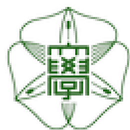

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INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on December 25, 2013 to 5 recipients.
The titles of theses and other information are as follows:

Studies on Immunoinhibitory Receptor and Viral Antigen Expression in Bovine Leukemia Virus Infection

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Bovine leukemia virus (BLV) induces B cell lymphoma named bovine leukosis in a subset of BLV-infected (BLV^{+}) cattle. In BLV^{+} cattle before the development of bovine leukosis, abnormally proliferating B cells induce immunoinhibition, resulting in economic loss and disease progression. No effective vaccine has been developed against BLV although the number of BLV^{+} cattle is increased. In this study, it is hypothesized that BLV-induced immunoinhibition is induced by the programmed-death-1 (PD-1) and PD-ligand-1 (PD-L1) system which is involved in the exhaustion of immune cells. To develop a novel therapy for BLV infection, expressions and function of PD-1 and PD-L1 were analyzed in BLV^{+} cattle. In addition, to clarify the mechanism of BLV antigen expression and the development of leukosis, a marker discriminating BLV-expressing from -silencing cells was identified and cellular functions of both of the B cell subsets were investigated in BLV^{+} cattle.

CHAPTER I: PD-L1 expression in B cells was investigated in BLV^{+} cattle by flow cytometry. Previous reports showed that PD-L1 was involved in immune evasion of infectious agents and tumors. In BLV infection, high frequency of PD-L1^{+} B cells was observed in blood collected from BLV^{+} cattle at the late stage of the infection and cattle with bovine leukosis. The increase in the proportions of PD-L1^{+} B cells in lymphocytes was positively correlated with disease progression and negatively with *interferon-gamma* (*IFN-γ*) mRNA expression.

CHAPTER II: The effect of PD-L1 cross-linking on cellular death was investigated using PD-L1-expressing cell lines and bovine lymphocytes. Inhibition of the interaction between PD-1 and PD-L1 by specific antibodies or recombinant proteins is known to reactivate various functions of exhausted T cells. The treatments with anti-PD-1 or anti-PD-L1 antibody are now undergoing on the clinical application for refractory infection and cancer. However, some researchers reported that cross-linking of PD-L1 induced an inhibitory signal into PD-L1^{+} cells, although details on the mechanism are unknown. In Chapter II, PD-1-Ig, which is the recombinant soluble bovine PD-1 fused with bovine IgG Fc, and anti-bovine PD-L1 mAb were used to analyze the influence of PD-L1 cross-linking on cellular functions. The treatment of PD-1-Ig or anti-PD-L1 mAb led to increased frequency of dead cells in PD-L1^{high} cells among PD-L1-expressing cell lines. When bovine lymphocytes were incubated with anti-PD-L1 mAb, cytokine production was augmented. Meanwhile, PD-1-Ig treatment upregulated the population of dead cells in PD-L1^{+} B cells, and reduced both cytokine production and cell proliferation in bovine lymphocytes.

CHAPTER III: Anti-bovine PD-1 mAb was

established to investigate PD-1 expression and the effect of PD-1 blockade on T cell re-activation in BLV infection. PD-1 is expressed on the surface of T cells and interacts with PD-L1, resulting in T cell exhaustion. The high level of PD-1 expression is observed in pathogen-specific T cells in chronic infection and tumor. In this chapter, it was observed that high frequency of T cells expressed PD-1 in BLV^+ cattle with bovine leukosis. *In vitro* PD-1 blockade by anti-PD-1 mAb increased IFN-γ production and cell proliferation, whereas decreased B cell activation and expression of BLV-gp51 in lymphocytes from BLV^+ cattle.

CHAPTER IV: The differences in BLV antigen expressions, gene expression profiles and cellular behavior were investigated in IgM^{high} and IgM^{low} B cells isolated from BLV^+ cattle. BLV particles and antigens are rarely detected in freshly isolated lymphocytes from BLV^+ cattle, although, viral genome is inserted into host genome as provirus. Meanwhile, the subpopulation of B cells can express BLV antigens after *ex vivo* cultivation, although the molecular mechanism for this expression remains unknown. In this chapter, it was observed that IgM^{high} B cells were increased in number in blood from BLV^+ cattle, and were prone to express BLV antigens as compared to IgM^{low} B cells. To identify the cellular behavior of IgM^{high} and IgM^{low} B cells, several parameters in these two subsets were investigated. IgM^{low} B cells showed higher expression levels of the *Tax*/*Rex* mRNA, PD-L1 and some proto oncogenes (e. g. *Maf*, *Jun* and *Fos*). These data indicated that IgM^{low} B cells are superior to evade from immune surveillance system and to become neoplastic clones.

In conclusion, this study suggests that IgM^{high} B cells are prone to express BLV antigens and IgM^{low} B cells contribute to the immune evasion and the production of neoplastic clones. This observation is valuable to clarify the mechanisms of BLV expansion *in vivo* and transformation by BLV. In addition, this study also clarified that the PD-1/PD-L1 pathway is involved in BLV-induced immunosuppression and disease progression. Both anti-bovine PD-1 and anti-bovine PD-L1 mAbs established in this study are suitable drug candidates to reactivate immune function in BLV^+ cattle.

The original papers of this thesis appeared in *Vet. Res.*, **42**: 103 (2011) and **44**: 59 (2013).

The studies on establishment of molecular targeted diagnosis and therapy based on expression kinetic analysis of canine histiocytic sarcoma-related factor

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Canine histiocytic sarcoma (or CHS) is an aggressive malignant neoplasm originating from dendritic cells and macrophages. Definitive diagnosis of CHS is relatively difficult by conventional histopathological examination because objective features of CHS are not well-defined. In addition, CHS is characterized by progressive local infiltration and a very high metastatic potential. In this study, it was tried to establish a novel diagnostic technique and therapeutic

approach for improvement of the clinical outcome.

For the establishment of genetic diagnostic technique, MHC class IIα, CD11b, CD11c, and CD86 as specific cellular surface antigens of CHS were selected, and mRNA expression levels of these surface antigens were comparatively analyzed between CHS cases and non-CHS cases. Specimens were collected from dogs with CHS (hereafter referred to as HS dogs) (n = 30) and non-HS dogs (n = 36), including lymphoma (n = 4), other malignant tumors (n = 26) and inflammatory diseases (n = 6), by excisional biopsy or needle biopsy. To determine the efficacy of the CHS detection, mRNA expression levels of the selected 4 surface antigens were quantitatively analyzed using real-time quantitative polymerase chain reaction (qPCR). The mRNA expression levels of each surface antigen in HS dogs were significantly higher than those in non-HS dogs ($p < 0.01$). Cutoff values for discriminating CHS from all diseases were calculated on the basis of receiver operating characteristic analysis. The diagnostic accuracy of these cutoff values was > 84%. The accuracy for lymphoma was the lowest in all non-HS dogs. These results suggest that quantitative analysis of mRNA expression of the selected surface antigen could be a highly reliable adjunctive diagnostic technique for CHS. However, substantial investigation is required for the exclusion of diseases with similar cell types of origin to lymphoma.

Survivin is the inhibitory factor of apoptosis that has major functions in cell proliferation, including inhibition of apoptosis and regulation of cell division. Survivin is expressed in most types of human and canine malignant neoplasms. In this study for the therapeutic approach, to investigate the hypothesis that survivin may be potential target of effective treatment for CHS, the relation between survivin expression and aggressive biological behavior of CHS was researched. Firstly, expression levels of selected 7 apoptosis-inhibitory genes, including survivin, in specimens of 30 HS dogs were comparatively analyzed, and the correlation between survivin expression and these clinical outcomes, including survival time, freedom from progression and lomustine (CCNU)-resistance were determined. Next, the effect on biological activities, including apoptosis, cell growth, chemoresistance, and phagocytic activities, through inhibition of the expression of survivin, including survivin siRNA and YM155 as a small molecule of survivin inhibitor, in 4 CHS cell lines and normal canine fibroblasts were evaluated. Annexin V staining for apoptosis, methylthiazole tetrazolium assays for cell viability, CCNU- and doxorubicin (DOX)-sensitivity, qPCR for expression analysis of chemoresistnce genes, including MGMT, ABCB1 and ABCC2, and latex beads assay for measurement of phagocytic activities were performed, respectively. Lastly, the effect of anti-tumor and CCNU-sensitivity by YM155 against 3 different types of murine xenograft models, which established from CHS cell lines with CCNU-resistance, were evaluated. The expression levels of the surviving gene in 30 HS dogs were significantly higher than those of the other 6 apoptosis-inhibitory genes. Enhanced expression of the survivin gene expression decreased CCNU-sensitivity of HS dogs, resulting in shorter freedom from tumor progression and survival time. After the transfection with survivin siRNA, apoptosis, cell growth inhibition, enhanced CCNU- and DOX-sensitivity, decrease of 3 chemoresistance genes expression levels, and weakened phagocytic activities were observed in all CHS cell lines. In contrast, fibroblasts were not significantly affected by survivin knockdown. After the treatment with YM155, these changes were observed in 2 out of the 4 HS cell lines. In contrast, fibroblasts and other 2 HS cell lines were not significantly affected by survivin knockdown. However, YM155 significantly suppresses tumor growth without abnormal behavior against murine all models of CHS. In addition, the combination of YM155 with CCNU increased CCNU-efficacy against murine models with CCNU-resistance by the induction of chemoresistance genes knockdown. These results suggested that survivin might

control aggressive biological activities of CHS, and enhanced survivin expression might induce poor prognosis and therapeutic resistance. In contrast, survivin suppression induced inhibitory biological activities, including anti-tumor effects and enhanced chemosensitivities, in CHS. In conclusion, survivin may be a potential target of effective treatment for CHS regardless of chemoresistance.

It were tried to establish technique of genetic diagnosis and molecular targeted therapy on the basis of expression kinetic analysis of multiple CHS-related factors. This study has demonstrated that genetic analysis of selected surface antigens could detect CHS with high diagnostic accuracy. Furthermore, it has showed that survivin inhibition using YM155 induced tumor growth inhibition and weakened chemoresistance against murine xenograft models of CHS, followed by a potential target of effective treatment. These findings might indicate the useful preclinical evaluation of CHS. There remains substantial investigation for improvement of diagnostic accuracy, or practical advancement of survivin-targeted therapeutics, including safety evaluation for clinical cases and synergistic effect by combinaton of other chemocherapy or radiotherapy.

The original papers of this thesis appeared in *J. Vet. Intern. Med.* **28**: 204–210 (2014) and *PLoS One* **8**: e79810 (2013).

Identification and characterization of an amiloride-sensitive Na^+ conductance and a hyperpolarization-activated, inwardly rectifying Cl^- conductance endogenously expressed in surface cells of rat rectal colon

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Surface epithelium of mucosa contributes to salt and water absorption of mammalian distal colon. Transepithelial Na^+ absorption in the surface epithelium is mediated by electrogenic and/or electroneutral transport systems, although the underlying mechanism of the absorptive process depends upon species, segments, and electrolyte and water balance of the body. The electrogenic Na^+ absorption involves the two-step process: Na^+ influx across the apical membrane followed by Na^+ efflux across the basolateral membrane via Na^+-K^+-ATPase. The apical Na^+ influx is believed to be mediated by the amiloride-sensitive epithelial Na^+ channel (ENaC), which is composed of α-, β-, and γ-ENaC subunits. Although these subunits are expressed at the mRNA and protein levels in the surface cells of the mammalian distal colon, basic electrophysiological properties of amiloride-sensitive Na^+ channels expressed in these cells remains unknown at the cellular level, and thus functional evidence for the involvement of the subunits in the native channels is incomplete. Therefore, the aim of the first chapter of the thesis was to identify and characterize an amiloride-sensitive Na^+ conductance endogenously expressed in surface cells of rectal colon (RC) of rats fed a normal Na^+ diet. In Ussing chamber experiments, apical amiloride inhibited a basal short-circuit

current (I_{sc}) in mucosal preparation of RC in a concentration-dependent manner with an apparent half-inhibition constant (K_i) value of 0.20 μM. Consistent with the results, whole-cell patch-clamp experiments in surface cells of intact crypts acutely isolated from rectal mucosa identified an inward cationic conductance, which was reversibly inhibited by amiloride with a K_i value of 0.12 μM at a membrane potential of −64 mV. Amiloride block of the current was weakly voltage-dependent, yielding an electrical distance (δ) of 0.24 sensed by the blocker. Ion selectivity sequence of the currents based on conductance ratios was Li^+ (1.9) > Na^+ (1) >> K^+ (≈ 0), respectively. Amiloride-sensitive current amplitude was saturated upon increasing extracellular Na^+ concentration, an apparent K_m value being likely << 15 mM at −60 mV. These results together have provided the first electrophysiological characterization of whole-cell currents most likely mediated by amiloride-sensitive Na^+ channels endogenously expressed in surface cells of RC of rats. Electrophysiological properties of the native Na^+ conductance matches those reported for a heterooligomer composed of α-, β-, and γ-ENaC in heterologous expression systems, supporting the hypothesis that these subunits may be the molecular basis of the native channels, which are responsible for amiloride-sensitive electrogenic Na^+ absorption in the mammalian distal colon.

In mammalian distal colonic surface epithelium, NaCl absorption driven by electrogenic Na^+ influx via luminal ENaC or electroneutral one via luminal Na^+/H^+ and Cl^-/HCO_3^- exchangers is believed to require the participation of some chloride transporting proteins including Cl^- channels in the basolateral membrane. ClC-2, a member of the voltage-gated Cl^- channel family is expressed in the distal colonic surface epithelial cells of various species, but its functional significance remains unclear. In the second chapter of the thesis, using electrophysiological techniques I have identified and characterized a ClC-2-like conductance naturally expressed by surface epithelial cells acutely dissociated from rectal colon of rats fed a standard diet. In whole-cell patch-clamp experiments, the surface cells, whether an amiloride-sensitive Na^+ conductance was present or not, exhibited a strong hyperpolarization-activated, inwardly rectifying Cl^- conductance. Electrophysiological properties of the native Cl^- current including voltage-dependent activation, anion selectivity sequence and Zn^{2+} sensitivity resembled those reported for recombinant rat ClC-2 (rClC-2) currents. Cell-attached patch recordings on the surface cells revealed that native ClC-2-like currents activated only at potentials at least 40 mV more negative than resting membrane potentials. In Ussing chamber experiments with rat rectal mucosa, either basolateral or apical application of Zn^{2+} (100 μM), which inhibited native ClC-2-like currents had little, if any, effects on basal amiloride-sensitive short-circuit current. These results not only demonstrate that a functional ClC-2-type Cl^- channel is expressed in rat rectal surface epithelium, but also suggest that the channel activity may be negligible and thus nonessential for controlling electrogenic Na^+ transport in this surface epithelium under basal physiological conditions. It is tempting to speculate that the native ClC-2-like channels might be activated under other physiological and pathophysiological conditions.

The original paper of this thesis appeared in *Am. J. Physiol. Cell Physiol.,* **286**: C380–390 (2004) and *J. Membr. Biol.,* **235**: 27–41 (2010).

Studies on the modulatory mechanisms of Hyperpolarization-activated cation current (I_h) in rat dorsal root ganglion cells: Cytosolic ATP-induced activation of I_h without involvement of phosphorylation by PKA.

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A hyperpolarization-activated cation current (I_h) has been observed in various neurons of the central and peripheral nervous systems and in cardiac tissues. The channel underlying I_h is activated by membrane hyperpolarization and slowly depolarizes the membrane potential. Therefore I_h has been proposed to control neuronal excitability and its functional role and modulating mechanism have been studied by many investigators. To date, four different genes encoding I_h channels have been cloned from mammals (HCN1-4). The presence of I_h and its modulation by cAMP have been described in rat dorsal root ganglion (DRG) neurons. However, in rat DRG neurons, the precise modulating pathway for I_h and the possibility that other intracellular modulating factors affect I_h channel activity have not been studied yet. In this study, to confirm the contribution of PKA-induced phosphorylation to the regulatory mechanisms of I_h and the effect of intracellular ATP on the electrophysiological properties of I_h, whole-cell current recording and immunocytochemical staining was performed in cultured rat DRG neurons.

Immunocytochemical experiments using specific antibodies against HCN channel subunits (HCN1, 2 and 4) indicated that cultured rat DRG neurons might express HCN2 or HCN4 channels. Under conventional whole-cell voltage-clamp conditions, hyperpolarizing voltage steps elicited slowly activating inward currents (I_h). Extracellular applications of 2 mM Cs^+ and 2 mM Ba^{2+} reduced the amplitude of I_h by 79.2% and 34.5%, respectively. The reversal potential of I_h was −20.3 mV. The activation curve of I_h in rat DRG neurons shifted by about 15 mV in the positive direction with an intracellular solution containing 1-mM cAMP. When 2-mM ATP was applied intracellularly, the half-maximal activation voltage (V_{half}) of I_h shifted from −97.4 to −86.8 mV. The V_{half} of I_h using the pipette solution containing both ATP (2 mM) and cAMP (1 mV) together was not significantly different from that obtained in the presence of ATP or cAMP alone. In the presence of 2-mM ATP, the V_{half} of I_h was not influenced by the application of an adenylate cyclase inhibitor SQ 22,536. Intracellular dialysis of non-hydrolysable ATP analogs, AMP-PNP and ATP-γ-S mimicked the effect of ATP on the voltage-dependence of I_h. Preincubation and external application of H-89, a PKA inhibitor failed to influenced the effect of intracellular ATP on the voltage-dependence of I_h.

I next examined the effect of the catalytic subunit of PKA (PKAc) on I_h and confirmed an effect of PKAc on Ca^{2+} channel currents carried by Ba^{2+} (I_{Ba}) in identical neurons as a positive control of PKA activity. In order to measure both I_h and I_{Ba} from the same neuron, Cs-rich pipette solution was used to minimize contamination of voltage-gated K^+ current. In neurons dialyzed with the Cs-rich pipette solution, the V_{half} of I_h was more negative by about 10 mV than that obtained with the K-rich pipette solution. However, similar to the result obtained with K-rich pipette solution, when ATP (2 mV) was added to the

Cs-rich pipette solution, the voltage-dependency of I_h was shifted about 10 mV in the positive direction. The amplitudes of I_{Ba} rapidly decreased with time (rundown) using the control pipette solution. With the pipette solution containing 2-mM ATP, the rundown of I_{Ba} was largely diminished. In the presence of PKI (50 μM) the rundown of I_{Ba} amplitude was significantly larger than that observed with ATP (2 mM) alone. On the other hand, ATP-induced depolarinzing shift of I_h activation was not affected by PKI. When PKAc (250 U/mL) was applied intracellularly, the rundown of I_{Ba} was markedly and significantly decreased and the current amplitude at 340 s was 86.1%. The bath application of okadaic acid (OA, 1 μM) enhanced the effect of PKA on the rundown of I_{Ba}. In some neurons dialyzed with PKAc, an increase in the amplitude of I_{Ba} was observed in the presence of OA. In the neurons dialyzed with the PKAc containing pipette solution and exposed to OA, the V_{half} of I_h was −108.7 mV, which was not significantly different from that without PKAc and OA. When the concentration of ATP was raised to 2 mM in the presence of PKAc, the activation curve of I_h significantly shifted in the depolarizing direction. In neurons dialyzed with PKAc in the presence of OA, activation time constant, τ_{fast} and τ_{slow} were not influenced between −90 and −130 mV.

Based on these results, it is clarified that the PKA-induced phosphorylation do not play a major role in the modulating mechanism of I_h and intracellular ATP can regulate the voltage-dependency of I_h activation by adenylate cyclase- and PKA- independent mechanisms in cultured rat DRG neurons.

The original papers of this thesis appeared in *J. Neurophysiol.*, **90**: 2115–2122 (2003) and *Biomedical research (Tokyo, Japan)*, **28**: 177–189 (2007).

Studies on neuropathogenesis and neuroanatomical distribution of disease-specific prion protein in cattle experimentally infected with bovine spongiform encephalopathy

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Bovine spongiform encephalopathy (BSE) was first identified in the United Kingdom in 1986. Subsequently, it spread to European, Asian, and North American countries. A variant form of Creutzfeldt-Jakob disease that was reported in 1996 is thought to be caused by the consumption of BSE-contaminated beef products. Therefore, it is important to understand the pathogenesis of the classical BSE (C-BSE) in cattle that first emerged in UK and spread all over the world, in order to reduce the C-BSE risk to public as well as to animals. However, due to the difficulty in using cattle for experimental infection, our current knowledge of the pathogenesis of the C-BSE in cattle is still inadequate. Therefore, the author investigated the relationship among the clinical manifestation, the accumulation of disease-specific prion protein (PrP^{Sc}) and the vacuolar changes of cattle experimentally infected with the C-BSE in order to clarify neuropathogenesis of the C-BSE. Furthermore, different type BSE cases from the C-BSE cases,

called atypical BSE, disclosed in 2003, and thereafter, the presence of atypical BSE cases was reported in many countries including Japan. In this thesis the author also analyzed properties of the BSE/JP24 isolate, the second L-type-like atypical BSE disclosed in Japan, by experimental infection in cattle.

In Chapter 1, to evaluate the relationship between the kinetics of PrP^{Sc} deposition in the central nervous system and the clinical course of the disease, temporal and special distribution of PrP^{Sc} was analyzed in the central nervous system of Holstein/Friesian cattle inoculated intracerebrally with 3 sources of C-BSE isolates. Cattle euthanized at 10 months post-inoculation (mpi) showed PrP^{Sc} deposits in the brainstem and thalamus, but no vacuolation. This suggested that the BSE prions might exhibit area-dependent tropism in the brain. At 16 and 18 mpi, a small amount of vacuolation was detected in the brainstem and thalamus, but not in the cerebral cortices. At 20 to 24 mpi, when clinical symptoms were apparent, heavy PrP^{Sc} deposits were evident throughout the brain and spinal cord. The mean time to the appearance of clinical symptoms was 19.7 mpi, and the mean survival time was 22.7 mpi. These findings indicate that PrP^{Sc} accumulation was detected approximately 10 months before the clinical onset.

Since the C-BSE-affected cattle showed auditory abnormality such as hypersensitivity to sound, in chapter 2, the author focused on neuropathological changes observed in the auditory brainstem of the C-BSE-challenged cattle. Before the appearance of clinical signs (i.e., at 3, 10, 12 and 16 mpi), vacuolar change was absent or mild and PrP^{Sc} deposition was minimal in the auditory brainstem nuclei. The two cattle sacrificed at 18 and 19 mpi without clinical signs showed mild vacuolar degeneration and moderate amounts of PrP^{Sc} accumulation in the auditory brainstem pathway. In the cattle showing clinical manifestation (i.e., after 20 mpi), spongy changes were more prominent in the nucleus of the inferior colliculus compared with the other nuclei of the auditory brainstem and the medial geniculate body. These pathological findings suggest that neuropathological changes characterized by spongy lesions accompanied by PrP^{Sc} accumulation in the auditory brainstem nuclei may be associated with hyperacusia in BSE-affected cattle.

It has been assumed that the causative agent of the C-BSE in cattle is a single strain that first emerged in UK. However, atypical BSE cases that show different neuropathological and molecular phenotypes have been recently reported in European countries, North America, and Japan. In Chapter 3, to clarify the characteristic of L-type like atypical BSE case that was disclosed in Japan, BSE/JP24, the author inoculated brain homogenates from cattle of the BSE/JP24 case into Holstein/Friesian cattle and examined biochemical and neuropathological properties as well as the clinical course of the disease. The BSE/JP24 isolate successfully transmitted to Holstein/Friesian cattle. Based on the incubation period, neuropathological hallmarks, and molecular properties of PrP^{Sc}, the BSE/JP24 prions were apparently distinguishable from the C-BSE prions and closely resemble bovine amyloidotic spongiform encephalopathy prions that were found in Italy.

In conclusion, this study clarified a part of the C-BSE pathogenesis by analyzing the association of the clinical symptom, the accumulation of PrP^{Sc} and the vacuolar changes of cattle experimentally infected with the C-BSE. In addition, this study clarified biochemical and neuropathological features of the BSE/JP24, the L-type like atypical BSE isolate in Japan. Results of this study provide important information on the risk analysis and management on BSE to reduce the risk of BSE to public as well as the risk of re-emergence of BSE.

Original papers of this thesis appeared in *Microbiol. Immunol.,* **53**: 704–707 (2009), *J. Comp. Pathol.,* **145**: 302–307 (2011) and *Jpn. J. Infect. Dis.,* **65**: 37–44 (2012).

Hokkaido University conferred the degree of Doctor of Philosophy on March 25, 2014 to 16 recipients.
The titles of thesis and other information are as follows:

Elucidation of the murine blood-testis barrier function — role as a "gatekeeper" regulating persistent spermatogenesis —

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Male germ cells, sperm, are produced in a cyclic and complicated process called spermatogenesis, which occurs in the seminiferous tubules in the testes. The differentiation of germ cells is supported, nurtured, and supervised by somatic Sertoli cells, which line the seminiferous tubules. During testis development in prepubertal period, Sertoli cells form the blood-testis barrier (BTB) which divides the seminiferous epithelium into the basal and adluminal compartments. The BTB restricts the entry of molecules in the interstitial space into the adluminal compartment, thereby BTB has been considered to play an important role in maintaining homeostasis of seminiferous epithelium. However, to accomplish germ cell differentiation, preleptotene/leptotene spermatocytes residing in basal compartment need to migrate across the BTB into adluminal compartment. Because the precise function of the BTB in spermatogenesis remains obscure, in this thesis, the author attempted to reveal the relationships between dynamics of BTB component proteins and meiosis progression during murine spermatogenesis.

At first, the author proved the dynamics of the BTB components during adult spermatogenesis focusing on spermatocyte migration across the BTB. Structural analysis revealed that the BTB component tight junction (TJ) proteins localized at the BTB throughout adult spermatogenesis. Although the TJ proteins including CLDN11, OCLN, and ZO1 were at the adluminal side of germ cells before their BTB migration, new TJ fibrils composed of these molecules are formed at the basal side of the preleptotene/leptotene spermatocytes preparing for migration across the BTB. Therefore, the intermediate compartment, a microenvironment sandwiched between TJ proteins from the apico-basal direction, is formed temporarily, and it is considered that the old TJ fibrils at the adluminal side are degraded to accomplish the passage of germ cells. Remarkably, unlike other BTB-constructing TJ proteins, CLDN3 localized to the basal portion of seminiferous tubules only around migration stages, in accordance with the emergence of the preleptotene/leptotene spermatocytes. These results indicated that the barrier function of the BTB is strictly maintained even in spermatocyte migration across the BTB, and the stage-specific localization of CLDN3 protein plays an important role in regulation of germ cell migration.

The author next examined the cell types expressing CLDN3 in the mouse testis and evaluated the integrity of spermatogenesis after *Cldn3* knockdown in order to verify the CLDN3 function in spermatogenesis. Astonishingly, besides Sertoli cells that form BTB, preleptotene/leptotene spermatocytes lacking TJ structure expressed the mRNA and protein of CLDN3. *Cldn3* knockdown caused a partial delay in spermatocyte migration

across the BTB, resulting in a prolonged period of the preleptotene phase during spermatogenesis. These data strongly indicated that Sertoli cells as well as preleptotene/leptotene spermatocytes participated in the regulation of BTB by expressing CLDN3, and that CLDN3 regulates the progression of spermatogenesis by promoting germ cell migration across the BTB.

Finally, the author attempted to validate the relationship between the BTB assembly and spermatogenic progression using vitamin A deficient mice showing spermatogenic arrest. Although vitamin A deficiency induced no changes in the initiation and progression of the first spermatogenic wave during prepubertal period, the numbers of preleptotene/leptotene spermatocytes derived from the second spermatogenic wave onwards were decreased in coincidence with the delay in the BTB assembly. Furthermore, BTB disruption in vitamin A deficient mice preceded testicular degeneration and complete spermatogenic arrest during adult age. From these results the author proposes that BTB integrity is regulated by vitamin A metabolism with control of meiosis and is required for persistent differentiation of germ cells rather than the initiation of spermatogenesis.

The present study suggests that Sertoli and germ cells coordinately establish an intimate and elaborate cellular network, morphologically observed as BTB, regulating their reciprocal functions during the germ cell development. Therefore, the author concluded that the BTB structure acts as "Gatekeeper" to regulate persistent spermatogenesis, and its functional and structural disruptions with altered dynamics of component TJ proteins could lead animals to male infertility.

The original papers of this thesis appeared in *Mol. Reprod. Dev.*, **77**: 630–639 (2010), *Biol. Reprod.*, **89**: 3, 1–12 (2013) and *J. Reprod. Dev.,* **59**: 525–535 (2013).

Studies on the pathogenesis of avian influenza in chickens

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Influenza A virus infections are found in a variety of birds and mammals including humans. These viruses are classified into subtypes on the basis of the antigenic specificity of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. To date, viruses of 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) have been isolated from avian species. Influenza A viruses infecting chickens are categorized into two pathotypes based on their virulence to chickens; namely, low pathogenic avian influenza virus (LPAIV) and highly pathogenic avian influenza virus (HPAIV). The HA of avian influenza virus is a primary viral protein to determine the pathogenicity in chickens. The HAs of HPAIVs have at least a pair of di-basic amino acid residues at their cleavage site, which permits cleavage activation by ubiquitous proteases such as furin and PC6, leading to systemic infection in chickens. On the other hand, the HAs of LPAIVs are cleaved only by trypsin-like proteases expressed in the respiratory and intestinal tracts, causing local infection in chickens. Highly pathogenic avian influenza caused by HPAIV has occurred worldwide and led to serious economic losses in poultry industry. The sporadic infections with H5 or H7 HPAIV in humans have been also reported and 648 people were infected

with H5N1 HPAIV and 384 deaths were confirmed for the last decade.

The pathogenicity of infectious agents is determined by the host-pathogen interactions. Innate immune system is one of the host factors to interact with pathogens and it is the first line of the defense against infection. Production of cytokines and chemokines in the innate immune system is critical response to induce inflammation and to eliminate pathogens in the early stage of infection. High levels of cytokines and chemokines were found in severe cases of H5N1 influenza virus infection in birds and mammals. Therefore, aberrant cytokine response called "cytokine storm" is hypothesized to be the main cause of high mortality in HPAIV infection in animals. However, H5 HPAIV infection was also lethal to mice genetically lacking cytokines or chemokines such as Tumor necrosis factor alpha (TNF-α), Interleukin (IL)-1, IL-6, and CC chemokine ligand 2 or those receptors, and immunosuppressive treatment was not always an effective therapy for H5 HPAIV infection. It has been still controversial whether aberrant cytokine response is the cause or effect of severity of influenza in birds and mammals.

In Chapter I, it is described that virus growth and cytokine response in chickens experimentally infected with two H7 HPAIVs and an H5 LPAIV were analyzed to examine the relationship between pathogenicity of avian influenza viruses and host responses. In Chapter II, it is described that the role of extensive response of cytokines, especially IL-6, in the pathogenesis of HPAIV infection in chickens was investigated using recombinant chicken IL-6 (rchIL-6) and anti-rchIL-6 antibodies. The findings obtained in the present studies should contribute to the better understanding of the pathogenesis of highly pathogenic avian influenza in chickens and the development of therapeutic drugs for proper treatment of severe cases of influenza in humans.

The original paper of Chapter I of this thesis appeared in *PLoS ONE*, **8**: e68375 (2013).

Studies on the interspecies transmission of influenza virus and vaccine preparation for the emergence of H5N1 highly pathogenic avian influenza virus infection

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Influenza A virus is widely distributed in birds and mammals including humans. On the basis of antigenic specificity of hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are divided into sixteen HA and nine NA subtypes (H1-H16 and N1-N9, respectively). Influenza A viruses of all of HA and NA subtypes have been isolated from water birds such as migratory ducks. There are four instances of the emergence of new pandemic strains in humans in the last one hundred years. A new pandemic strain is defined as a virus possessing new HA and/or NA subtypes that humans have not experienced for the last several decades. It is revealed that the A/Hong Kong/68 (H3N2) pandemic influenza virus emerged in 1968 via genetic reassortment in pigs concurrently infected with an H3Nx influenza virus circulating in migratory ducks

through domestic ducks and the H2N2 virus that was prevailing among humans at the time. The A/Hong Kong/68 (H3N2) pandemic strain bare PB1 and HA genes from the H3Nx virus of duck origin and the others from a human H2N2 Asian flu virus strain. Avian influenza viruses possess HA which preferentially binds to sialic acid α2,3-galactose sialyloligosaccharides (SAα2,3Gal), whereas swine and human influenza viruses bind to sialic acid α2,6-galactose sialyloligosaccharides (SAα2,6Gal). Pigs play an important role in the generation of pandemic influenza viruses since they have both SAα2,3Gal and SAα2,6Gal receptors and are susceptible to infection with both avian and human influenza viruses. Receptor specificity of influenza A viruses is the key determinant of host range. It is assumed that binding specificity to SAα2,6Gal receptor is required for efficient human-to-human transmission. However, the mechanisms by which avian influenza viruses acquire binding specificity to SAα2,6Gal receptor and cause pandemic influenza are not fully understood. In Chapter I, to assess whether viruses recognizing SAα2,6Gal receptor are selected, H3 avian influenza virus was consecutively passaged in pigs. Variants that preferentially bind to SAα2,6Gal receptor were selected from the nasal swabs of pigs after three passages. The present finding shows that avian influenza can acquire the potential to infect humans after serial passages in pigs.

H5N1 highly pathogenic avian influenza (HPAI) viruses has spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China. The WHO/OIE/FAO H5N1 Evolution Working Group has previously identified 20 new clades of viruses and established specific criteria for naming H5N1 clades. In Japan, H5N1 HPAI viruses belonging to clade 2.3.2.1 were isolated from dead whooper swans in 2008, fecal samples of ducks that flew from Siberia in October 2010, and from wild birds and domestic poultry in 2011. Recently, antigenic variants of H5N1 HPAI viruses have appeared in poultry in Asian countries and Egypt under immunological selection pressure. It is previously demonstrated that an inactivated avian influenza vaccine prepared from non-pathogenic avian influenza virus, A/duck/Hokkaido/Vac-1/04 (H5N1) (Dk/Vac-1/04), conferred protective immunity against the challenge with H5N1 HPAI viruses isolated until 2008. Vac-3 vaccine from A/duck/Hokkaido/Vac-3/07 (H5N1) (Dk/Vac-3/07), which is antigenically closely related with Dk/Vac-1/04, was prepared. Growth potential of Dk/Vac-3/07 in embryonated chicken eggs was higher than that of Dk/Vac-1/04. In Chapter II, the potency of the Vac-3 vaccine was assessed by the challenge with antigenically drifted HPAI viruses prevailing recently in Asia. Vac-3 vaccine conferred sufficient immunity to protect from infection with variant H5N1 HPAI viruses in chickens. The present results show that Vac-3 vaccine is useful as a stockpile vaccine for the emergence of H5N1 HPAI. Since the misuse of vaccines lead to the silent spread of antigenically drifted viruses, it is recommended that avian influenza vaccine should be applied very carefully in addition to the stamping-out policy.

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Study on the pro-inflammatory cytokine expression profile in dogs with inflammatory bowel disease

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Canine inflammatory bowel disease (IBD) is a group of disorders that is characterized by persistent gastrointestinal signs and histological evidence of intestinal inflammation. That is a common cause of chronic vomiting and diarrhea in dogs and must be differentially diagnosed from other possible causes such as infection, food allergy, neoplasia, and exocrine pancreatic insufficiency. Lymphocytic-plasmacytic enteritis (LPE) and Lymphocytic-plasmacytic colitis (LPC) are the most common form of canine IBD. Histopathological features of LPE are diffuse lymphocytic-plasmacytic infiltration to the lamina propria in the duodenal mucosa and that of LPC are lymphocytic-plasmacytic infiltration to the lamina propria in the colonic mucosa. It has been suggested that dysfunction of mucosal immune system with loss of tolerance to luminal antigens possibly plays important roles in the pathogenesis of canine IBD. Inflammatory colorectal polyps (ICRPs) in Miniature Dachshunds are recently recognized in Japan as a major cause of large bowel diarrhea in this breed. The most typical clinical signs of ICRPs are hematochezia, tenesmus, and mucoid feces. ICRPs are characterized by the formation of multiple small polyps (small polyps) in the large bowel mucosa, accompanied by a large space-occupying solitary polyp (large polyp) formation. ICRPs are thought to be a novel form of canine IBD because of the unknown etiology, the existence of idiopathic inflammation, and the effectiveness of immunosuppressive therapy.

In human IBD, Crohn's disease (CD) and ulcerative colitis (UC), intestinal immune disorder plays a central role of the pathophysiology. Both CD and UC, pro-inflammatory cytokines, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*, are significantly increased in the inflamed mucosa when compared to healthy controls, and thought to be important in the pathogenesis. On the other hand, in dogs with LPE and LPC, there are few reports about investigation of pro-inflammatory cytokine expressions, and it is still controversial. Furthermore, there was no report about investigation of pro-inflammatory cytokine expressions in dogs with ICRPs. Therefore, the aim of this thesis was to investigate pro-inflammatory cytokine expressions in dogs with LPE, LPC, and ICRPs.

In Chapter 1, the author investigated pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*, in the intestinal mucosa of dogs with LPE and LPC by quantitative real-time RT-PCR. As a result, TNF-α, IL-12p35, IL-12/23p40, and IL-23p19 mRNA expression was significantly decreased in the duodenal mucosa from dogs with LPE when compared to those in controls. IL-23p19 mRNA expression was only significantly increased in the colonic mucosa from dogs with LPC when compared to that in controls. Thus, different from human IBD, no distinct cytokine profile was found in canine IBD at the level of mRNA expression in the inflamed intestinal mucosa. No clear evidence was obtained for the contribution of cytokine profile to the pathogenesis of canine IBD.

In Chapter 2, to explore key mediators in the pathogenesis of ICRPs, the author investigated pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19* in the colorectal mucosa of dogs

with ICRPs by quantitative real-time RT-PCR. Among these cytokines, *IL-8* expression was markedly up-regulated in large polyps. To examine IL-8 protein expression, the author analyzed IL-8 protein level and its localization in colorectal mucosal specimens of ICRP dogs by ELISA and immunofluorescence microscopy. IL-8 protein was significantly increased in large polyps and serum in dogs with ICRPs when compared to controls. By immunofluorescence microscopy, IL-8 was only localized in macrophages, but not in mucosal epithelial cells and neutrophils. The number of IL-8-positive macrophages was significantly increased in large polyps compared to controls. These results suggest that IL-8 is produced mainly by macrophages and may induce neutrophil infiltration in the colorectal area of ICRP dogs.

In conclusions, involvement of pro-inflammatory cytokines in dogs with LPE and LPC may be different from human IBD. On the other hand, there is clear evidence for the up-regulation of pro-inflammatory cytokine expressions, in particular IL-8, in dogs with ICRPs. Further study is needed to clarify the etiology of canine IBD.

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A study on effects of non-steroidal anti-inflammatory drugs (NSAIDs) on differentiation capacity of canine osteogenic cells

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Non-steroidal anti-inflammatory drugs (NSAIDs) have been used for pain management in orthopedic patients of animals as well as human beings with osteoarthritis or fracture. However, safety concerns for using NSAIDs in fracture patients have been remained as a controversy. Purpose of the present study was to evaluate effects of NSAIDs on differentiation capacity of canine osteogenic cells.

In Chapter 1, effects of short-term treatment with NSAIDs on osteogenic differentiation were estimated. Canine osteosarcoma cell line (POS) that could be spontaneously differentiated into osteoblastic cells were used for osteogenic model. During different stages of osteoblastic differentiation, POS cells were treated with carprofen and meloxicam for 72 hours. Morphological change of nodule formation, protein synthesis of alkaline phosphatase (ALP) and gene expressions of ALP and osteocalcin were evaluated in a time-course manner. Differentiation of osteoblasts was suppressed by NSAIDs in transitional stage between pre-osteoblastic and mature osteoblastic stages, which was correlated with delayed non-calcified nodule formation and decreased expression level of osteocalcin mRNA. However, fully calcified nodule formation was observed in all experimental groups during post-medication period. These results indicated that NSAIDs reversibly suppressed osteoblastic differentiation, which would have occurred by intrinsic potentials to restore insufficient PGE_2 in the cells.

In Chapter 2, to clearly demonstrate that compensatory responses to PGE_2 deficiency exist

in normal ostegenic cells, canine bone marrow derived mesenchymal stem cells (BMSCs) were used. Osteogenic induction medium was supplemented with recombinant human interleukin (rhIL) -1β (1 ng/ml) as an inflammatory stimulator. Various classes of NSAIDs, including carprofen, meloxicam, indomethacin, and robenacoxib, were used to treat osteogenic cells during 20 days of osteogenic period in which matrix fully calcified. Levels of gene expressions were measured including osteoblast markers (ALP and osteocalcin), PGE_2 related enzymes (COX-1, COX-2, cPGES and mPGES-1) and PGE_2 receptors (EP2 and EP4). Levels of protein production levels of ALP, osteocalcin and PGE_2 were quantified. Morphologically, differentiation of ALP positive cells was observed and level of calcification was quantified. Decreased ALP expression and delayed differentiation into ALP positive cells by each of the NSAIDs were detected on day 4 when ALP expression was highest. Level of calcium deposition was somewhat suppressed by NSAIDs on day 20, while osteocalcin production showed no significant suppression. Gene expression levels of PGE_2 related receptors and enzymes were up-regulated on day 4. Furthermore, levels of PGE_2 synthesis were restored at 48 hours under each of the NSAIDs. Channels for PGE_2 synthesis were utilized differently depending on the classes of NSAIDs, supported by differences in expression levels of EP2/EP4 and COX-1/COX-2 mRNA and in rates of PGE_2 restoration among the groups. These results indicated that intracellular compensatory responses occurred under PGE_2 deficiency derived by NSAIDs and inhibition of osteogenic differentiation would be more limited by using specific classes of NSAIDs.

In conclusion, canine osteogenic cells would have compensatory capacity to restore PGE_2 deficiency under NSAIDs treatment, which could prevent derailed osteogenic differentiation. Suppressive effects of NSAIDs on osteogenic differentiation would be minimal, suggesting that NSAIDs could be recommended for orthopedic patients as analgesics.

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Treatment of rabies by intrathecal immunization and pathogenesis of myocardial necrosis in rabid rabbits

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Rabies is a fatal zoonotic disease for which no effective treatment measures are currently available. However, pre- and post-exposure prophylaxes are empowered to control the rabies. Previously, humoral and cellular immunities are orchestrated in RABV clearance from the CNS and recovery in rabies patients and animal. In addition, we previously described that SC immunization prior to IT immunization induced a more rapid and higher antibody response in the CSF than IT immunization alone. The VNA, which is crucial in RABV clearance, is originated both from serum and de novo locally produced in CNS. We considered that SC immunization prior to IT immunization might be applicable in rabid animal. In this study, we apply SC immunization prior to IT immunization to treat the experimentally induced rabies rabbits and also described the

neurogenic cardiomyopathy in rabid rabbits as seen in rabid patients.

In the first experiment, the rabbits exhibited neuromuscular symptoms of rabies within 4–8 dpi of RABV inoculation. All of the rabbits died within 8–12 dpi with the exception of one rabbit in the SC group and all four rabbits in SC/IT group, which recovered and started to respond to external stimuli and eat and drink by hand feeding at 11–18 dpi and survived until the end of the experimental period. RABV was eliminated from the CNS of the surviving rabbits. Humoral and cellular immunities were involved in RABV clearance and recovery. We report here a possible, though still incomplete, therapy for rabies using IT immunization in animal previously received SC immunization. Our protocol may rescue the life of rabid patients and prompt the future development of novel therapies against rabies.

In the second experiment, experimentally induced rabies, which had brain lesions, showed myocardial lesions and the severity of the cardiac lesions was proportional to that of the brain lesions. Rabies virus antigen was not found in the hearts of any rabbits. Neither the frequency nor the cumulative dose of anesthesia was related to the incidence or the severity of the myocardial lesions. The myocardial lesions were consisted with the neurogenic cardiomyopathy in human being. The rabies-induced brain lesions were most prominent in the brain stem, which controlled the heart function and autonomic nervous system. Take together, the myocardial lesions were classified as neurogenic cardiomyopathy.

In conclusion, our IT immunization protocol can be applicable to treat rabies animal previously received SC immunization when the rabies signs occurred. However, neuronal complication is still the obstacle for this protocol. Furthermore, neurogenic cardiomyopathy should be considered and treated in rabies patients. Finally, we purposed that our protocol might be rescue the life of rabid patients and prompt the future development of novel therapies against rabies.

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Studies on *in vitro* maturation/fertilization/development and mitochondrial activity of *in vitro*-grown bovine oocytes derived from early antral follicles

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The development to blastocysts of bovine oocytes derived from *in vitro*-growth (IVG) culture is low. For improving developmental competence of IVG oocytes, the present studies were conducted to use culture system supplemented with phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine: IBMX) immediately before IVM (pre-IVM) and to clarify the optimal duration of IVG culture and the effects of pre-IVM with IBMX on nuclear and cytoplasmic maturation of IVG oocytes.

In chapter I, the viability and growth of oocytes after different IVG durations (10, 12 and 14 days) were examined. Nuclear maturation

(metaphase II: M II) of oocytes at 10-, 12- and 14-day IVG was also examined after IVM with or without pre-IVM culture. The viability of oocytes after 14-day IVG was lowest. The mean diameters of 12- and 14-day IVG oocytes were larger than that of 10-day IVG oocytes. M II rate of 10-day IVG oocytes was lowest, regardless of pre-IVM culture. M II rates of 12- and 14-day IVG oocytes with pre-IVM were higher than those without pre-IVM. Also, after 12- and 14-day IVG, the developmental competence and nuclear status of IVG oocytes as well as the membrane integrity of cumulus cells were examined. After being subjected to pre-IVM and IVM, the blastocyst rate of 12-day IVG oocytes was higher than that of 14-day IVG oocytes, but was lower than that of *in vivo*-grown oocytes. The percentage of oocytes arrested at the germinal vesicle (GV) stage derived from 12-day IVG was higher than that from 14-day IVG immediately after pre-IVM culture. The proportion of 12-day IVG oocytes having cumulus cells with intact membrane was also higher than that of 14-day IVG oocytes. These results demonstrate that 12-day IVG culture is suitable for oocyte growth, and prolonging IVG culture reduces the integrity of cumulus cells, and the viability and developmental ability of IVG oocytes.

In chapter II, 12-day IVG oocytes with normal appearance were subjected to examination of GV structure before IVM with and without pre-IVM. In addition, percentages of M II were examined after IVM. The proportions of GV 3 and M II stages of IVG oocytes with pre-IVM were higher than those without pre-IVM. Also, the fertilizability and developmental competence of IVG oocytes with and without pre-IVM were examined. Regardless of pre-IVM, normal fertilization rates of IVG oocytes were similar, but were lower than that of *in vivo*-grown oocytes. Cleavage and blastocyst rates of IVG oocytes with pre-IVM were higher than those without pre-IVM. These results demonstrate that pre-IVM with IBMX improved the maturational and developmental competences of IVG oocytes, probably due to promotion of their chromatin transition and synchronization of meiotic progression.

In chapter III, mitochondrial activity in IVG oocytes were investigated at different pre-IVM duration. Moreover, meiotic progression and development to blastocysts of IVG oocytes were examined. Mitochondrial activity in IVG oocytes at 10 h pre-IVM was higher than those at 0 and 20 h pre-IVM. After 10 h IVM, M I rate of IVG oocytes with 10 h pre-IVM tended to be higher than that with 0 h, and similar to that of *in vivo*-grown oocytes. After IVM, M II rate of IVG oocytes with 10 h pre-IVM was higher than those with 0 h and 20 h pre-IVM, and similar to that of *in vivo*-grown oocytes. Cleavage and blastocyst rates of IVG oocytes with 10 h pre-IVM were higher than those with 20 h pre-IVM, and similar to those of *in vivo*-grown oocytes. These results demonstrate that IVG oocytes with 10 h pre-IVM showed higher development to blastocysts associated with the increase of mitochondrial activity in IVG oocytes. When blastocysts derived from IVG oocytes with 10 h pre-IVM were transferred to recipient cows, more than half of transferred embryos (7/11) developed to elongated embryonic stage at day 16 of estrus and one pregnancy (1/2) was confirmed at day 62.

In conclusion, the present studies demonstrate that 12 days of IVG followed by 10 h pre-IVM culture with IBMX is considered as the optimal IVG system for bovine growing oocytes derived from early antral follicles, and that this IVG system can produce the oocytes having the high maturational and developmental competences as same as *in vivo*-grown oocytes.

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Studies on the aging-related changes in bovine oocytes during *in vitro* maturation

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Nuclear and cytoplasmic maturation are essential for oocytes to acquire developmental competence during *in vitro* maturation (IVM). After the completion of nuclear maturation, oocytes gradually accomplish cytoplasmic maturation and acquire developmental competence. Then, they maintain that competences of embryonic development for a certain period, but eventually undergo deterioration in their quality and lose developmental competence. This phenomenon is called "oocyte aging". In this thesis, the author conducted the study to estimate the timing when bovine oocytes acquire and lose their developmental competence during IVM culture and investigate aging-related changes in *in vitro*-matured bovine oocytes, especially focusing on the functions of mitochondria during IVM culture and early embryonic development.

In chapter 1, the objective was to estimate the timing when bovine oocytes acquire and lose their developmental competence during IVM culture. The embryonic development of *in vitro*-matured bovine oocytes based on the times of nuclear maturation and sperm penetration was investigated. Firstly, cumulus oocyte complexes were subjected to IVM for 14 to 22 h. The timing when 50% of oocytes achieved nuclear maturation was estimated to be 17.5 h after IVM start. Secondly, using oocytes subjected to IVM for 12 to 30 h, sperm penetration was examined after 4 to 18 h of *in vitro* fertilization (IVF). A significant negative correlation between IVM duration and the timing when 50% of oocytes were penetrated by sperm after IVF start was observed. Finally, oocytes subjected to 12 to 30 h of IVM were inseminated and cultured for 6 days to examine embryonic development. It was showed that bovine oocytes had their highest developmental competence at around 12 h after achieving nuclear maturation (i.e., around 30 h after the initiation of IVM culture), and began to lose gradually their developmental competence after this timing.

In chapter 2, the objective was to clarify the aging-related changes in *in vitro*-matured bovine oocytes. The oocytes subjected to IVM for 20 h (oocytes immediately after nuclear maturation), 30 h (fully matured oocytes with high developmental competence) and 40 h (aged oocytes) served in the determination of reactive oxygen species (ROS) production, mitochondrial activity and ATP content. ROS in the oocytes after 30 h of IVM was lower than in the oocytes after 20 h of IVM, and this result showed that the oocytes after 30 h of IVM had high competence to protect against oxidative stress. The mitochondrial activity and ATP content in bovine oocytes subjected to 40 h of IVM were highest among all IVM durations. In addition, mitochondrial activities at 3 days after IVF in embryos derived from the oocytes subjected to 22 h (penetrated by sperm at around 30 h after starting IVM) and 34 h (penetrated by sperm at around 40 h after starting IVM) of IVM were evaluated. In the group subjected to 34 h of IVM, high-polarized mitochondria were frequently observed at the periphery of blastomeres. These high-polarized mitochondria at the periphery of blastomeres might have already been extruded from blastomeres. It is suggested that high mitochondrial activity observed in oocytes at prolonged duration after IVM start and localization of high-polarized mitochondria at the periphery of blastomeres during early embryonic development

may be associated with the low developmental competence in aged bovine oocytes.

The present study demonstrates that bovine oocytes acquire their highest developmental competence at around 30 h after the initiation of IVM culture and then begin to lose their developmental competence gradually. Moreover, it is showed that, in contrast to the past reports, the mitochondria activity increases with aging of *in vitro*-matured bovine oocytes. Therefore, this depression of developmental competence in *in vitro*-matured bovine oocytes is possibly related with the enhanced mitochondrial activity.

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The mechanism of radiation-induced endothelial nitric oxide synthase activation and its role in tumor reoxygenation after X-irradiation

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Low oxygen conditions drastically decreases cellular radiosensitivity and facilitate the adaptive responses of tumor. Hence, hypoxic regions in solid tumor are well-known obstacle for successful radiotherapy. It occurs mainly as a result of the excess proliferation of tumor cells and accompanying deficiency in blood and nutrient supplies. It has been reported that ionizing radiation decreases hypoxic regions in tumor, leading to the improvement of radiosensitivity of solid tumor. However, the mechanism of radiation-induced tumor reoxygenation has not been elucidated. In the first study, to elucidate the mechanism of radiation-induced reoxygeneration, the effect of nitric oxide (NO) on radiation-induced tumor reoxygeneration in murine SCCVII tumors was studied. Hoechst33342 perfusion assay and electron spin resonance oxymetry showed that X-irradiation increased tissue perfusion and pO_2 in tumor tissue. Immunohistochemical analysis using two different hypoxic probes showed that X-irradiation decreased hypoxic regions in tumors; treatment with a nitric oxide synthase (NOS) inhibitor, L-NAME, abrogated the effects of ionizing radiation. Moreover, X-irradiation increased endothelial NOS (eNOS) activity without affecting its mRNA or protein expression levels in SCCVII-transplanted tumors. Tumor growth delay assay showed that L-NAME decreased the anti-tumor effect of fractionated radiation (10 Gy $\times$ 2). These results suggested that X-irradiation increased eNOS activity and subsequent tissue perfusion in tumors. Increases in intratumoral circulation simultaneously decreased tumor hypoxia. As a result, the radiation-induced NO increased tumor radiosensitivity. In the second study, to clarify the involvement of ataxia telangiectasia mutated (ATM) and heat shock protein 90 (HSP90) in the radiation-induced endothelial nitric oxide synthase (eNOS) activation, bovine endothelial cells (BAEC) were irradiated with X-rays. The activity of nitric oxide synthase (NOS) and the phosphorylation of serine 1179 of eNOS (eNOS-ser1179) in BAEC were significantly increased after irradiation.

Ku-60019 (an ATM inhibitor) or geldanamycin (an HSP90 inhibitor) significantly reduced the radiation-induced increases of NOS activity and eNOS-Ser1179 phosphorylation. Furthermore, geldanamycin suppressed the radiation-induced phosphorylation of ATM-Ser1181. Therefore, ATM and HSP90 regulated the radiation-induced eNOS activation. In conclusion, this study provides new insight for mechanisms in radiation-induced tumor reoxygenation. Ionizing radiation increases eNOS activity via DNA damage response. Activation of eNOS increases tissue perfusion through the production of NO and reoxygenates the hypoxic regions in solid tumor. This radiation-induced tumor reoxygenation increases tumor radiosensitivity toward subsequent irradiation, providing the new insight for the improvement of radiotherapy by modulating eNOS activity.

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3-Metyl pyruvate enhance the radiosensitivity through increase of mitochondria-derived reactive oxigen species in the tumor cells

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Considerable interest has recently been focused on the special characteristics of cancer metabolism, and several drugs designed to modulate cancer metabolism have been tested as potential anticancer agents. To date, however, very few studies have been conducted to investigate the combined effects of anticancer drugs and radiotherapy. In this study, to evaluate the role of mitochondria-derived reactive oxygen species (ROS) in the radiation-induced cell death of tumor cells, we have examined the effect of 3-methyl pyruvate (MP). MP is a membrane-permeable pyruvate derivative that is capable of activating mitochondrial energy metabolism, on human lung carcinoma A549 cells and murine squamous carcinoma SCCVII cells. Pretreatment with MP significantly enhanced radiation-induced cell death in both cell lines, and also led to increases in the mitochondrial membrane potential, intracellular adenosine triphosphate content, and mitochondria-derived ROS production following the exposure of the cells to X-rays. In A549 cells, MP-induced radiosensitization was completely abolished by vitamin C. In contrast, it was partially abolished in SCCVII cells. These results therefore suggest that the treatment of the cells with MP induced radiosensitization via the production of excess mitochondria-derived ROS in tumor cells.

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Avian cytochrome P450 enzymes as a protection system to environmental chemical compounds

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Since long time ago, environmental chemical compounds derived from natural or anthropogenic sources have threatened avian species. Researches about the environmental chemicals and the effect on avian species have been performed more than 50 years, but still, the risk of chemicals on avian species, or the species difference in sensitivity to chemicals among bird species have not yet been thoroughly understood. One of the major reasons is the speciation history of avian species. Avian species are majorly classified into three groups, i.e., *Paleognathae*, *Galloanserae* and *Neoaves*. *Paleognathae* is comprised of about 50 species including ostrich and emu. *Galloanserae* is comprised of about 500 species including most poultry species, and poultry species such as chicken and quail are the common species used for toxicological assays. The largest group is *Neoaves*, which about 95% of avian species belong to. Thus, the problem is that the most species examined in toxicological tests belong to *Galloanserae*, and the knowledge is limited to *Galloanserae*, although most endangered species by chemicals belong to *Neoaves*.

In this study, I focused on cytochrome P450 (CYP) enzyme, which is a major enzyme metabolizing xenobiotics and determines the sensitivity to chemicals. In mammalian species, CYP 1-3 family isoforms are known to be responsible of most xenobiotic metabolism. In avian species, CYP is also expected to have important roles in xenobiotic metabolism, although the knowledge is limited in respect to species, functions and even information of existing isoforms.

In chapter 2, I aimed to identify and classify all the CYP 1-3 genes of three avian species, i.e., chicken (*Gallus gallus*), zebra finch (*Taeniopygia guttata*) and turkey (*Meleagris gallopavo*) by using public genome information. The results showed that CYP complements of avian species were mostly orthologous to those of mammalian species, at the subfamily level. Based on the genetic information, I performed quantitative real-time RT-PCR to identify dominantly expressed isoforms. The result showed CYP2C45 is the most highly expressed gene in chicken liver, followed by CYP1A5, CYP2D49, CYP2C23a, and CYP3A37. CYP2C45 gene was suggested to be the most important isoform in xenobiotic metabolism of chicken.

In chapter 3, I focused on CYP2C23 gene, which had been recognized as “avian specific isoform”. I cloned CYP2C23 genes from eight avian species, to assess the species differences in genes and the protein expression levels of CYP2C23. Phylogenetic analysis and homology analysis showed unique characteristics of *Galloanserae* CYP2C23 genes such as *Galloanserae*-specific lack of amino acid residues. These results suggested CYP2C23 genes of *Galloanserae* may have evolved special selection pressures, compared to *Neoaves* and *Paleognathae*.

In chapter 4, I selected warfarin, an anticoagulant rodenticide, as a model chemical to assess the species differences in xenobiotic metabolism ability and sensitivity to chemicals. Warfarin is a major rodenticide used all over the world, and the relative compounds including warfarin or bromadiolone have caused secondary poisoning incidents in avian species. The poisoning are often observed in raptor species, while the *in*

vivo toxicological assays examining the toxicity of warfarin to avian species have suggested "LD_{50} of warfarin is extremely high in poultry species". I selected several avian species to examine the *in vitro* warfarin metabolism ability by CYPs. The results showed that chicken had the highest metabolic ability of warfarin, while owls have extremely low metabolic ability. This result indicated that the species difference in sensitivity to warfarin may be derived of the species difference in metabolic ability. Moreover, I assessed the species difference in vitamin K epoxide reductase (VKOR), a target protein of warfarin. The result showed that chicken VKOR was resistant to warfarin inhibition (Ki = 11.3 μM), while ostrich VKOR is strongly inhibited by warfarin (Ki = 0.64 μM). With these results, I could summarize that there are large interspecies differences in CYP-mediating metabolic ability and VKOR inhibition by warfarin, which is leading to the large species difference in sensitivity.

In conclusion, this study provided basic information as for the species difference in avian CYP genes, in addition to the dominant genes in avian xenobiotic metabolism. Moreover, with a model compound warfarin, I could clearly demonstrate the large interspecies difference in xenobiotic metabolism ability, which possibly leads to the species difference in sensitivity to chemical compounds.

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Male reproductive characteristics and genetic polymorphism of feral raccoons (*Procyon lotor*) in Hokkaido

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The raccoon (*Procyon lotor*) is a mammal indigenous to North America, and has become feral since 1979 in Hokkaido as an invasive alien species. Until now, an eradication program has been conducted under the Invasive Alien Species Act; however control has not been successful yet. To decrease the raccoon population size efficiently, it is necessary to gather basic biological information. Particularly to understand the reproductive characteristics in the habitat is crucial. And it is also important to infer dispersing process and to reveal population units with geographical or genetic barrier for effective management. Thus this whole study was performed to elucidate one aspect of reproductive characteristics and genetic background of feral raccoons in Hokkaido.

First, timing of puberty and seasonal reproductive physiology were clarified in male raccoons with using histological and endocrinological method in total 219 male raccoons. For the majority of raccoons, which were born in spring, prepubertal development began in May in yearling stage, and spermatozoa production began in October prior to their second mating season in winter. On the other hand, some larger juveniles, which could achieve enough body growth, attained puberty during the juvenile period earlier than other many individuals. After reaching puberty, adult males exhibited active spermatogenesis with high peripheral testosterone concentration in winter mating season. In many seasonal breeding mammals, males generally

exhibited inactive spermatogenesis along with low testosterone concentration in non-breeding season. However in this study, some raccoon adult males were found to produce spermatozoa actively despite low plasma testosterone concentrations in the summer non-breeding season. Such an indefinite seasonal reproductive change in gonad is unique to raccoon males. Besides, to reveal the factors that regulate testosterone production and contribute to differences in spermatogenetic activity in summer, changes in the biosynthesis, metabolism and reactivity of testosterone were investigated with evaluating intensity of steroidogenic enzymes and the androgen receptor (AR) in 15 adult males. As a result, seasonal changes in testosterone concentration were correlated with 3βHSD expression, suggesting that 3βHSD may be important in regulating the seasonality of testosterone production in raccoon testis. Immunostaining of P450arom and AR was detected in testicular tissues that exhibited active spermatogenesis in summer, while they were scarce in aspermatogenic testes. This suggests that spermatogenesis might be maintained by some mechanism that regulates P450arom expression, in synthesizing estradiol, and AR expression, in controlling reactivity to testosterone.

Second, mitochondrial DNA (mtDNA) polymorphism was clarified with using 526 DNA samples from 44 municipalities throughout Hokkaido. In most samples, sex was determined with *os penis* at the time of captured. In the other sex undetermined samples, a genetic method for sex identification was applied, which was developed based on nucleotides differences between *ZFX* and *ZFY* genes. With sequence analysis of 682-bp fragment in D-loop of mtDNA, 7 haplotypes with 26 polymorphic sites were detected and their geographical distribution in Hokkaido was observed. Including one haplotype which had been found previously in other report but not in the present study, founder event in Hokkaido was established from at least 8 female haplotypes. In 7 haplotypes in this study, 3 of them were distributed widely over subprefectures in Hokkaido and 5 haplotypes including these 3 were detected also in Honshu and Kyushu, indicating that raccoons were introduced at several remote places in Japan, or re-introduction as second release over prefectures happened after first foundation. The other 2 haplotypes were detected only in limited close regions in Hokkaido, thus, these haplotypes were thought to be introduced in only small area and dispersed around there. Besides, a haplotype found in this study was identified as a report in non-native area in Europe and also native-range North America. An introduction of raccoons across countries by export from native habitat was revealed genetically in this study, which is the first report in Japan. Further study with using a microsatellite DNA can help to clarify genetic variability among/within population, geographical genetic barrier, and evidence of population bottlenecks at founder events.

Reproductive characteristics detected in this study, which showed a possibility of early-maturation and a perennial reproduction in male raccoons, are explaining one aspect of high reproductive potential of raccoons. And detecting geographical genetic distribution could partly infer a background of founder introduction. Further studies which investigate how and why raccoons could have been increasing and dispersing throughout Hokkaido are required for efficient eradication control against this invasive alien species.

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Studies on steric shielding of cell surface proteins by filovirus envelope glycoproteins

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Filovirus infection causes severe hemorrhagic fever in human and nonhuman primates. Despite extensive research, the molecular basis for the pathogenicity of filoviruses remains elusive. Previous studies showed that the viral envelope glycoprotein (GP) is involved in the pathogenicity of filoviruses. Recently, a novel GP function designated steric shielding, by which cell surface molecules are sterically shielded and functionally impaired on the surface of GP-expressing cells, has been reported. In this study, I investigated the possible contribution of GP-mediated steric shielding to the pathogenicity of filoviruses and the impact of the steric shielding against Fas-mediated apoptosis.

In chapter I, I found that the steric shielding efficiency differed among filovirus strains and the difference was correlated with their relative pathogenicities. While the GP mucin-like region (MLR) that is known to be the functional domain for the steric shielding was indispensable, the difference of shielding efficiency among filovirus GPs did not depend on the primary structure of the MLR. In addition, I mapped the GP regions responsible for the different shielding effects observed among the Marburg virus strains tested and found that the amino acid residue at position 547 in GP2 was important for the efficiency of the steric shielding.

In chapter II, I found that the Fas molecule was prominently shielded on the surface of GP-expressing cells, resulting in the suppression of Fas-mediated apoptotic signaling and the increase of viability in GP-expressing cells. This apoptosis suppressive effect via steric shielding by GPs was observed in all tested filoviruses, representative of each genus of the family *Filoviridae*, suggesting that GPs of all filoviruses thus far known may have a common ability of the steric shielding to interfere with host proteins.

The present study suggests that the steric shielding by GPs may play important roles in impairing the functions of cellular molecules and be involved in the pathogenicity of filoviruses.

Hence, information on the impact of the steric shielding effect against cell surface molecules important for host-cell homeostasis and/or antiviral activity may be helpful in developing antiviral therapeutics.

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Heterosubtypic antiviral activity of IgA antibodies against influenza A virus hemagglutinins

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Both IgA and IgG antibodies are known to play important roles in protection against influenza A viruses. In this research, I analyzed the properties of IgA and IgG antibodies cross-reactive to influenza A viruses of multiple hemagglutinin (HA) subtypes and discussed the potential roles of IgA antibodies in heterosubtypic immunity.

In chapter I, I found that both intranasal and subcutaneous immunization induced IgA and IgG antibodies cross-reactive to multiple HA subtypes, whereas IgA was not detected remarkably in mice immunized subcutaneously. There was no fundamental difference in the overall spectrum of cross-reactivity between the antibodies produced by intranasal and subcutaneous immunization. Though cross-neutralizing activities of those antibodies were not detected, a remarkable inhibition of virus budding or release from infected cells was observed in the presence of cross-reactive IgA antibodies. These results suggest that the majority of HA-specific cross-reactive antibodies do not block cellular entry of viruses, but non-neutralizing IgA may have the potential to inhibit viral release from infected cells.

In chapter II, I demonstrated the difference in antiviral activity between IgA and IgG, using monoclonal antibodies (MAbs) S139/1 IgA and IgG which recognized the same epitope on HA. These MAbs were originated from mice immunized with a virus of H3 subtype, and had cross-neutralizing activity against viruses of H1, H2, and H13 subtypes. Against the homologous H3 virus, S139/1 IgA and IgG antibodies similarly showed strong binding activities and there was no significant difference in their hemagglutination inhibiting (HI) and neutralizing activities. On the other hand, S139/1 IgA showed higher cross-binding, HI, and neutralizing activities against the heterologous H1, H2, or H13 viruses than S139/1 IgG. Furthermore, S139/1 IgA significantly suppressed the extracellular release of the heterologous viruses from infected cells, but S139/1 IgG did not. Electron microscopy of cells infected with the H3 virus and then cultured in the presence of S139/1 MAbs, revealed that S139/1 IgA deposited newly produced viral particles on the cell surface, most likely by tethering the particles. The polymeric structure of IgA is considered to be important for this tethering activity.

Taken together, anti-HA IgA showed greater potential in antiviral activities against influenza A viruses than IgG antibodies. The advantage of IgA is likely due to increased avidity and cross-linking activity conferred by its multivalency. Particularly, the results obtained in the present study suggest that tethering and depositing newly budded virus particles on the infected cell surface may be one of the IgA-mediated protective mechanisms important for heterosubtypic immunity against influenza A viruses.

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Identification of novel virus from vervet monkey in Zambia and analysis of its viral assembly

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Recently, emerging and reemerging infectious diseases, including zoonoses, are constantly appearing worldwide and become a major concern to public health for humans and nonhumans. Because of increased contact between humans and wildlife along to development of human society, zoonotic diseases are increased.

Primary infections of human PyVs, such as JC virus and BK virus, usually occur in childhood and cause subclinical infections. When the host becomes immunocompromised, the viruses reactivate and cause diseases, including nephropathy and progressive multifocal leukoencephalopathy. It is controversial as to whether PyVs can be transmitted from wild animals to humans and thereafter cause disease. To examine potential threats of the zoonotic transfer of PyVs between nonhuman primates (NHPs) and humans, the surveillance of PyVs in wildlife is important. In this thesis, I examined PyVs in wild NHPs in Zambia with permission from the Zambia wildlife authority.

In chapter 1, I analyzed 200 DNA samples from the spleens and kidneys of NHPs (n = 100). I detected seven PyV genome fragments (7/200; 3.5%), and identified five full-length viral genomes. Phylogenetic analysis revealed that four PyVs were closely related to known PyVs, african green monkey PyV and simian agent 12. Only one virus detected from a vervet monkey spleen was found to be related, with relatively low nucleotide sequence identity (74%), to the chimpanzee PyV, which shares 48% nucleotide sequence identity with the human merkel cell PyV identified from merkel cell carcinoma. This virus was named vervet monkey PyV 1 (VmPyV1) as a novel PyV.

In chapter 2, I focused on further characterization of the VmPyV1. To examine whether the VmPyV1 genome produce viral proteins in cultured cells, the whole VmPyV1 genome was transfected into mammalian cells. I detected VP1, a major capsid protein, in expression in the transfected cells. Because VmPyV1 encodes the unique extended C-terminal VP1, I generated virus-like particles (VLPs) to examine the role of VmPyV1 VP1 in virion formation. Furthermore, I generated VLPs consisting of the deletion mutant (ΔC) VP1 lacking the C-terminal 116 amino acid residues, and compared its VLP formation efficiency and morphology to those of VLPs from wild-type (WT) VP1. WT and ΔC VLPs were similar in size, but the number of ΔC VLPs was much lower than that of WT VLPs in VP1-expressing cells. These results suggest that the length of VP1 is unrelated to virion morphology; however, the C-terminal region of VmPyV1 VP1 affects the efficiency of its VLP formation.

In this thesis, I detected PyV genomes in NHPs in Zambia and also identified a novel PyV, which was designated as VmPyV1. Moreover, I confirmed the formation of VLPs in the transfected cells with the plasmid encoding the VmPyV1 VP1. Although the pathogenicity of VmPyV1 and function(s) of the extra C-terminal region of VmPyV1 are still unclear, these findings provided information about the PyV prevalence in NHPs in Zambia and the C-terminal of VmPyV1 VP1 may have some function for efficient VLP formation. The surveillance of wildlife needs to be continued to examine the transmission possibility of infection agents.

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Investigation for the mechanism of quinolone antibacterial agent ofloxacin-induced chondrotoxicity in juvenile rats

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Quinolone antibacterial agents (quinolones) have been known to induce arthropathy in juvenile animals with some unknown mechanism, and therefore the prescription of quinolones has been contraindicated for children and adolescents. The purpose of the present study was to elucidate some factors leading to the onset of the chondrotoxicity. To do this, chondrotoxicity was investigated in male juvenile rats administered the quinolone ofloxacin (OFLX) as follows: 1) gene expression profile was assessed in the distal femoral articular cartilage of juvenile rats treated orally with OFLX by GeneChip, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), and *in situ* hybridization (ISH), 2) expression of genes which were changed in the articular cartilage of juvenile rats receiving OFLX was evaluated in juvenile rats given DC-159a or DX-619 that did not cause chondrotoxicity in juvenile rats, and 3) tail-suspended juvenile rats were studied to examine the effect of body-weight loading onto the articular cartilage on the occurrence of the chondrotoxicity.

OFLX was orally administered by gavage once to male juvenile Sprague-Dawley (SD) rats, 3 weeks of age. In the GeneChip analysis, the expression of 134 gene probes in the OFLX-treated group showed statistically significant differences with at least 1.5-fold difference from the control. qRT-PCR analysis for these genes exhibited that the expression of 7 genes including Tnfrsf12a, Ptgs2, Plaur, and Mmp3 were up-regulated, while that of Sstr1 and Has2 were down-regulated with dose dependency in the articular cartilage of juvenile rats. ISH analysis also showed that the expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 was noted in chondrocytes around the cartilage lesions. These results suggested that cytokines, chemokines and/or proteases produced by up-regulation of cell death-, inflammatory response-, stress response- and proteolysis-related genes play important roles in the onset of OFLX-induced chondrotoxicity in juvenile rats.

Secondly, the chondrotoxic potential between new synthesized quinolones DC-159a and DX-619, and OFLX were orally administered and the distal humerus and femur were subjected to microscopic examination. Moreover, concentrations of these quinolones in the femoral articular cartilage and gene expression of Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, Mmp3, Sstr1, and Has2 in the articular cartilage of the distal femur were investigated. In a microscopic examination, no changes in the articular cartilage were observed in any animal administered DC-159a or DX-619, whereas cavity formation and chondrocyte cluster in the cartilage of distal humerus and femur were noted in animals that received OFLX. In toxicokinetic analysis, the maximum cartilage concentration (cartilage C_{max}) values of DC-159a or DX-619 were little lower than or almost comparable to that of OFLX.

Furthermore, the area under the cartilage concentration-time curve up to 24 h post-dose (cartilage $AUC_{0-24\,h}$) value of DC-159a or DX-619 was higher than that of OFLX. In qRT-PCR analysis, up-regulation of Dusp1, Fos, and Mt1a, and down-regulation of Sstr1 and Has2 genes were observed in the femoral articular cartilage. However, Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes, which were up-regulated in the distal femoral articular cartilage exposed to OFLX, did not increase or slightly changed. In conclusion, the penetration of DC-159a or DX-619 into the cartilage was low compared with that of OFLX, and no obvious changes in Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes were observed in the articular cartilage of juvenile rats treated with DC-159a or DX-619, which was likely to be responsible for non-chondrotoxic potentials of DC-159a and DX-619.

Finally, the effect of body-weight loading onto the articular cartilage on the occurrence of chondrotoxicity was investigated in male juvenile SD rats given OFLX orally. Just after dosing of OFLX, hindlimb unloading was performed for 0, 2, 4, or 8 h by a tail-suspension method. Animals were sacrificed at 8 h post-dose, and then the distal femoral articular cartilage was subjected to a histological examination and an investigation for gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 by qRT-PCR analysis. As a result, cartilage lesions and up-regulations of these 4 genes that were seen in rats without the tail suspension were not observed in rats with the 8-h tail suspension, and a tendency to decrease in the incidence of the cartilage lesions and the gene expression was noted in a tail-suspension time dependent manner.

In conclusion, Tnfrsf12a, Plaur, Ptgs2, and Mmp3 genes whose expressions were considered related to the cartilage lesions increased in the articular cartilage of juvenile rats treated orally with OFLX. Our results also indicate that body-weight loading onto the cartilage is necessary to induce gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 and cartilage lesions caused by OFLX.

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