of the enzymes showed detectable incision of DNA containing TFT paired to adenine, and TDG and MBD4 very slowly excised DNA containing TFT paired to guanine. Comparison of the cytocidal actions of TFT on normal, TDG- and MBD4-depleted HeLa cells, revealed no significant differences among the cell types. In contrast, TDG- and MBD4-depleted HeLa cells were less sensitive to 5-fluoro-2'-deoxyuridine, an analogue of 5FU, than control cells. These results suggest that DNA containing TFT is relatively resistant to incision by DNA glycosylase enzymes and that such incision might not be involved in the cytocidal action of TFT.

In summary, I demonstrated that the antitumor nucleobase-analogue TFT is incorporated well into cellular DNA, probably by DNA polymerase α, and that DNA containing TFT is relatively resistant to incision by a number of DNA glycosylases and possibly serves as a template for DNA replication. Although the precise cytocidal mechanisms of TFT are still obscure, the present results suggest that accumulation of TFT in the cellular DNA is important for the cytocidal actions of this nucleobase analogue. As there are differences in the DNA incorporation and incision repair pathways between TFT and 5FU analogues, drugs containing TFT, such as TAS-102, may offer great promise for chemotherapy of patients with tumors resistant to ordinary treatments.

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In vivo pharmacodynamic studies of carbapenem antibiotics against *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*

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For antimicrobial agents, PK-PD analysis has become a standard method to predict clinical efficacies. Although the method is basically established, there still remain difficulties to evaluate the PK-PD index required for efficacy and its target value properly when half-lives of the drugs in mice are much shorter than those in humans such as carbapenem antibiotics. To solve the issue, the author used cilastatin to obtain a longer half-life by inhibition of murine dehydropeptidase-I, a degradation enzyme of carbapenems and also chose the appropriate range of administration doses which can obtain an adequate amount of T $>$ MIC at each administration. Through the application of these approaches, it was demonstrated that the efficacy of tomopenem is driven by the T $>$ MIC and the magnitude necessary for a static effect against *P. aeruginosa* is similar to that of MEM. It was also revealed that the magnitudes required for the efficacy against both *P. aeruginosa* and MRSA were almost the same.

To estimate clinical efficacies more in detail, the author evaluated *in vivo* efficacy of tomopenem by simulating human-exposures in mice. The pharmacokinetics in mice were considered to be well simulated for approximating the *f*%T $>$ MIC observed in humans. The *in vivo* efficacy of human-simulated exposures of MEM at 1,000 mg TID showed bacteriostatic or bactericidal effects against almost all strains of *P. aeruginosa* with MICs $\leq$ 4 μg/ml. As the results of MEM correlated with the breakpoint defined by CLSI, this model is considered to be a valuable method to predict clinical efficacy in humans. In this model, tomopenem is expected to be effective at 750 mg TID against *P. aeruginosa* and MRSA strains with MICs of $\leq$ 8 μg/ml and at 1,500 mg against *P. aeruginosa* and MRSA strains with MICs of $\leq$ 16 μg/ml.

Although the dosing regimens were significantly different between the two studies, 4

to 90 mg/kg/dose and 50 to 800 mg/kg/dose, the efficacy of tomopenem was correlated with $f\%T > MIC$ and a bacteriostatic effect was achieved with 30–40 $f\%T > MIC$ against *P. aeruginosa* in both studies. These results demonstrated that $T > MIC$ should be the major factor for the efficacy of tomopenem and C_{max}/MIC and AUC/MIC cause little impact on the efficacy of tomopenem. As antimicrobial agents are generally thought not to interact with host, the results from PK-PD analysis evaluated with the appropriate dosing regimen in the murine thigh infection model are thought to correlate with clinical efficacies in humans even when dosing regimen and PK profiles of compounds in mice are different from those in humans. From these results, the author concluded that clinical efficacies in humans can be predicted by *in vivo* pharmacodynamic studies.

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