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INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy (Ph.D) in Veterinary Medicine on March 23, 2007 to 13 recipients.

The titles of theses and other information are as follows :

Effects of leptin on bovine immune cell functions

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Leptin, the *obese* gene product, is produced and secreted mainly from adipocytes and acts centrally via its specific receptor in the hypothalamus to regulate body weight and energy expenditure. It is also known that leptin plays a role in the regulation of neuroendocrine, reproductive, haematopoietic and immune functions, in rodents and humans. In ruminant, circulating leptin concentrations are considerably altered by changes in nutritional and physiological conditions such as parturition and lactation, in which periods it is known that the susceptibility to infectious insults in cows increase. To examine the roles of leptin in bovine immune system, I investigated direct effects of leptin on functions of bovine peripheral T lymphocyte, polymorphonuclear neutrophil (PMN) and monocyte/macrophges.

I first tested the effects of leptin on mitogenic response of peripheral T lymphocytes. Leptin alone did not influence but slightly suppressed proliferation of T lymphocytes in the presence of concanavalin A (Con A). Leptin also inhibited proliferation of T lymphocytes induced by anti-CD3 antibody. Con A treatment activated some protein kinases such as JAK2, while anti-CD3 antibody treatment increased mRNA expression of suppressor of cytokine signaling (SOCS) 3, interferon (IFN)-γ, interleukin (IL)-2 and IL-4 in T lymphocytes. Leptin alone increased only SOCS3 mRNA expression. Simultaneous treatments with the mitogens and leptin enhanced IFN-γ mRNA expression but decreased IL-2 mRNA expression, without any synergistic effect on phosphorylation of the protein kinases or mRNA expression of SOCS3 and IL-4. These results suggest that leptin modulates bovine T lymphocyte functions, possibly at the transcription level.

I next tested the effects of leptin on superoxide production and degranulation of peripheral PMN. Leptin, tumor necrosis factor (TNF)-α, phorbol myristate acetate (PMA) and opsonized zymosan particles (OZP) did not stimulate degranulation responses, while zymosan-activated serum (ZAS) did. Neither leptin nor TNF-α enhanced the ZAS-induced degranulation responses. TNF-α, PMA, OZP and ZAS increased superoxide production in different magnitudes, whereas leptin did not. These results indicate that leptin does not have direct effect on degranulation and superoxide production in bovine PMN, although TNF-α influences superoxide production.

I finally tested the effect of leptin on cytokine expression and prodction of monocyte/

macrophage. Of cytokines examined, monocyte/macrophage constitutively expressed TNF-α, IL-1β, IL-12p35, IL-18 mRNA, and faintly expressed IL-12p40 mRNA. Monocyte/macrophage treated with leptin augmented the mRNA expression of TNF-α and IL-12p40, but not the other genes tested, to comparable levels of the cells treated with lipopolysaccharide. Monocyte/macrophage treated with leptin, however, induced the mRNA expression of caspase-1, which is shown to mediate conversion of latent IL-β and IL-18 to their active forms, and actually increased production of IL-β as well as TNF-α These results suggest that leptin directly acts on monocyte/macrophage to produce factors that could modify T lymphocytes function, such as IL-12 and IL-18 through IL-12p40 and caspase-1 inductions.

To further elucidate the role of monocyte/macrophage derived factors, peripheral blood mononuclear cells (PBMC) containing both monocyte/macrophage and T lymphocytes were treated with ConA and leptin. PBMC treated with either leptin alone or combination of leptin and ConA enhanced their proliferative activity by 10-40%, compared with those treated with ConA alone. This is quite contrast the result on isolated T lymphocytes and indicate that leptin produced some cytokines such as IL-12 from monocyte/macrophage that enhanced T lymphocyte.

Taken together, this study has demonstrated that leptin is a key molecule in the regulation of bovine immune systems. That is, leptin directly modifies the T lymphocyte functions toward Th1 phenotype and the monocyte/macrophage functions to produce a variety of cytokines. Therefore, leptin might indirectly regulate immune functions through the cytokines produced.

Original papers of this thesis appeared in *J. Vet. Med. Sci.,* 69 : 125-131 (2007), *J. Vet. Med. Sci.,* 69 : 513-518 (2007) and *Vet. J.,* (in press) (2007).

Roles of adipocyte-secreted factors in bovine mammary gland development

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Mammary gland undergoes a cycle of morphogenic and functional changes accompanied with the process of pregnancy, lactation, and involution under regulation by systemic hormones. Recent evidences indicate that stromal adipocytes are no longer considered to be inert cells only functioning as energy stores, but are emerging as endocrine cells that secrete various factors including leptin, and that stromal cell-derived factors play critical roles in mammary epithelial development.

Hepatocyte growth factor (HGF) is produced by stromal cells and induces proliferation, differentiation and motility of adjacent epithelial cells by binding to the receptor c-Met. It was proved that HGF induces tubulogenesis of mammary epithelial cells. However, limited information of HGF about the expression and function in stromal adipocytes is currently available, especially in ruminant.

In this thesis, first, I demonstrated the molecular cloning of bovine HGF and c-Met cDNAs. Both HGF and c-Met mRNAs were expressed in various bovine tissues, including adipose tissue and mammary gland. HGF mRNA was detected only in the inactive stage of bovine mammary gland development, but not in the developing, lactating, and involuting stages, while c-Met mRNA was detected in the inactive and involuting stages. Immunohistochemical analysis demonstrated that c-Met protein was found in mammary epithelial cells in the inactive, developing, and involuting stages.

The expression of HGF mRNA was also investigated in stromal adipocytes cultured *in vitro*. HGF mRNA was hardly detected in bovine and human preadiocytes, but significantly increased after differentiation to mature adipocytes. In contrast, HGF mRNA was expressed in murine and rat preadipocytes and diminished after the differentiation. Analyses of the promoter activity and mRNA stability of *hgf* genes in these cells suggest that the mRNA stability might affect the expression level of HGF mRNA in murine 3T3-L1 cells and that the expression in bovine adipocytes may be regulated by other mechanisms.

Next, I examined the effects of adipocyte-secreted factors on morphogenesis of bovine mammary epithelial cells (BMEC) by three-dimensional culture method using collagen gel *in vitro*. Conditioned medium (CM) from bovine mature adipocytes induced morphogenesis of BMEC, while CM from bovine preadipocytes had no effect. On the other hand, CM of 3T3-L1 preadiocytes induced morphogenesis although CM of 3T3-L1 mature adipocytes did not. Recombinant HGF induced tubulogenesis of BMEC. Anti-HGF antibody or NK4 HGF antagonist prevented the morphogenesis by CM of bovine mature adipocytes and 3T3-L1 preadipocytes. HGF protein was more abundantly detected in the CM of bovine mature adipocytes than that of preadipocyte. In contrast, HGF concentration in the CM of 3T3-L1 preadipocytes was higher than that of mature adipocytes. These results indicate that secretion of HGF from adipocyte is controlled in species-specific manners.

Finally, I examined the effect of systemic hormones (insulin, cortisol and prolactin) and leptin, another adipocyte-secreted factor, on the morphogenesis of BMEC. Neither the mixture of hormones non leptin induced the morphogenesis. However, the hormone mixture enhanced HGF-induced morphogenesis, resulting in developing larger ductal and alveolar organoids. On the other hard, the HGF-induced formation of organoids was dose-dependently inhibited by simultaneous addition of leptin. The results suggest that the systemic hormones and adipocyte-secreted factors such as HGF and leptin intricately regulate mammary epithelial morphogenesis.

In conclusion, expression of HGF and c-Met mRNA were altered during the development of bovine mammary gland and HGF production in stromal cells was regulated in the adipocyte differentiation-dependent and species-specific manners. Adipocyte-secreted HGF induced mammary epithelial morphogenesis that was positively or negatively regulated by the mixture of systemic hormones and leptin, respectively.

Original papers of this thesis appeared in *Domest. Anim. Endocrinol.,* 30 : 239-246 (2006) and *Jpn. J. Vet. Res.,* 54 : 183-189 (2007).

Studies on the pathogenesis and rapid diagnosis of bovine viral diarrhea

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Original papers of this thesis appeared in *J. Virol. Methods,* 138 : 140-146 (2006) and *Virus Res.,* 116 : 78-84 (2006).

Pharmacological profile of a novel phosphodiesterase (PDE) 7 A and PDE 4 dual inhibitor, YM-393059

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YM-393059 is a novel phosphodiesterase (PDE) inhibitor that inhibited PDE7A with high potency (IC_{50} = 14 nM) and PDE4 with moderate potency (IC_{50} = 630 nM), but did not inhibit PDE1, PDE2, PDE3, or PDE5 at all at concentrations up to10 μM. In a cell-based assay, YM-393059 was found to inhibit interleukin (IL)-2 production induced by anti-CD3 antibody, *Staphylococcal enterotoxin B,* and phytohaemagglutinin in mouse splenocytes with IC_{50} values ranging from 0.48 to 1.1 μM. It also inhibited anti-CD3 antibody-induced interferon (IFN)-γ and IL-4 production in splenocytes with IC_{50} values of 1.8 and 2.8 μM, respectively. When orally administered, YM-393059 inhibited anti-CD3 antibody-induced IL-2 production in mice. In addition, YM-393059 inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor-α production *in vivo* more potently than IL-2 (ED_{50} values of 2.1 and 74 mg/kg). YM-393059 was also found to inhibit LPS-induced IL-1β production with ED_{50} value of 11.8 mg/kg, but it had only a slight affect on IL-6 production.

To characterize the pharmacological profile of YM-393059, its effects on several acute and chronic inflammation models were examined. In acute inflammation models, YM-393059 significantly suppressed the delayed-type hypersensitivity reaction to sheep red blood cells in mice with an ED_{50} value of 17.1 mg/kg. YM-393059 failed to suppress paw edema in the carrageenin-induced edema model in rats. These pharmacological effects were similar to those of cyclosporine, a typical T-cell immunosuppressant. However, YM-393059, but not cyclosporine, significantly inhibited zymosan-induced neutrophil accumulation in mice with an ED_{50} value of 25.7 mg/kg. In mouse toluene-2,4-diisocyanate-induced contact dermatitis, a chronic inflammation model, YM-393059 and cyclosporine significantly suppressed ear edema at doses of 30 and 20mg/kg, respectively. In this model, YM-393059also tended to reduce serum IgE antibody level, whereas cyclosporine dramatically

potentiated it. These results suggest that YM-393059 inhibits both Th1- and Th2-cell-dependent reactions and also the function of neutrophils.

Furthermore, the anti-rheumatic effect of YM-393059 was measured in a mouse collagen-induced arthritis model. The acute rejection of cardiac allograft in rats and the α_2-adrenoceptor agonist-induced sleep in mice were used to examine the side effects. YM-393059 and cyclosporine significantly suppressed arthritis development at doses of 30-100 and 20 mg/kg, respectively, in the prophylactic treatment of collagen-induced arthritis mice. At these dose ranges, YM-393059 significantly inhibited increases in the serum IgG level that occurred in response to autoantigenic collagen in arthritic mice, whereas cyclosporine did not. In contrast, cyclosporine completely suppressed the acute rejection of cardiac allografts in rats, whereas YM-393059 did not, even at a dose of 100 mg/kg. These results suggest that YM-393059 shows the anti-rheumatic effect via suppressing autoimmune response without affecting the response to alloantigens. PDE4 inhibitors cause the dose-limiting side effects of nausea and emesis. In contrast to PDE4 inhibitors, YM-393059 did not shorten the duration of α_2-adrenoceptor agonist-induced sleep in mice, which is a model for the assessment of the side effects caused by PDE4 inhibitors.

In conclusion, the pharmacological profile of YM-393059 suggests that a PDE7A/PDE4 dual inhibitor would be a novel and attractive approach for the treatment of a wide variety of inflammatory and autoimmune disorders, such as rheumatoid arthritis.

The original paper of this thesis appeared in *Eur. J. Pharmacol.,* 541：106-114 (2006), *ibid.,* 550：166-172 (2006), and *ibid.,* 559：219-226 (2007).

Taxonomical and epizootiological study of *Trichinella* infections among wild animals in Hokkaido, Japan

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Studies on the novel diagnosis and cerebrospinal fluid biomarkers of canine and feline lysosomal disease

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The lysosomal diseases encompass more than 40 distinct diseases, most of which are caused by the deficient activity of a lysosomal hydrolase leading to the progressive, intralysosomal accumulation of substrates such as sphingolipids, mucopolysaccharides, and oligosaccharides. In the thesis, I primarily focus on GM1 and GM2 gangliosidoses, included in sphingolipidosis. GM1 gangliosidosis is lethal lysosomal disease that affects progressively the brain and multiple systemic organs and is caused by a defect of acid β-galactosidase, resulting in the intralysosomal accumulation of the specific physiological substrates, GM1 ganglioside. GM2 gangliosidosis is autosomal recessively inherited lethal disease that causes mainly progressive neurodegeneration and brain dysfunction which results from massive neuronal storage of GM2 ganglioside. The hydrolysis of GM2 ganglioside is catalysed by lysosomal β-hexosaminidase (Hex) in cooperation with GM2 activator protein. There are three isoenzymes of Hex : Hex A, a heterodimer of αβ subunits, Hex B, a homodimeric structure ββ, and Hex S, a homodimeric structure αα, and only Hex A can act on the complex of GM2 ganglioside and GM2 activator protein. GM2 gangliosidosis is classified, based on gene deficiency, into three major variant forms that differ in expression pattern of these isoenzymes and GM2 activator protein.

In veterinary clinics, many lysosomal diseases including gangliosidosis in animals are not investigated to a definitive diagnosis. Generally, it is needed both to identify the storage materials in the central nervous system (CNS) and to demonstrate the defect of a specific enzyme for the definitive diagnosis of lysosomal diseases. Therefore, these diseases are diagnosed by postmortem examination in most cases. An early diagnosis using samples obtained with minimal invasiveness from living animals results in a quick determination of the prognosis of the affected animals.

At present, only symptomatic therapy is available for patients with GM1 and GM2 gangliosidoses in both human and veterinary medicine. Some promising therapies have been investigated now. These novel therapies are expected to be effective and safe, but all of these therapies need further investigation using appropriate animal models prior to application to humans. Canine and feline models show definite neurological features similar to those of humans. Especially, canine GM1 gangliosidosis is an excellent model of human disease because canine and human β-galactosidases are structurally similar. However, dogs are not as fertile, making it more difficult and expensive for many individuals to be used in therapeutic trials. If it is possible to evaluate the degree or extent of degeneration in the CNS using samples obtained from living individuals, it will lessen the number of dogs necessary to determine the efficacy of potential therapeutic programs for GM1 gangliosidosis and simultaneously contribute to the welfare of experimental animals.

In the thesis a method for the diagnosis

of canine and feline GM1 and GM2 gangliosidoses was established using cerebrospinal fluid (CSF). The concentration of GM1 ganglioside in CSF was determined by thin-layer chromatography (TLC)-enzyme staining using biotin-conjugated cholera toxin B, which specifically binds with GM1 ganglioside. The concentration of CSF GM1 ganglioside was increased in Shiba dogs with GM1 gangliosidosis, and the increased level was approximately proportional to the age of the dogs. The concentration was high in the affected dog even at 5 months of age, when Shiba dogs with GM1 gangliosidosis first manifest neurologic signs. Similarly, the concentrations of CSF GM2 ganglioside were increased in Golden retriever dog and Japanese domestic cats with GM2 gangliosidosis 0 variant (Sandhoff disease). These were determined by TLC-enzyme immunostaining using mouse anti-GM2 ganglioside antibody (IgM). From these results it was concluded that this method enables a definitive and early diagnosis of canine GM1-gangliosidosis, and probably of GM2 gangiosidosis, even if tissues and organs cannot be obtained. However, other ganglioside than major storage substrates related directly to deficient enzyme can also be elevated in CSF of cases of GM1 and GM2 gangliosidoses. So, it is necessary to assay for specific enzyme deficiencies to definitively separate each gangliosidosis from another gangliosidosis.

Also, the present study investigated CSF biomarkers for estimating degeneration of the CNS in experimental dogs with GM1 gangliosidosis and preliminarily evaluated the efficacy of long-term glucocorticoid therapy for GM1 gangliosidosis using the biomarkers identified here. Periodic studies demonstrated that GM1 ganglioside concentration, activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), and concentrations of neuron specific enolase (NSE) and myelin basic protein (MBP) in CSF were significantly higher in dogs with GM1 gangliosidosis than those in control dogs, and their changes were well related with the months of age and clinical course. In conclusion, GM1 ganglioside, AST, LDH, NSE and MBP could be utilized as CSF biomarkers showing CNS degeneration in dogs with GM1 gangliosidosis to evaluate the efficacy of novel therapies proposed for this disease. In addition, I preliminarily treated an affected dog with long-term oral administration of prednisolone and evaluated the efficacy of this therapeutic trial using CSF biomarkers determined in the present study. However, this treatment did not change either the clinical course or the CSF biomarkers of the affected dog, suggesting that glucocorticoid therapy would not be effective for treating GM1 gangliosidosis.

Studies on the prevention of milk fever using dietary anion salts in dairy cows

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Risk factors of injury of the superficial digital flexor tendon (SDFT) in the Thoroughbred race horses, and the effects of uphill exercise on the force in the SDFT during running

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Molecular basis of cell tropism of JC virus infection

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The original papers of this thesis appeared in *Acta. Neuropathol. (Berl).,* 111 : 379-387(2006), *Microbiol. Immunol.,* 51 : 327-337 (2007) and *Microbiol. Immunol.,* 51 : 339-347 (2007).

Molecular pathogenesis for dominant hereditary spherocytosis associated with band 3 deficiency in cattle due to a nonsense mutation of the *AE1* gene

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Various mutations in the *AE1* (anion exchanger 1, band 3) gene cause dominant hereditary spherocytosis, a common congenital hemolytic anemia associated with deficiencies of AE1 of different degrees and loss of mutant protein from red blood cell membranes in humans and animals. However, the mechanisms underlying decreases in AE1 protein levels and the dominant inheritance of red cell phenotypes observed in various types of hereditary spherocytosis still remain unknown. A nonsense mutation R664X of the *AE1* gene in cattle (band $3^{Bov.Yamagata}$) also causes dominant hereditary spherocytosis with marked membrane instability associated with total lack or remarkable reduction of red cell AE1 contents in homozygous or heterozygous animals, respectively.

To determine the mechanisms underlying decreases in AE1 protein levels, in Chapter 1, bovine wild-type AE1 and R664X mutant AE1 were expressed in K562 and HEK293 cell lines and *Xenopus* oocytes and analyzed for their expression, turnover, and intracellular localization. R664X mutant protein underwent rapid degradation and caused specifically in-

creased turnover and impaired trafficking to the plasma membrane of the wild-type protein through hetero-oligomer formation in K562 cells. Consistent with those observations, co-expression of mutant and wild-type AE1 reduced anion transport by the wild-type protein in oocytes. Transfection studies in K562 and HEK293 cells revealed that the major pathway mediating degradation of both R664X and wild-type AE1 employed endoplasmic reticulum (ER)-associated degradation through the proteasomal pathway.

Then, in order to analyze the mechanisms underlying the ER-associated degradation of R664X AE1, possible roles of ubiquitylation and *N*-glycosylation were examined in K562 and HEK293 cells in Chapter 2. Proteasomal degradation of R664X AE1 appeared to be independent of *N*-glycosylation since bovine AE1 was shown to lack a potential *N*-glycosylation site and the *N*-glycosylated mutant P661S/R664X exhibited a profile for proteasomal degradation and steady state levels similar to those for R664X AE1 in HEK293 cells. In addition, the proteasomal degradation of R664X AE1 occurred without ubiquitylation at a demonstrable level in both K562 and HEK293 cells. Moreover, in HEK293 cells, proteasome inhibitors did not cause aggresome formation of R664X AE1 but remarkably increased its retention in the ER. These characteristics were contrary to that the ΔF508 mutant of cystic fibrosis transmembrane-conductance regulator was ubiquitylated and was found as juxtanuclear aggresomes upon proteasome inhibition.

Finally in Chapter 3, the effect of R664X AE1 on the expression of ankyrin was examined. To do so, intracellular distribution and association with AE1 of EGFP-tagged AnkN90, *N*-terminal AE1-binding domain of bovine ankyrin were examined in HEK293 cells. When transfected with wild-type AE1, EGFP-AnkN90 was principally localized to the plasma membrane, suggesting the association with wild-type AE1 and trafficking to the cell membrane. By contrast, EGFP-AnkN90 was retained in the ER in the cells expressing ER-retarded R664X AE1. Immunoprecipitation analyses demonstrated specific association of EGFP-AnkN90 with wild-type and/or R664X AE1 to form hetero-oligomers in the transfected cells. A proteasome inhibitor caused remarkable accumulation of EGFP-AnkN90 in the cells co-transfected with the wild type or R664X AE1.

In conclusion, the present study shows that AE1 R664X protein, which is associated with dominant hereditary spherocytosis, has a dominant-negative effect on the functional expression of wild-type AE1 and ankyrin through increased degradation by the proteasomal ER-associated degradation pathway, leading to decreases in AE1 protein levels and dominant inheritance of the disease. The ER-associated degradation for AE1 has some novel characteristics including ubiquitylation- and *N*-glycosylation-independence.

Application of the risk assessment framework for the microbiological hazards and marine biotoxins in food

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Food Safety and health standards and related texts should be based on risk analysis, and WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) articulated the need of risk assessment for setting sanitary measures. It is important for risk managers to make transparent , scientific information based decision. In order to ensure food safety on pesticide residues, food additives, veterinary drug residue, environmental contaminant, risk assessment has been utilized for more than 40 years.

In 1997, Codex Alimentarius Commission (Codex) recommended to introduce risk assessment in the area of microbiological hazards in food. Consequently FAO/WHO convened the first expert consultation on this issue, and further initiated international microbiological risk assessments (MRA) from January 2001 in response to the request from Codex Committee on Food Hygiene. MRA conducted by FAO/WHO focused on pathogen-commodity combinations, and utilised quantitative, probabilistic risk assessment models, and estimated risk reduction associated with different risk mitigation scenarios.

In this study, MRA on *Salmonella* Enteritidis in egg was conducted to evaluate the effects of time and temperature restrictions assuming a flock prevalence of 25%. Restricting shelf-life to less than 14 days reduced the predicted risk of illness per serving by a negligible amount (~1%). However, keeping retail storage temperature at no more than 7.7℃ reduced risk of illness per serving by about 60%. If shelf-life were reduced to 7 days, risk per serving would also be reduced by about 60%. The *Listeria* (*L.*) *monocytogenes* in ready-to-eat foods risk assessment demonstrates that the vast majority of cases of listeriosis resulted from the consumption of high numbers of *L. monocytogenes* and foods where the level of the pathogen did not meet the current criteria, whatever they might be (0.04 or 100 CFU/g). The model also predicted that the consumption of low numbers of *L. monocytogenes* had a low probability of causing illness. Eliminating higher levels of *L. monocytogenes* at the time of consumption had a large impact on reducing the number of predicted cases of illness.

Marine toxins are produced by algae and are accumulated and concentrated in filter feeder molluscan shellfish. Once people consume contaminated shellfish, develop neurological, gastrointestinal, and cardiovascular syndromes, some of which result in high mortality and long-term morbidity. In particular, paralytic shellfish poisoning can cause death within hours after consuming exceeding level of the toxins and may require immediate intensive care. Risk management strategies utilized worldwide include periodic monitoring of toxin levels in shellfish, and stop harvesting and distribution of the contaminated shellfish when the levels in shellfish are exceeding regulatory levels, and rapid notification of public health authorities to prevent illnesses. Codex, however, has not established international regulatory levels on marine

biotoxins. Regulatory levels and targeted toxins are different among member countries and these facts might cause trade disputes.

In this thesis, risk assessment on marine biotoxins was conducted by utilizing the procedure of chemical risk assessment based on animal data and human epidemiological data, and tried to establish acute reference dose. As a result, the derived provisional acute reference dose for the Azaspiracid, Okadaic Acid, Saxitoxin, and Domoic Acid toxin groups were 0.04 μg/kg bw, 0.33 μg/kg bw, 0.7 μg/kg bw, and 100μg/kg bw, respectively. For the yessotoxin (YTX) group, a Non-Observed-Adverse-Effect-Level was established based on animal studies. Applying a safety factor of 100, a provisional acute RfD of 50 μg/kg bw was suggested for the YTX group. The database for cyclic imines, brevetoxins, and pectenotoxins was insufficient to establish provisional acute RfDs for these three toxin groups.

A food safety risk assessment is an objective and systematic evaluation of relevant scientific knowledge to help the risk manager make an informed, transparent decision about how to reduce the human health risk. The risk assessments can provide a clear estimation of risk, and a decision support tool to identify effective mitigation strategies by linking data from entire food chain and the various data on human disease. From now on, risk managers should appropriately utilize outcomes of risk assessments on microbiological hazards and marine biotoxin to identify appropriate interventions along with entire food chain from primary production to consumer, and to reduce the food safety risk.

The original papers of this thesis appeared in *Marine Pollution Bulletin,* 53 : 579-590(2006), and *Marine Pollution Bulletin,* 52 : 1735-1745 (2006).

Enhancement of radiation-induced cell killing by inhibiting G2 checkpoint with purvalanol A.

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The combination treatment of solid tumors with an anticancer drug and radiation has been clinically used to improve cure rate. Previous studies have demonstrated that X-ray-induced apoptosis and reproductive cell death were enhanced by combining with a low dose of 1-(3-C-ethynyl-β-D-*ribo*-pentofuranosyl) cytosine (ECyd), which was synthesized as an anti-tumor drug targeting RNA synthesis, and X irradiation in various tumor cells. In ECyd-enhanced radiation-induced apoptosis, the downregulation of G2 checkpoint-related proteins and anti-apoptotic proteins was found to be important in various cell lines. However, it is still unclear which molecule among these proteins plays a crucial role in sensitization of radiation-induced apoptosis by ECyd because of its nonspecific inhibitory effects on RNA synthesis in tumor cells. Recently, it was reported that a G2 checkpoint regulator, Cdc2, controlled the activity of an anti-apoptotic protein, survivin. Survivin is strongly expressed in the G2/M phase and undergoes cell cycle-dependent phosphoryla-

tion on Thr34 by a Cdc2-cyclin B1 complex to make a complex with caspase-3 on the mitotic apparatus.

In the present study, to obtain direct evidence that the activity of Cdc2 kinase is associated with the maintenance of an anti-apoptotic system in X-irradiated tumor cells, a purine analog derivative purvalanol A, which is a cyclin-dependent kinase inhibitor, was employed.

Human gastric adenocarcinoma MKN45 cells exposed to 20 Gy of X rays increased the Cdc2 kinase activity and the expression of regulatory proteins (phospho-Cdc2 and cyclin B1) of the G2 phase, followed by the activation of G2 checkpoint, whereas the treatment of X-irradiated MKN45 cells with 20 μM purvalanol A suppressed the increase in the Cdc2 kinase activity and the expression of G2 phase regulatory proteins, and reduced the fraction of G2/M phase in the cell cycle. Furthermore, this treatment resulted in not only the significant increase in radiation-induced apoptosis but also the loss of clonogenicity in both MKN45 (p53-wild) and MKN28 (p53-mutated) cells. The expression of anti-apoptotic proteins, inhibitor of apoptosis protein (IAP) family members (survivin and XIAP) and Bcl-2 family members (Bcl-XL and Bcl-2), in X irradiated cells followed by the treatment of purvalanol A was significantly lower than that of cells exposed to X rays alone. X irradiation alone induced the release of cytochrome c from mitochondria to cytosol but not the formation of active fragments of caspase-3 (p17). This fact strongly suggested that the treatment with purvalanol A released the inhibitory machinery against mitochondrion/caspase-3-dependent apoptotic signal pathways in X-irradiated cells.

In summary, the cyclin-dependent kinase inhibitor purvalanol A was shown to efficiently inhibit the radiation-induced Cdc2 kinase activity and the expression of regulatory proteins of G2 phase, which were related to the G2 checkpoint. Furthermore, the treatment with purvalanol A resulted in a significant increase in radiation-induced cell death. These data suggest that the inhibition of radiation-induced Cdc2 kinase activity could be a clinical target for cancer radiotherapy.

The original papers of this thesis appeared in *Radiation Research,* 164 : 36-44 (2005) and *Radiation Research,* 167 : 563-571 (2007).

Behavioral analysis and seroepidemiological survey of infectious diseases in Kuril harbor seals (*Phoca vitulina stejnegeri*) around Hokkaido

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