Japanese Journal of Veterinary Research 58(3&4): 185–194, 2010

INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy on September 24, 2010 to 6 recipients.
The titles of theses and other information are as follows:

Molecular characterization of tetraspanins of *Echinococcus multilocularis* and studies for their potential as vaccine antigens

Dang Zhisheng

Department of Collaboration and Education, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Echinococcus multilocularis is one of the most important pathogens in genus *Echinococcus*, family Taeniidae, which causes zoonotic alveolar echinococcosis. In this study, cDNAs of 7 tetraspanin (TSP) proteins from *E. multilocularis* larva cDNA library were cloned and characterized and the vaccine efficacies of recombinant TSPs were evaluated.

In chapter I, 7 TSP cDNAs were cloned from *E. multilocularis* larva cDNA library which had been constructed by the vector-capping method. The full-length cDNAs of TSPs were amplified and sequenced. By BLAST searching of TSP sequences, the homologues in other parasite species were obtained and sequence identity analysis was carried out. Especially, the sequence comparison between *Echinococcus* TSP5 and *Taenia* T24, a diagnostic antigen, showed very high identity. In order to investigate the location of TSP5 in *Echinococcus* larva, the large extracellular loop (LEL) was expressed in *E. coli* and anti-recTSP5 antibody was prepared. By immunohistochemistry, it was found that TSP5 is expressed on the surface/outer membrane of cyst and protoscolex, which implied that the protein is exposed to host immune system and should posses well immunogenicity. From the results in this chapter, it can be expected that, like those TSPs from other parasites, tetraspanin of *E. multilocularis* may be the potential candidates for diagnosis and vaccine development against echinococcosis.

In chapter II, the vaccine efficacies of 7 recTSP proteins were evaluated in mice immunized and challenged with *E. multilocularis* eggs. BALB/c mice were immunized subcutaneously with recTSPs. ELISA analysis revealed strong IgG immune response against recTSPs as well as cross-reaction between them. Immunized mice were then challenged with infective eggs. The cyst lesion numbers formed in liver were counted to determine the vaccine efficacies of 7 TSPs. The result showed that TSPs displayed varying protective efficacies (37–88%) against *E. multilocularis* metacestode primary infection.

In chapter III, the protective efficacies of TSPs by intranasal administration were evaluated primarily. Here, TSP1 and TSP3, which showed high protective efficacies in section 1 of chapter II, were selected as the antigens for intranasal vaccination. ELISA analysis showed significant IgA induction in nasal cavity, intestine and liver. Cyst lesion number reduction in liver of TSP1 and TSP3 immunized mice were 37% and 62% separately. The protective efficacies between

different administration routes (subcutaneous and intranasal) of TSP3 were comprised to investigate effects of different antibody response against *E. multilocularis* infection. The results revealed 82% and 61% cyst lesion reduction of TSP3 when separately administrated subcutaneously and intranasally. Subcutaneous administration of TSP3 displayed higher protective efficacy than intranasal one.

In conclusion, this study focused on the characterization of *E. multilocularis* TSPs and evaluation of their vaccine efficacies in different administration routes, which indicated that TSPs are the potent vaccine candidates against alveolar echinococcosis.

The original papers of this thesis appeared in *Mol. Biochem. Parasitol.*, **168**: 117-119 (2009) and *Vaccine*, **27**: 7339–7345 (2009).

Studies on the molecular mechanisms underlying host lymphocyte-transformation by *Theileria parva*

Kyoko Hayashida

Department of Collaboration and Education, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Theileria parva is a tick-transmitted intracellular protozoan parasite that causes East Coast fever, a fatal bovine lymphoproliferative disease. *T. parva*-infected lymphocytes behave like tumor cells, and the cellular transformation that results from infection is thought to be due to molecules secreted from schizonts into the host cytoplasm or exposed on the parasite surface, which affect the host's signaling pathway. The aim of this study is to reveal the mechanisms underlying host cell transformation by *Theileria* parasites.

In chapter I, a novel *T. parva* schizont-derived cytoskeleton binding protein (TpSCOP) expressed in the schizont stage of *T. parva* was biochemically characterized. TpSCOP was shown to interact with F-actin *in vitro*. Expression of TpSCOP in a murine lymphocytic cell line resulted in the activation of NF-κB signaling pathways, leading to apoptosis resistance. The activation of mitogen-activated protein kinase (MAPK), including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), was also detected. Furthermore, the introduction of TpSCOP into *T. parva*-infected cells also enhanced the activation of NF-κB. This is the first report to demonstrate that a parasite-derived molecule has the ability to activate the host NF-κB pathway. Based on these results, TpSCOP likely plays an important role in apoptosis inhibition during *Theileria* infection.

In chapter II, in the process of screening anticancer compounds library, TIBL (trans-4-iodo, 4'-boranyl-chalcone), a specific inhibitor of mouse double minute 2 (MDM2), was found to markedly inhibit proliferation and induce apoptosis of *T. parva*-infected lymphocytes in a dose-dependent manner. MDM2, which acts as an ubiquitin ligase for p53, plays a central role in regulating the stability of p53 and has oncogenic activity when overexpressed in cells. MDM2 was overexpressed in *Theileria*-infected lymphocytes, and several alternatively spliced isoforms were identified. The overexpression of MDM2 was associated with impaired accumulation of functional p53 in *T. parva*-

infected lymphocytes, despite normal transcription of p53 mRNA. Treatment of *T. parva*-infected lymphocytes with TIBL stabilized p53 protein accumulation, suggesting that MDM2 interfered with the functional activity of p53. The findings indicate that the abnormal p53 response in *T. parva*-infected cells is a consequence of aberrant expression of MDM2. Expression of MDM2 may therefore be a key factor in maintaining uncontrolled proliferation of *T. parva*-infected lymphocytes.

In conclusion, these studies have provided evidence that the expression of TpSCOP protein from the *Theileria* schizont and the aberrant MDM2 expression in *Theileria*-infected cells, are involved in the transformation process during *Theileria*-infection.

The original papers of this thesis appeared in *Mol. Biochem. Parasitol.*, **174**: 8–17 (2010).

Identification of DELE, a novel DAP3-binding protein which is crucial for death receptor-mediated apoptosis induction

Tanenobu Harada

Department of Bioresources, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Apoptosis is known as a form of programmed cell death, essential for the organogenesis of individuals, maintenance of the immune system, and elimination of injured cells and tumor cells. In cases when the regulatory mechanism of apoptosis becomes imbalanced, it induces severe diseases; for example, tumor development, breakdown of the immune system or neurodegeneration.

Members of the death receptor (DR) family including tumor necrosis factor (TNF) receptor type1, Fas, DR3, TNF-related apoptosis inducing ligand receptors (TRAILR1/DR4 and TRAILR2/DR5) and DR6 are responsible to transduce apoptosis signal. These receptors are important for the regulation of many physiological and pathological events related to several human diseases. DR member family molecules induce apoptosis by the trimerization and aggregation of DR molecules on the plasma membrane. This receptor aggregation leads to recruitment of several subcellular proteins such as Fas-associated death domain protein (FADD) to the cytoplasmic domain of these receptors. The recruitment of FADD to DR is mediated through each death domain (DD) of DR to induce activation of caspases, cysteine proteases resulting in apoptosis induction.

Death associated protein 3 (DAP3) is a GTP binding protein originally identified as a molecule which is responsible for activating interferon γ-induced apoptosis, and it has been reported that DAP3 is also important as a signal transducer for apoptosis induced by DR stimulation. In TRAIL induced apoptosis, DAP3 binds to TRAIL receptors, DR4 and DR5, and forms a complex with caspase-8 through FADD to activate caspase-8 depending on the stimulation of TRAIL. Thus, DAP3 is functional in cytoplasm for activation of TRAIL-mediated signaling pathway immediately downstream of DR4 and DR5. On the other hand, DAP3 is also observed abundantly in mitochondria, and the deletion mutant of DAP3 which lacks N-terminal region containing mitochondrial localization signal is not able to activate an apoptosis

induced by the stimulation of FasL and TNF-α. These observations suggest that the mitochondrial localized DAP3 is also responsible for induction of apoptosis. However, molecular mechanisms of mitochondrial DAP3 for induction of apoptosis are poorly understood.

From this point of view, initially, the mitochondrial localized proteins were selected by using the prediction program of subcellular localization (PSORT II; http://psort.ims.u-tokyo.ac.jp/) from positive clones obtained by yeast two-hybrid screening, previously performed in our laboratory to identify DAP3-binding proteins. The results showed that the subcellular localization of one of the positive clones which encoded a functionally unknown protein was predicted to be localized in mitochondria. Consequently, focused on this clone designated as DELE (death ligand signal enhancer), the molecular functions of DELE were analyzed. The DELE protein is a 55 kDa protein consists of 515 amino acids, and found to contain a mitochondrial targeting sequence at the N-terminus, two tetratrico peptide repeat (TPR) motifs.

In this study, the molecular functions of DELE on the apoptosis induced by the stimulation of TNF-α, FasL and TRAIL were characterized. The co-immunoprecipitation analysis revealed that DELE actually binds to DAP3 in mammalian cells. The subcellular localization of DELE was analyzed by confocal laser scanning microscopy. The result shows that the subcellular localization of DELE is mainly observed in mitochondria, as was predicted by the analysis using PSORT II.

The A549 cell lines in which the DELE gene is stably expressed were found to become susceptible to the apoptosis induction by the stimulation of TNF-α, anti-Fas, and TRAIL. In addition, knockdown of DELE gene expression by siRNA treatment significantly protected the HeLa cells from the apoptosis induction by the stimulation of these cytokines. Moreover, activation of caspase-3, -8, and caspase-9 by these stimulations was significantly suppressed by the knockdown of DELE gene expression.

In conclusion, this study demonstrated that the newly identified DELE regulates the death receptor-mediated apoptosis through the regulation of caspase activity.

The full text of this thesis (PDF) appears at http://eprints.lib.hokudai.ac.jp/dspace/handle/2115/43931
The original papers of this thesis appeared in *Apoptosis*, In press.

Studies on the Resistance to Diminazene Aceturate in *Babesia gibsoni In Vitro*

Shiang-Jyi Hwang

Laboratory of Veterinary Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Canine babesiosis caused by *Babesia gibsoni* is treated with diminazene aceturate (DA). DA can temporarily improve the clinical signs of canine babesiosis but is unable to eliminate the parasites from infected dogs, and relapses often occur. Therefore, it is believed that *B. gibsoni* might achieve the resistance against DA. However, there is no report clearly demonstrating the DA resistance in *B. gibsoni*. Therefore, in this study, a DA-resistant *B.*

gibsoni strain was developed and the differences between the DA-resistant *B. gibsoni* strain and wild-type *B. gibsoni* were compared. First, a DA-resistant *B. gibsoni* strain was developed *in vitro* by the gradual increase of the DA concentration from 1 to 200 ng/ml. The parasites survived and proliferated in the medium containing 200 ng/ml DA, which is much higher than the 50% inhibitory concentration (IC_{50}) of DA for *B. gibsoni*. Subsequently, these parasites were removed from erythrocytes and exposed directly to 200 ng/ml DA. They survived and invaded fresh erythrocytes, though wild-type *B. gibsoni* did not survive. Based on these results, the parasites cultured with 200 ng/ml DA were determined as a DA-resistant *B. gibsoni* strain. Thereafter, to investigate the characteristics of the DA-resistant *B. gibsoni* strain, the effects of other antibabesial drugs, including clindamycin, doxycycline, metronidazole and pentamidine, on the DA-resistant *B. gibsoni* strain were examined. The DA-resistant *B. gibsoni* strain showed strong resistance against pentamidine, and weak resistance against clindamycin and doxycycline. Moreover, the IC_{50} values of clindamycin, doxycycline and pentamidine for the DA-resistant strain at day 7 were higher than those for the wild-type *B. gibsoni*, respectively. These results indicated that the DA-resistant *B. gibsoni* strain could have resistance not only to DA, but also to other antibabesial drugs. Especially the DA-resistant *B. gibsoni* strain exhibited resistance against pentamidine, which shares similar structure with DA. In other protozoan, the mechanisms of drug resistance through mutations and/or amplification in drug transporters or drug targets were demonstrated. Therefore, the analysis for those metabolic pathways in the DA-resistant *B. gibsoni* strain will lead to elucidate the mechanism of the action of DA against *B. gibsoni*.

Consequently, to characterize the DA-resistant *B. gibsoni* strain, the transcription level of *B. gibsoni* heat shock protein 70 (*BgHsp70*) gene, which plays important roles in cell proliferation and the control of cellular function, was measured by quantitative real-time reverse transcription-polymerase chain reaction. In *Plasmodium falciparum*, Hsp70 has been proposed to contribute to the development of drug resistance. Therefore, the change in the transcription levels of the *BgHsp70* gene was analyzed in DA resistance. During the development of the DA-resistant *B. gibsoni* strain, DA-resistant *B. gibsoni* variants, which were maintained in culture with DA from 1 to 175 ng/ml for more than 8 weeks, were also obtained. The copy number of the *BgHsp70* transcripts in the DA-resistant variant cultured with 1 ng/ml DA was significantly lower than in wild-type *B. gibsoni* while those in DA-resistant variants increased with escalating doses of DA from 1 to 75 ng/ml, though they were lower than in wild-type *B. gibsoni*. Moreover, those in DA-resistant variants cultured with $>$ 125 ng/ml DA were almost the same as wild-type *B. gibsoni*. It is hypothesized that the transcription level of the *BgHsp70* gene would be reduced during the selection of the DA-resistant *B. gibsoni* strain under the long-term weak pressure of DA, and then would be returned to the normal level after achieving resistance against DA. However, since the reason why the transcription level of the *BgHsp70* gene was reduced is still unclear, further study will be necessary to confirm this hypothesis.

In conclusion, it was clearly demonstrated the development of DA resistance of *B. gibsoni in vitro*. The DA-resistant *B. gibsoni* strain obtained resistance against other antibabesial drugs. Moreover, the transcription level of the *BgHsp70* gene was reduced by the weak DA pressure and then recovered when *B. gibsoni* had achieved resistance against DA. However, the role of BgHsp70 for the DA resistance in *B. gibsoni* remains unclear. Further studies of BgHsp70 might prove to determine the mechanism of the DA resistance of *B. gibsoni*. Finally, the results obtained from this study could contribute to a better understanding of the DA resistance in *B. gibsoni in vitro*.

The full text of this thesis (PDF) appears at http://eprints.lib.hokudai.ac.jp/dspace/handle/2115/43997
The original papers of this thesis appeared in *J. Vet. Med. Sci.* **72**: 765–771 (2010) and *Jpn. J. Vet. Res.*, **58**: 155–164 (2010).

A novel regulatory role of retinoid in lipid metabolism in mammalian cells

Mabrouk Attia Abd Eldaim Abd Elkader

Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Vitamin A is essential for a variety of physiological processes, including vision, immune functions, reproduction, embryonic development as well as cellular growth and differentiation. It must be ingested from a diet containing retinol, retinyl esters, and/or β-carotene, and stored in the liver. When necessary, it is delivered by associating plasma carrier protein, RBP4. Lately, a novel role of RBP4 in energy metabolism is reported in rodent and human.

In this study, I first assessed how plasma RBP4 and retinol levels were regulated in ruminants by measuring the RBP4 and retinol concentrations in the plasma, colostrum, and milk of cows in various physiological and pathophysiological conditions. The plasma RBP4 levels in non-pregnant non-lactating (control) cows were around 45 μg/ml, which were sustained during 60 h fasting, but decreased significantly 4 h after LPS administration. The basal plasma retinol concentration was around 30 μg/dl, but this decreased to approximately one-third and one half of these values during fasting and 8 h after LPS challenge, respectively. The plasma RBP4 and retinol levels in cows 3 to 6 days before parturition were comparable to those of the controls. However, on the day of parturition both were significantly decreased and had returned to basal levels by two weeks after calving. Interestingly, RBP4 was clearly detected in colostrum (16.4 ± 5.6 μg/ml), but was only faintly detected in milk from cows at 7 and 15 days after calving. The retinol concentrations in colostrum were almost 10-fold higher than those in plasma, while those in milk were comparable to those in plasma. These results suggest that RBP4 and retinol levels are independently regulated under physiological and pathophysiological conditions and that RBP4, like retinol, is transferred from maternal stores to calves through colostrum.

Retinoic acid (RA), an active metabolite of retinol, is shown to suppress adipogenesis both *in vivo* and *in vitro*, but there are also some contradictory reports. Here I further examined the cause of the contradictory results regarding effects of RA. Bovine intramuscular preadipocytes cultured in medium containing a high glucose concentration (HG, 17.5 mM) accumulated lipids strongly, but to a lesser extent in medium containing a normal glucose concentration (NG, 5.5 mM). Treatment with RA during the culture period decreased and increased lipid accumulation in the presence of NG and HG, respectively. Similar opposing effects of RA that were dependent on the glucose concentration were observed in 3T3-L1 cells. Moreover, the changes in lipid accumulation were accompanied by parallel alterations in fatty acid synthase (FAS) and sterol regulatory element

binding protein (SREBP)-1 gene expression in 3T3-L1 cells and bovine cells. Transfection of SREBP-1 siRNA into 3T3-L1 cells cultured with HG abolished RA-induced enhancement of lipid accumulation and FAS expression. Transfection of nuclear form of SREBP-1a cDNA into 3T3-L1 cells cultured with NG abrogated RA-induced suppression of lipid accumulation and FAS expression. These results suggest that RA suppresses and stimulates lipid accumulation of mouse adipocytes and bovine intramuscular adipocytes through extracellular glucose concentration-dependent modulation of SREBP-1 expression.

To clarify the mechanism(s) by which RA alters SREBP-1a expression glucose concentration-dependently, I first tested the possible involvement of oxidative stress in the expression because hyperglycemic conditions per se could be an oxidative stress. The cells treated with H_2O_2 (2 μM), an oxidative stressor, induced increased cleavage of SREBP-1a, without affecting amounts of SREBP-1a mRNA and precursor protein, and enhanced expression of FAS gene and lipid accumulation. Increased cleavage of SREBP-1a by H_2O_2 was also observed even in the presence of RA. These results suggest that H_2O_2 treatment enhances the cleavage of SREBP-1a precursor protein, which independently occurs with the RA suppression of SREBP-1a gene expression. These results also show clear difference from the finding that RA enhancement of SREBP-1a expression in high glucose medium, suggesting oxidative stress induced by high glucose is unlikely to be involved in the SREBP-1a gene transcription.

Next, I examined the involvement of nuclear receptors in the regulation of SREBP-1a expression. The cells treated with RA in normal glucose medium for 6 days increased and decreased expression of retinoic acid receptor (RAR) α and RARγ genes and expression of SREBP-1a, retinoid X receptor (RXR) α, peroxisome proliferator-activated receptor (PPAR) γ, PPARβ/δ, and liver X receptor (LXR) α genes, respectively, whereas the cells treated with RA in high glucose medium increased and decreased expression of SREBP-1a, RXRβ, PPARβ/δ, and LXRβ genes and expression of RARα gene, respectively. Interestingly, glucose concentration-dependent opposing effects of RA on expression of SREBP-1a gene were observed when the cells cultured only for 24 h. Moreover, gene expression of the nuclear receptors in the cells cultured for 24 h basically followed the pattern of respective gene expressions in the cells cultured for 6 days. Expression of carbohydrate-responsive element binding protein was not detected in 3T3-L1 cells cultured in normal and high glucose medium for 1 and 6 days.

Adipogenic differentiation of 3T3-L1 cells was induced by treating the cells for the first 2 days with a mixture of insulin, isobutylmethylxanthine and dexamethasone (Dex, a synthetic glucocorticoid). In normal glucose medium, RA suppressed SREBP-1a expression even without treatment with the mixture for 1day. In high glucose medium, RA increased SREBP-1a expression only in the presence of Dex treatment, suggesting that glucocorticoid plays a role in modulation of RA responsiveness in high glucose condition. Moreover, in the cells cultured with high glucose and Dex, RA increased PPARγ, PPARβ/δ, RXRα, RXRβ and LXRβ genes, but not RARα and LXRα, although in normal glucose medium Dex alone had no apparent effects on the expression of these genes even in the presence of RA.

To clarify roles of nuclear receptors in glucocorticoid- and RA-dependent induction of SREBP-1a mRNA in the cells cultured in high glucose medium, effects of nuclear receptor agonists and antagonists were examined. Treatment of the cells with RA (RAR and RXR agonist) and 9-cis-RA (RXR agonist), but not TTNPB (RAR agonist), glucocorticoid-dependently enhanced expression of SREBP-1a gene. Treatment with HX531 (RXR antagonist) and T0070907 (PPARγ antagonist) inhibited the glucocorticoid- and RA-dependent induction of SREBP-1a mRNA. Treatment with troglitazone

(PPARγ agonist) and T0901317 (LXR agonist), but not GW0742 (PPARβ/δ agonist), also enhanced expression of SREBP-1a gene glucocorticoid-dependently. Furthermore, transfection with LXRβ and PPARγ siRNA, but not LXRα and PPARβ/δ siRNA, abrogated glucocorticoid- and high glucose-dependent enhancement of SREBP-1a expression in the presence of RA. In addition, LXRβ siRNA also suppressed enhancement of expression of PPARγ and RXRβ genes by RA and Dex, while PPARγ siRNA inhibited the enhancement of RXRβ gene expression but not of LXRβ gene expression. These results indicate the pivotal roles of LXRβ, PPARγ and RXR in glucocorticoid-, retinoic acid-, and high glucose-dependent induction of SREBP-1a mRNA, and suggest that LXRβ is up-stream regulator for expression of PPARγ.

In summary of the latter part, RA displays opposing effects on SREBP-1a gene expression and subsequent lipid accumulation in cultured mouse 3T3-L1 cells in an extracellular glucose concentration dependent manner. These opposing effects occurred at the early phase of adipogenesis, and were dependent on glucocorticoid to induce LXRβ, PPARγ and RXR, the key nuclear factors influencing the SREBP-1a gene expression. As LXRβ is shown to be a glucose sensor, this nuclear factor might play a role in the glucose-dependency.

The original papers of this thesis appeared in *J. Dairy Res.*, 77:27–32 (2010) and *Jpn. J. Vet. Res.*, **58**: 149–154 (2010).

Biological defense system against xenobiotics in meat-producing animals

Wageh Sobhy Abdelrahem Abdallah Darwish

Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Meat-producing animals are frequently exposed during their lifetime to a lot of xenobiotics which affect on their biological systems, growth, disease response and lead to changes on the carcass quality. These changes may have some public health impact if people consumed such contaminated meat or meat products. Meat-producing animals have developed enzyme systems which help them to metabolize such xenobiotics. Studying of the profile of the different enzymes used in xenobiotics metabolism may be a good tool to reflect the pre-slaughter exposure to xenobiotics in the meat-producing animals. As many of these enzymes tend to increase upon exposure to xenobiotics, so these enzymes are considered as biomarkers for xenobiotics exposure.

Cytochrome P450 superfamily and other phase II enzymes are considered as major enzyme systems that play important roles in xenobiotics metabolism. Thus, in this thesis, I studied the biological response to xenobiotics in meat-producing animals by studying the characterization of different phase I and II enzymes, mainly CYP1A, UGT1A1 and GSTA1 subfamilies, in these animals.

In chapter I, I investigated the tissue-specific mRNA expression of different cytochrome P450 (CYP) isoforms, UDP glucuronosyl transferase 1A1 (UGT1A1) and glutathione-S-transferase (GSTA1) in the different tissues of cattle using quantitative real-time polymerase

chain reaction (qPCR). CYP1A1-like mRNA was expressed in all of the tissues examined including liver, with the highest expression level in kidney. CYP1A2-, 2E1- and 3A4-like mRNAs were only expressed hepatically. Interestingly, significant expression of CYP2B6-like mRNA was recorded in lung tissue, while CYP2C9-like mRNA was expressed in liver and kidney tissues of the examined cattle. UGT1A1- and GSTA1-like mRNA were expressed in all of the examined tissues, except the mammary glands, and the highest expression levels were recorded in kidney. The high expression of UGT1A1 in lung tissue and GSTA1 in liver tissue was unique to cattle, this has not been reported for rats or mice. The findings of this chapter strongly suggest that the liver, kidneys and lungs of cattle are the major organs contributing to xenobiotic metabolism. Moreover, induction of CYP1A1, UGT1A1 and GSTA1 are considered as good biological biomarkers for pre-slaughter exposure to xenobiotics.

In chapter II, I extended my study to include other growing sources for meat production such as deer and horses. Thus, I investigated and characterized the metabolic activities of CYP1A in deer, cattle and horses in comparison to those of rats using ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-demethylation (MROD) assays. Also, I performed an inhibition study for these activities using anti-rat CYP1A1 antibody and identified that these activities were due to the CYP1A subfamily. Interspecies differences in the CYP1A-dependent activities were highly observed in this chapter. In particular, I found that the horse had the highest EROD and MROD activities among the examined animal species. In the kinetic analysis, the horses showed the highest Vmax and catalytic efficiency (Vmax/Km), followed by the cattle, deer and rats.

In chapter III, I compared the mutagenic activation activity of hepatic microsomes from the three meat-producing animals (cattle, deer and horses) with those of rats as a reference species. In the Ames *Salmonella typhimurium* TA98 assay, the liver microsomes of all examined animals mutagenically activated benzo[*a*]pyrene, an ideal promutagens, in terms of production of histidine-independent revertant colonies. The microsomes of horses had the highest ability to produce revertant colonies of the examined animals under both low and high substrate concentrations. Inhibition of this mutagenic activity using α-naphthoflavone, anti-rat CYP1A1, anti-rat CYP3A2 and anti-rat CYP2E1 antibodies suggested that this activity was mainly because of CYP1A1 in these animals as well as in rats. The addition of co-factors for two phase II enzymes, microsomal UDP glucuronosyl transferase (UGT) and cytosolic glutathione-S-transferase (GST), reduced the production of the revertant colonies in a concentration-dependent manner. Interestingly, horses had the highest reduction rate among the examined animals, suggesting that phase II enzymes play a great role in producing a state of balance between the bioactivation and detoxification of xenobiotics in these meat-producing animals.

In chapter IV, I elucidated that accumulation of carotenoids is a possible cause for inter-species difference in CYP1A-dependent activity in this group of animals. The relationship between inter-species differences in CYP1A-dependent activity and the accumulated carotenoids and retinoids as candidates of dietary CYP1A inducers in ungulate species was clarified. Interestingly, there were positive correlations between the accumulated carotenoids, such as β-carotene, with both EROD activity and CYP1A protein expression. These correlations were negative with the accumulated retinoids, such as retinol. The β-carotene was major component of carotenoids in ungulates, and known as an inducer of CYP1A. On the other hand, the retinol is reported as the reducer of CYP1A. Other factors which affect CYP1A1 expression, such as polycyclic aromatic hydrocarbons, were also analyzed. To cancel the effects of inter-species difference in CYP1A

induction signal cascade among these animals, the rat cell line (H4-II-cells) was treated with the extracted carotenoids from the examined animals. CYP1A expression and dependent activities in the treated cells had confirmed that the carotenoid accumulation is, at least in part, a regulator for the inter-species differences in CYP1A expression and activities.

In chapter V, I determined a partial sequence of CYP1A1 in the camel and its phylogenetic position. The deduced amino acid sequence of camel CYP1A1 showed the highest identity 94% with those of sheep and cattle CYP1A1. In a phylogenetic analysis, the camel CYP1A1 isoform was located beside sheep and cattle CYP1A1. When I studied the distribution of camel CYP1A1 mRNA in different tissues, I found that this isoform was expressed in all tissues except the hump. Interestingly, the lungs of all the camels and tongues of two of the three animals showed high expressions of CYP1A1 mRNA, and this may indicate exposure to ligands of aryl hydrocarbon receptor such as environmental pollutants or flavonoids.

In conclusion, in this thesis, I clarified the biological defense systems to xenobiotics in the meat-producing animals. I confirmed the inter-species differences in CYP1A expression and dependent activities. Subsequently, I explained the mechanism of the protection of these animals against the mutagenic activation of promutagens and procarcinogens. Also, I declared a possible cause for the inter-species differences in CYP1A dependent activities and expression. Moreover, I characterized cytochrome P450s and phase II enzymes in some ungulates such as camel, cattle, deer, horses and deer in comparison to rats.

The full text of this thesis (PDF) appears at http://eprints.lib.hokudai.ac.jp/dspace/handle/2115/43888

The original papers of this thesis appeared in *J. Vet. Med. Sci.*, **72**: 221–224 (2010), *J. Vet. Med. Sci.*, **72**: 561–566 (2010), *J. Vet. Med. Sci.*, In press, Animal, In press and *Food. Chem. Toxicol.*, In press.