HOKKAIDO UNIVERSITY

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INFORMATION

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The titles of theses and other information are as follows:

Research on autoimmune glomerulonephritis model mouse — Candidate gene on the telomeric region of chromosome 1 derived from MRL/MpJ —

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Autoimmune glomerulonephritis (AGN), a complication of systemic autoimmune disease, is caused by glomerular deposition of immune complexes. Previous studies have reported that the telomeric region of chromosome 1 (Chr1) in some disease model mice contains several AGN susceptibility loci. In this study, we created B6.MRLc1(82-100) strain which was MRL/MpJ-type homozygous in the telomeric region of Chr1 (82-100 cM) by applying serial backcrosses between C57BL/6 and MRL/MpJ and confirmed the application of this mouse in AGN models.

The B6.MRLc1(82-100) showed glomerular damage with IgG depositions and these lesions were more severe in females. Furthermore, the proliferation of CD3-or B220-positive cells were observed in the spleen of the B6.MRLc1(82-100). Serum levels of blood urea nitrogen (BUN) and the anti-double strand DNA (dsDNA) antibody in the B6.MRLc1(82-100) were significantly higher than those of C57BL/6 at same age. From these findings, it became evident that the B6.MRLc1(82-100) had developed AGN.

This MRL/MpJ-derived AGN susceptibility loci were named "*Mag*" (*MRL autoimmune glomerulonephritis*). In order to reduce the number of regions of AGN susceptibility loci, we newly produced B6.MRLc1(82-86), B6.MRLc1(92-100), and B6.MRLc1(100) strains, and assessed their pathology. Only the B6.MRLc1(92-100) showed AGN and these results confirm the localization of AGN candidate genes on Chr1 (92-100 cM). As the candidate genes, the expressions of *Fcgr2b* (*low-affinity Fc receptor for IgG IIB*) and *Fcgr3*, which are major immune-associated genes present in Chr1 (92.3 cM) and code the inhibitory and active Fc gamma receptors, respectively, were assessed in kidney and immune organs of B6.MRLc1(92-100). Both receptors were localized on glomerular mesangial regions and dendritic cells, and elevated *Fcgr3*/*Fcgr2b* ratio were observed in these organs of B6.MRLc1(92-100). From these findings, it was strongly suggested that the imbalance between those genes had a great impact on the pathogenesis of AGN.

Female showed severe AGN in B6.MRLc1(82-100). To clarify the effects of sex hormones to AGN, we castrated sex organs of both sexes of the B6.MRLc1(82-100). The effects of gonadectomy in the male mice were more apparent than those in the female mice. The male gonadectomy group (GG) showed severer glomerular damage than the male sham operated groups (SG). Interestingly, CD3-positive cells were observed in both the thymic cortex and medulla, in all animals except the males in the SG.

These results suggested that the pathological sex-related differences in AGN were strongly affected by the inhibitory roles of male sex hormones.

The elevated expressions of inflammatory mediators were recently clarified by the array analysis using biopsy samples from patients. We attempted to identify the aggravating factors for AGN in the B6.MRLc1(82-100) strain by studying the expressions of inflammatory mediators by comprehensive PCRarray analysis. In the kidney of the B6.MRLc1(82-100), *Il1f6* (Interleukin 1 family member 6) and *Cxcl5* [chemokine (C-X-C motif) ligand 5] showed the highest expressions. These expressions were remarkably elevated with the developments of AGN in the kidney of B6.MRLc1(82-100). The *Il1f6* and *Cxcl5* proteins were mainly localized on distal parts of renal tubules and glomerular podocytes, respectively. These results suggest that the *Il1f6* and *Cxcl5* play important roles as aggravating factors of glomerular damage and tubulointerstitial lesions.

Thus, by research on disease model mice, it was concluded that the quantitative changes in candidate genes, sex hormones, and local aggravating factors were closely related to the pathogenesis of AGN. These findings will provide fundamental knowledge that will enable early diagnosis and subsequent therapy of AGN in the fields of medicine and veterinary science.

Original papers of this thesis appeared in *Histol. Histopathol*., **23**: 411-422 (2008), *Kidney Int*., **74**: 339-347 (2008) and *Lupus*, in press.

Studies on intracellular regulation and physiological function of the electrogenic Na^+-HCO_3^- cotransporter NBCe1-B in bovine parotid acinar cells

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Involvement of enteric glial cells in functional changes of enteric neurons in inflammatory bowel disease: Study using *in vitro* model system

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To identify the action of BK in the enteric nervous system (ENS), the effects of bradykinin (BK) on the enteric neuron and glial cells and on the interaction between them were examined by using primary culture of myenteric plexus cells from the rat intestine. Furthermore, the impacts of lipopolysaccharide (LPS) or inflammatory cytokines, which are elevated in serum of inflammatory bowel disease (IBD) patients were also investigated.

Immunocytochemical analysis showed that cells in primary culture of myenteric plexus were composed of enteric neurons (30%) and glial cells (68%). BK evoked $[Ca^{2+}]_i$ rise in both neurons and glial cells in a dose-dependent manner, which were abolished by a B2 receptor antagonist HOE140, but not by a B1 receptor antagonist lys-des-Ag^9-HOE140. BK evoked a sustained depolarization in neurons. B2 receptor expression in both neurons and glial cells was indicated by immunochemical and RT-PCR analyses.

In neurons, a non-selective cyclooxygenase (COX) inhibitor indomethacin significantly suppressed both the $[Ca^{2+}]_i$ increase and depolarization in response to BK. Prostaglandin E_2 (PGE_2), but not PGI_2, significantly potentiated the neural responses to BK. An EP_1 receptor antagonist SC19220 suppressed the neural responses to BK, whereas an EP_3 receptor agonist sulprostone did not potentiate it. Furthermore, BK evoked PGE_2 secretion from cultured myenteric plexus cells, the amount of which was declined by either indomethacin or lowering the number of glial cells in culture.

LPS or IL-1β, but not TNF-α, potentiated $[Ca^{2+}]_i$ responses to BK in both neurons and glial cells, the increment of which were suppressed by either pretreatment with an interleukin-1 receptor antagonist IL-1ra or reducing the number of enteric glial cells in culture. LPS increased IL-1β secretion from pure culture of glial cells.

After cultured with IL-1β, the augmented $[Ca^{2+}]_i$ response to BK in both cell types were partly inhibited by the B1 receptor antagonist. The B2 receptor antagonist completely abolished the responses to BK in neurons but not glial cells. Two different B1 receptor agonists, Lys-des-Arg^9-BK and BK fragment 1-8, evoked substantial $[Ca^{2+}]_i$ increase in glial cells but not neurons. Quantitative RT-PCR and immunocytochemical analyses showed that IL-1β potentiated B1 receptor but not B2 receptor expression in enteric glial cells.

The amount of PGE_2 release by BK from myenteric plexus cells was increased by IL-1β via activation of B1 receptor in glial cells. The enhanced PGE_2 release was suppressed completely by indomethacin and partly by nimesulide, a selective COX-2 inhibitor. These drugs also suppressed the neural responses to BK enhanced by IL-1β. Double immunostaing analysis showed that IL-1β increased COX-2 expression in glial cells but not neurons.

These results show that BK causes a $[Ca^{2+}]_i$ increase and depolarization in rat myenteric neurons through the activation of B2 receptors, which was partly associated with PGE_2 released from glial cells in response to BK. Furthermore, LPS

up-regulates B1 receptors and COX-2 expression in enteric glial cells via secretion of IL-1β in an autocrine fashion. The enhancement of PGE_2 production results in the alteration of cross-talk of the neuron-glial interaction. Such functional changes of enteric glial cells may cause dysfunction in the gut of IBD patients.

The original papers of this thesis appeared in *Neuroscience*, **145**: 642–653 (2007), *Neuroscience*, **151**: 222–231 (2008) and *J. Neurosci. Res.*, in press.

Studies on Immune responses in chickens infected with virulent and avirulent strains of Marek's disease virus

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Marek's disease (MD) is a progressive lymphoproliferative disease of chickens caused by Marek's disease virus (MDV). MD has been controlled by the vaccination with avirulent strains of MDV. Though the protective mechanism of MD vaccines has not been clarified, cell-mediated immunity (CMI) may be effective to prevent the proliferation of MDV. In this study, immune responses were compared between virulent MDV-challenged chickens with and without the vaccination with a vaccine strain, CVI988.

A comprehensive transcriptional analysis using DNA microarray was conducted with spleens from virulent MDV-challenged chickens with or without the vaccination at 7 and 21 days post-infection (dpi). In MDV-challenged chickens, the expressions of many genes related to "host-pathogen interaction" were up-regulated at 7 dpi. However, many of them were down-regulated while the amount of the MDV genome detected was increased, suggesting the immune suppression in chickens challenged with virulent MDV. Only few genes showed significant changes of their expressions in chickens only vaccinated. On the other hand, the expression levels of many genes were increased in vaccinated-challenged chickens at 21 dpi, suggesting the activation of immune responses effective to prevent the viral reactivation.

The expression levels of cytokine genes and interferon (IFN)-related genes were quantified by real-time PCR in MDV-infected chickens with or without the vaccination. Consistent with the results obtained from DNA microarray, the expression of genes related to CMI were up-regulated at the early phase of the infection but not at 10–14 dpi, the latent phase in challenged-chickens. Though the MDV genome load was increased at 21 dpi, indicating the viral reactivation, no apparent increase in the expression of those genes was observed in challenged-chickens. On the contrary, the MDV genome load was very low, but the levels of cytokine gene expressions were dramatically changed in vaccinated-chickens with or without challenge during the experimental period. The expression of IFN-γ gene was specifically upregulated in challenged-vaccinated chickens at 10 dpi. Enzyme-linked immunesorbent assay showed the active production of IFN-γ from splenocytes upon the stimulation with mitogen in vaccinated-challenged chickens at 7 and 14 dpi but not in challenged-chickens. In addition, the expressions of IFN-γ receptor (IFNGR) 2 and IFN regulatory factor (IRF)-3

mRNA were also upregulated in vaccinated-challenged chickens. No increase in the expression of these genes was observed in challenged-chickens. These results suggested that IFN-γ could play an important role in the protective mechanism induced by the MD vaccine, especially in the latent phase of the infection.

Consistent with the results obtained from DNA microarray, the induction of T cell receptor (TCR) 1-related genes was confirmed in vaccinated-challenged chickens at the late latent phase by real-time PCR. Moreover, the flow cytometric analysis revealed that the major subpopulation of TCR1$^+$ cells expressed CD8α brightly, called CD8α^{high} TCR1$^+$ cells. The number of this subset was increased specifically in vaccinated-challenged chickens in the late latent phase of the infection. Analysis of quantitative real-time PCR suggested that CD8α^{high} TCR2$^+$ cells, which is a major subset of CD8α^{high} cells, could function as cytotoxic T lymphocytes and a source of IFN-γ. On the other hand, the expression levels of IFNGR2 and IRF-3 were increased in CD8α^{high} TCR1$^+$ cells. Although the major function of CD8α^{high} TCR1$^+$ cells was not clarified in this study, this subset may be activated by IFN-γ produced by CD8α^{high} TCR2$^+$ cells to prevent the reactivation of MDV from the latent phase.

In conclusion, the present study showed the induction of IFN-γ and CD8α^{high} TCR1$^+$ cell population at the late latent phase in vaccinated-challenged chickens. They are possibly important key factors involved in the protective mechanism induced by a vaccine strain, CVI988. Further studies are necessary to clarify how the vaccine works. The data of this study will provide novel insights into the immunology of MD and valuable information to create a new MD vaccine which is desired to be safer and more effective than present ones.

The original papers of this thesis appeared in *Microbiol. Immunol.*, in press and *J. Vet. Med. Sci.*, in press.

Comparative immuno-epidemiological assessment of swamp and riverine type water buffaloes

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Epidemiological survey of bovine viral diarrhea virus (BVDV), bovine leukemia virus (BLV), *Anaplasma marginale* and *Babesia bigemina* infection in water buffaloes was performed in the Philippines. This is to get a rough estimate of the prevalence of these diseases in swamp and riverine type water buffaloes and somehow confirm that swamp type is more disease resistant/tolerant than riverine type water buffaloes. Result showed a 7.5%, 21.2%, 16.8% and 5.1% prevalence of BVDV, BLV, *Anaplasma marginale* and *Babesia bigemina* infection, respectively. Additional findings revealed the identification of new BVDV isolates belonging to BVDV type 1b group. These findings led to more in-depth immuno-epidemiologiocal investigation to compare the immune responses of swamp and riverine type water buffaloes.

Comparative assessment of interleukin (IL)-2, IL-12p35, IL-12p40, interferon γ (IFNγ) and tumor necrosis factor α (TNFα) was done by molecular cloning, sequencing and phylogenetic analysis. Multiple sequence comparison showed a high homology between the bubaline breeds, which

ranged from 98.6% to 100% similarity, whereas from 98.6% to 99.0% compared to cattle.

Cytokine response assessment was also done using an established real-time PCR quantification assay. Cytokine expression was measured in water buffaloes vaccinated with inactivated FMD vaccine as well as in naturally infected water buffaloes in the field. The counteraction between IFNγ and IL-4, and the possible suppressive action of IL-10 to that of IL-2 or IL-12 was noted, which is a common phenomenon between Th1 and Th2 cytokines. This study could aid in determining the immune responses that may be related to disease progression and pathogen-host specific pathogenesis.

Furthermore, to know whether the disparity in cytokine responses between swamp and riverine type water buffaloes contributes to disease resistance and susceptibility, an immuno-molecular investigation was conducted. Cytokine expression from Concanavalin A-stimulated PBMC revealed that both IFNγ and TNFα were highly expressed in swamp than in riverine type water buffalo. Single nucleotide polymorphism was found in IFNγ while 8 polymorphisms in TNFα promoter/enhancer regions. Luciferase assay confirmed that IFNγ and TNFα transcription activities were highly affected by the nucleotide difference in the cytokine promoter region. This suggests that disease tolerance or susceptibility of these buffaloes could be due to their transcription differences.

The original papers of this thesis appeared in *Vet. Immunol. Immunopathol.*, **113**: 348–356 (2006), *Comp. Immunol. Microbiol. Infect. Dis.*, **30**: 119–131 (2007), *J. Basic Microbiol.*, in press, *Res. Vet. Sci.*, in press and *Cytokine*, in press.

Development of anti-tick vaccine based on male tick molecules

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The magnitude of the negative impact exerted by ticks and tick-borne diseases (TBD) on livestock production requires the incorporation of several tick-control methods into livestock management practice. Among them, anti-tick vaccines, as an alternative to the current use of acaricides, have been considered to be an effective control method against ticks which transmit a variety of pathogens. This research focused on *Theileria parva*, the causative agent of East Coast fever, which is known as important TBD in Africa, and its vector tick *Rhipicephalus appendiculatus*, and molecular epidemiological survey of *T. parva* and analysis of host immune responses against *T. parva* infection were performed in the first part. Then, in the second part, candidate molecules for anti-tick vaccine were identified, and characterized from *R. appendiculatus* in order to establish the better methods to control ticks in the field.

A molecular-epidemiological study of TBD was conducted in Zambia. The infection rate for *T. parva* was relatively high in both Sanga cattle and ticks in Zambia. To investigate the relationship between the attachment duration of *R. appendiculatus* and the transmission of *T. parva* from the tick, infected adult ticks were allowed to attach to naive mice for variable lengths of time. The transmission of *T. parva* from the ticks to mouse skin started within 72 hours after tick

biting, and the transmission rate was increased with the duration of tick attachment. Furthermore, increased expressions of pro-inflammatory cytokine, interleukins 1 and 6, were observed in *T. parva*-infected cattle during the acute phase when the parasite proliferated. These results showed that ticks transmit *T. parva* to the host in their mid-feeding stage, and high protozoan load and resultant intense inflammatory responses in cattle could be involved in the disease progression of the *T. parva* infection, including the severity of clinical signs observed in infected cattle. In addition, a high infection rate of *T. parva* in both cattle and ticks in Zambia may cause problems in susceptible cattle if they are newly introduced.

Two testis-derived molecules, RAMP and voraxin, were identified from the *R. appendiculatus* tick, and characterized. RAMP, a male-specific molecule in the spermatophore, was considered to be related to female attachment behavior in *R. appendiculatus* ticks. When microinjected with recombinant RAMP, virgin female ticks showed significantly prolonged attachment duration during feeding, but no effect was observed on their fed weight. The second molecule, voraxin has been reported as a pair of testicular proteins, voraxins α and β, which are transferred to female via copulation, and stimulate female blood feeding in the lone star tick, *Amblyomma hebraeum*. In order to determine if voraxin α can be a candidate antigen for anti-tick vaccine, the *voraxin* α gene was cloned from fed-male *R. appendiculatus* ticks, and the protective effect of the *E. coli*-produced recombinant voraxin α (r-voraxin α) was assessed against the ticks. Ticks fed on r-voraxin α-immunized rabbit showed the reduced weight gain and oviposition rate. This result suggested that anti-tick vaccine using voraxin α can be a potential strategy to the reduced the size of vector population. In addition, voraxin α is likely to show this effect on the broad-spectrum of tick species because predicted amino acid sequence of voraxin homologues are highly conserved among the different tick genera.

In conclusion, this research provided fundamental information for the development of effective anti-tick vaccine by using male-derived molecules. The identification of testis-derived molecules related to female reproduction would be essential to improve the conventional design of anti-tick vaccines.

The original papers of this thesis appeared in *Exp. Parasitol.*, **120**: 337–342 (2008), *J. Vet. Med. Sci.*, **71**: 49–54 (2009) and *Vet. J.*, in press.

Studies on infection and propagation of neurotropic viruses in cultured dorsal root ganglia cells

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Many viruses which invade the central nervous system (CNS) via the peripheral nervous system (neural spread) have been reported ever since and they induced serious damage of central and peripheral nervous system to both animal and human. The studies on the infection and propagation mechanisms of these viruses are useful to understand the characters of them and finally they may be related to the development of therapeutic or preventive measures.

Sensory neurons are most probably involved in the virus transfer from the site of the primary

infection to the CNS. Dorsal root ganglia (DRG) are crucial in the initial events or the ground of secondary viral propagation of the *in vivo* and *in vitro* infection by neurotropic viruses, since they can act as gates for the virus entrance into the CNS. DRG has been useful in the understanding of these initial virus-host interaction events. In this study, I chose three neurotropic viruses; swine hemagglutinating encephalomyelitis virus (HEV), pseudorabies virus (PRV) and rabies virus (RV), which show neural spread in animals.

In chapter 1, I compared neurotropism and the roles of neuronal cytoskeletal components in the neural spread of HEV and PRV using cultured nerve cells and non-neuronal cells from DRG of newborn mice. HEV infected nerve cells but did not infect non-neuronal cells, whereas PRV infected both cell types. By using cytoskeletal inhibitors, it was suggested that infectivity of HEV and PRV within and among nerve cells depended on microtubules and intermediate filaments of nerve cells. Therefore, the viruses may be transported between the cell body and axonal terminals of neurons by fast axonal flow.

In chapter 2, the cell tropism of RV and the roles of neuronal cytoskeletal components in the production of viral protein were examined, using cultured nerve cells and non-neuronal cells from DRG of newborn mice. The infectivity of RV in DRG nerve cells was low, and intracellular viral nucleoprotein synthesis was independent from neuronal cytoskeleton. In addition, RV infected non-neuronal cells originated from DRG, suggesting that Schwann cell could be another candidate supporting neural spread of RV.

The present results suggest a diversity of modes in infection and neural spread, and these finding may be important for the development of preventive and therapeutic measures to the diseases caused by the neurotropic viruses.

Original papers of this thesis appeared in *J. Vet. Med. Sci.*, in press and *Jpn. J. Vet. Res.*, **56**: 181–188 (2009).

Mode of hantavirus infection among host rodents and characterization of a vaccine candidate strain of Puumala virus

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Hantavirus is widely distributed in the continents of Eurasia and Americas carried by rodent species, which play an important role to transmit the virus to humans. The two human illnesses caused by hantavirus are Hemorrhagic fever with renal syndrome (HFRS) and Hantavirus pulmonary syndrome (HPS). It is generally known that HFRS caused by Hantaan virus (HTNV) and Seoul virus (SEOV) is distributed in Eastern Asia, while HFRS by Puumala virus (PUUV) is distributed in wide areas of Europe and Western Russia. The field surveys for hantavirus infection in indigenous rodents species have been conducted in Hokkaido, Japan for more than 25 years, and antibodies to hantavirus have been detected in *M. rufocanus*. Previous studies revealed that the hantavirus carried by *M. rufocanus* is most closely related to PUUV among the known hantavirus types and designated as Hokkaido virus (HOKV).

The eradication of HFRS is extremely difficult since large number of wild rodents carry hantaviruses pathogenic to humans. Therefore, understanding of virus transmission among rodents and development of the vaccine are most

effective measures to control HFRS. One of the purposes of this study was to reveal the mode of HOKV infection in *M. rufocanus*. The mode of the HOKV transmission in the host animals was analyzed by detection of antibodies to HOKV, virus antigen, and virus RNA in 199 *M. rufocanus* captured in Nakagawa and Tobetsu, Hokkaido. The results indicate that males are more infected than females among *M. rufocanus*. HOKV RNA of infected animals was identified from different organs (lungs, spleen, kidney and blood clot), that may serve as the site for virus replication or maintenance. It was also revealed that acute infected animals have more than 10 times RNA copy numbers in their lungs than persistently infected animals. Therefore, acute infected animals may have more capability to transmit the virus to the other susceptible animals and humans. Higher population density of rodents is related with higher incidence of HFRS in Europe and may be related with increased number of acute infected animals. Such situation is an important risk factor for HFRS outbreak. Therefore, monitoring rodent population density and understanding the mode of hantavirus infection among rodents are quite useful measures to prevent and predict HFRS.

Until now no vaccines have been developed against PUUV infection. The other goal of the study is to obtain the basic information of a PUUV strain DTK/Ufa-97 having well growing feature particularly by genetic and antigenic analyses before this strain is chosen as the vaccine strain. The PUUV strain DTK/Ufa-97 replicates in Vero E6 cells cultured in serum free medium (SFM) better than Sotkamo as well as in minimum essential medium (MEM) containing fetal bovine serum (FBS). The virus yields in the culture fluid of infected Vero E6 cells were 8.3×10^4 ffu/ml for DTK/Ufa-97 strain and 3.5×10^2 ffu/ml for Sotkamo strain at 14 days post infection in SFM. Advantages of using SFM were free from animal or human origin components which could potentially carry viruses or prions. The data also show that the PUUV strains are genetically diversed but antigenically conserved. Anti-DTK/Ufa-97 antibody neutralized other PUUV strains. Therefore, DTK/Ufa-97 strain is a candidate strain for PUUV vaccine which can be widely used over to the 12.5 million populations at risk for PUUV infection of European countries and European Russia.

The information obtained in this study is important in understanding the epidemiology of hantavirus infection in the host animals and the basis of disease control of HFRS.

The original paper of this thesis appeared in *Microbiol. Immunol.*, **51**: 1081–1090 (2007) and *Jpn. J. Vet. Res.*, **56**: 151–165 (2008).

Role of survivin in radiation-induced apoptosis and its redox regulation

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Ionizing radiation is a useful tool for cancer therapy. In hematopoietic cell lines such as malignant lymphoma and leukemia cells, apoptotic cell death is prone to be induced by ionizing radiation. In these cell lines, ionizing radiation is known to easily promote apoptotic

signaling such as cytochrome *c* release from mitochondria, followed by activation of caspase and caspase-activated DNase. However, in solid tumor cell lines derived from adenocarcinoma, squamous cell carcinoma or melanoma, the constitutive or inducible antiapoptotic proteins such as Bcl-2 family and the inhibitor of apoptosis protein (IAP) family proteins seem to inhibit this apoptotic signaling. Survivin is a IAP family, which is intensively expressed in the G2/M phase and phosphorylated by cell division cycle 2 (CDC2), and known to have strongly antiapoptotic activity through interaction with Smac/DIABLO which acts as a proapoptotic protein. Its expression is prominently upregulated in most human cancer cells, but is undetectable or very low in normal tissues. Therefore, survivin seems to be an ideal target for radiosensitization in solid tumor cells. In this study, I firstly examined whether dominant negative vectors encoding survivin mutants, T34A (phosphorylation site by CDC2) and D53A (binding domain for smac/DIABLO) abrogate the function of constitutive survivin and facilitate radiation-induced apoptotic cell death in tumor cells.

In first experiment, we prepared two adenoviral vectors for pAd-T34A and pAd-D53A to investigate the mechanism of radioresistance of solid tumor cells. When T34A and D53A were overexpressed in human lung carcinoma A549 and human cervical carcinoma HeLa cells, radiation-induced apoptosis was significantly enhanced. Furthermore, we examined the binding capability of survivin with Smac/DIABLO in the cells overexpressing these mutants. Coimmunoprecipitation analysis revealed that mutant forms of survivin, D53A and T34A, could bind to Smac/DIABLO but with much less affinity compared to the authentic form. These results suggest that radiation-induced apoptosis of tumor cells is increased by inhibition of the interaction between survivin and Smac/DIABLO through overexpression of T34A and D53A. These results indicated that radiation-induced apoptosis of tumor cells is increased by inhibition between survivin and Smac/DIABLO through overexpression of T34A and D53A, suggesting that survivin may be effective for therapeutic treatment in radioresistant solid tumors.

Recently, it has been reported that apoptosis in human leukemia Molt-4 cells is remarkably inhibited if antioxidants such as N-acetyl-L-cystein (NAC) and Trolox are added to the medium within several minutes after X irradiation. This suggests that the secondary production of ROS occurs as a late event after irradiation and that these secondary ROS play an important role in radiation-induced apoptotic signaling. Mitochondria in mammalian cells are well-known to play an important role in the intrinsic pathway of genotoxic-agent-induced apoptosis by releasing cytochrome *c* into cytosol and to be a major source of reactive oxygen species (ROS).

The second aim of this study was to examine whether mitochondrial ROS are involved in radiation-induced apoptotic signaling in A549 cells. Post-irradiation treatment with NAC inhibited cytochrome *c* release from mitochondria but did not affect expression levels of Bcl-2, Bcl-X_L and Bax, suggesting that the late production of ROS triggered cytochrome *c* release. This treatment also inhibited radiation-induced apoptosis in T34A-and D53A-survivin-overexpressed A549 cells. Experiments using DCFDA (a classical ROS fluorescence probe) and MitoAR (a novel mitochondrial ROS probe) demonstrated that intracellular and mitochondrial ROS were enhanced 6 h after X irradiation. Furthermore, the ROS production ability of mitochondria isolated from A549 cells was evaluated by ESR spectroscopy combined with a spin-trapping reagent (CYPMPO). When isolated mitochondria were incubated with NADH, succinate and CYPMPO, an ESR spectrum due to CYPMPO-OOH was detected. This NADH/succinate-dependent ROS production from mitochondria of irradiated cells was significantly increased in comparison with that of unirradiated cells. Oxymetry using litium 5, 9, 14, 23, 27, 32, 36-octa-n-butoxy-2, 3-naphthalocyanine (LiNc-BuO) and ESR spectroscopy showed that oxygen consumption in irradiated A549 cells,

which was inhibited by rotenone, a mitochondrial complex I inhibitor, was increased in comparison with that of unirradiated cells. These results indicate that ionizing radiation enhances the ROS production from mitochondria to trigger cytochrome *c* release in A549 cells.

In summary, present experiments clearly demonstrated that abrogation of the function of constitutive survivin facilitate radiation-induced apoptotic cell death in radioresistant solid tumor cells such as A549 and HeLa cells and radiation-induced secondary ROS from mitochondria play as an essential role in cytochrome *c* release from mitochondria in their apoptotic signal pathways.

The original papers of this thesis appeared in *Cancer Lett*., **277**: 64–71 (2009) and *Cancer Lett*., **259**: 71–81 (2008)

The genetic bases of inter-and intrastrain differences in CYP2D-dependent drug metabolism in rats

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The rat has frequently been used in the fields of pharmacology and toxicology. However, inter-and intrastrain differences in drug response are often observed among rat strains, and are an important issue to be overcome. Still, the fundamental mechanism of inter-and intrastrain differences regarding drug metabolizing enzymes have been overlooked. In particular, the highest impact among drug metabolizing enzymes is exerted by the cytochrome P450 (CYP), the major phase I enzyme. In this thesis, I focused on the CYP2D subfamily that is a key enzyme involved in individual differences in drug metabolism.

In chapter 1, I elucidated the mechanism of low expression of CYP2D2 mRNA in Dark Agouti (DA) rats. DA rats have been considered as the animal model for the poor metabolizer phenotype of human CYP2D6 due to the low expression of CYP2D2 protein. I elucidated a single nucleotide substitution in the promoter region of the CYP2D2 gene, resulting in its attenuated expression of mRNA in DA rats. This is the first report that a mutation alters the expression level of CYP2D2 in rats. In addition, I identified heterogeneous nuclear ribonucleoprotein K, which serves as a molecular scaffold to bind to the core promoter of the CYP2D2 gene where the mutation was observed in DA rats.

In chapter 2, I clarified the relationships between genetic polymorphisms of the CYP2D3 gene and inter-and intrastrain differences in diazepam *p*-hydroxylation, the major metabolic pathway of diazepam at low concentration in rats. I identified the enzyme involved in diazepam *p*-hydroxylation to be CYP2D3 using yeast recombinant CYP2D isoforms. In addition, the polymorphism of diazepam *p*-hydroxylation among four rat strains (Sprague-Dawley, Brown Norway, DA, and Wistar rat) was revealed to be due to inter-and intrastrain differences in expression levels of CYP2D3 protein. Then, I clarified that single nucleotide mutation in coding region of CYP2D3 gene caused the nonfunctional truncated protein in poor metabolizer rats.

In this thesis, I clarified the genetic bases of inter-and intrastrain differences in CYP2D-dependent drug metabolism in rats. Clarification of inter-and intrastrain differences in rats will be further useful for predicting variability in human pharmacokinetics. Consequently, it is worth-

while to fully characterize animals used in pharmacokinetics studies from the point of view of the genetic expression of metabolic enzymes. Therefore, my work will strongly support the strain consideration in selection of a rat strain for drug metabolisms.

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Changes of reproductive physiology during conception period and maintenance of pregnancy in Hokkaido sika deer (*Cervus nippon yesoensis*)

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In the present thesis, the author focused on the period during conception and maintenance of pregnancy in Hokkaido sika deer. There was no insight into physiological factor influences to the conception date and basic reproductive physiology such as ovarian dynamics and hormonal changes were also unclear in sika deer. Therefore author conducted the study to understand basic reproductive physiology around the period of conception and pregnancy.

In chapter 1, the objective was to characterize ovarian dynamics and hormonal changes during seasonal transition. The follicular and luteal dynamics and changes in steroid hormones of eight captive female Hokkaido sika deer were examined and those were compared between the three periods: before, during and after the first estrous cycle. There was at least one ovulation without estrus and subsequently transient formation of corpus luteum (CL) and low increases in progesterone were observed just before the first estrus of the estrous season in all females. Since progesterone is known to induce estrus, transient increase in progesterone may be involved in the onset of estrous season. Majority of number of inter-estrus follicular waves were two and three during estrous season. The crystallization of cervical mucus suggested being a indicator of determining the peak of estrus since it was related to day of estrus.

In chapter 2, the objective was to characterize the ovarian dynamics and hormonal changes during conception and early gestation, and reveal the origin and timing of formation of multiple CLs, a unique characteristic of pregnant sika deer, to discuss its significance. Follicular and luteal dynamics together with changes in steroid hormones of six captive female Hokkaido sika deer were examined. Two CLs were formed in three females conceived at the first estrus of the estrous season. Second CL was formed from ovulated dominant follicle of follicular wave emerged soon after ovulation associated to the first estrus of the estrous season. On the other hand, first dominant follicle did not ovulate and only one CL formed in three females conceived at the second estrus of the estrous season. Therefore, it was suggested that second CL forms in case that female conceive at the first estrus of the season. Since progesterone concentrations did not differ dependent on number of CL, second CL was assumed to support the first CL by secreting progesterone otherwise insuffi-

cient for the maintenance of pregnancy.

In chapter 3, the objective was to characterize the early fetal development and produce the formula for precise fetal age estimation during early pregnancy. Fetal growth in five captive female Hokkaido sika deer was examined by transrectal ultrasonography until about 59–61 days of pregnancy. Fetus could be detected by Day 20–26 in all females. Straight crown-rump length (SCRL), curved crown-rump length (CCRL), head length (HL), trunk depth (TD) and heart rate (HR) of fetus were measured each examination and those increased linearly as pregnancy progress. By regression analysis, it was concluded that precise estimation of fetal age in early gestation can be done best by measurement of SCRL since it was highly correlated and measurable earlier than others.

In chapter 4, the objective was to characterize the action site of progesterone and estrogen during pregnancy. The spatio-temporal localization of estrogen receptor alpha (ERα) and progesterone receptor (PR) in the uteri of 24 Hokkaido sika deer derived from wild were examined immunohistochemically. Fetal ages of early pregnancy period were estimated based on chapter 3. Distribution of ERα and PR could be observed until Day 25 of pregnancy in sika deer and it was relatively long time compared to dominated ruminant. Therefore, not only progesterone but estrogen may secrete and act until Day 25 of pregnancy.

During seasonal transition, transient increase in progesterone concentration just before the first estrus of the season was observed and this is considered to be important for the onset of estrous season. In addition, the origin and timing of formation of second CL in pregnant females reveal and it was hypothesized that second CL forms in case that female conceived at the first estrus of the season. Taking all into consideration, ovulation is likely the key incident which involve in determining conception date. Therefore, the hypothalamic-pituitary-gonadal axis must be investigated for further understanding of detail physiological factor influences conception date in sika deer.

The present study provided the useful method for utilizing carcasses. Therefore, many carcasses are available for studying the physiological changes during early pregnancy more in detail than before. Owing to the method, involvement of estrogen in early pregnancy was suggested. Together with steroid hormone receptor distribution, peripheral steroid hormone concentration must be measured for understanding relationship between them. Further, examinations of other factors which may involve in pregnancy such as interferon tau and the distribution of oxytocin receptor in uterus are needed for understanding the mechanism for successful conception in sika deer.

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Studies on the establishment of a therapeutic model for prion diseases

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Prion diseases are fatal neurodegenerative disorders in humans and animals, and are strongly associated with the conversion of PrP^{C} to PrP^{Sc}. Therefore, compounds and/or strategies that

inhibit PrP^{Sc} formation are of therapeutic interest. However, no effective treatment for these diseases is currently available.

Anti-PrP mAbs have been shown to inhibit PrP^{Sc} formation in cells persistently infected with prion and passive immunization could protect the animals from prion infection through peripheral challenge when anti-PrP mAbs were administered in the early stage of infection. These results suggest that the mAbs are candidates for the treatment of prion diseases, however, the effect of mAbs on disease progression at middle-late stage of the disease remains unclear. Therefore, in Part I of this thesis, I have evaluated the therapeutic effect of anti-PrP mAbs by intraventricular infusion before and immediately after the clinical onset of the disease in mice infected with prion. The intraventricular infusion of anti-PrP mAbs reduced PrP^{Sc} formation to 70–80% of mice treated with negative-control mAb even when the treatment was started at the clinical onset. Additionally, spongiform changes, microglial activation and astrogliosis also appeared milder in mice treated with anti-PrP mAbs than in those treated with a negative-control mAb. Kinetic analysis revealed that anti-PrP mAbs decelerated the PrP^{Sc} formation rather than active degradation of PrP^{Sc}. Furthermore, their infusion initiated after clinical onset prolonged the survival of mice infected with the Chandler strain by 12 days compared with those infused with negative-control mAb without apparent adverse effects. These results suggest that immunotherapy can be a candidate for the treatment of prion diseases.

Although the intraventricular infusion of anti-PrP mAbs prolonged the survival of mice infected with the Chandler strain, the distribution of anti-PrP mAbs was relatively restricted to the hippocampus and thalamus. Thus, it is possible that an improved delivery of anti-PrP mAbs may enhance the therapeutic effects. In addition, the fact that antagonizing PrP^{Sc} formation is not sufficient to restore degenerated lesions necessitated a search for ways to promote regeneration of degenerated neuronal tissues. Bone marrow-derived mesenchymal stem cells (MSCs) have been reported to migrate to brain lesions in experimental models of ischemia, tumors and neurodegenerative diseases, and to ameliorate functional deficits. Therefore, in Part II, I have evaluated the therapeutic potential of MSCs for treating prion diseases. Transplantation of immortalized human MSCs (hMSCs) into the hippocampus prolonged the survival of prion-infected mice by 8 days compared with nontransplanted mice. The hMSCs preferentially migrated to the prion-specific brain lesions after intracerebral transplantation and even after intravenous injection. In addition, a subpopulation of hMSCs in the brains of prion-infected mice produced various trophic factors such as brain derived neurotrophic factor and vascular endothelial growth factor, and differentiated into cells of neuronal and glial lineages. These results suggest that MSCs may help to regenerate neuronal tissues damaged by prion propagation and furthermore act as a cellular vehicle for the delivery of therapeutic genes to the brain lesions.

In this thesis, I showed that anti-PrP mAbs and MSCs are candidates for treatments of prion diseases. However, neither of them could completely arrest disease progression nor ameliorate the disease when used alone. The mechanisms of their effects on prolongation of survival are expected to differ and the combination of MSCs with anti-PrP mAbs may therefore have a synergistic effect in the treatment of prion diseases. Although further studies are required on the possibility of a synergistic effect, I believe the results described in this thesis will help to promote further efforts to establish therapeutic agents for prion diseases.

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