Japanese Journal of Veterinary Research 56(1): 9-20, 2008

INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy (Ph.D.) in Veterinary Medicine on March 25, 2008 to 11 recipients.
The titles of theses and other information are as follows:

Role of uncoupling protein 1 in the anti-obesity effect of β3-adrenergic stimulation in the dog

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Obesity is the most common nutritional disorder, and the major risk factor for a number of diseases such as non-insulin dependent diabetes in companion animals. Anti-obesity programs including food restriction and/or exercise therapy would be applied to reduce body fat content, but the feasibility of programs is largely dependent on motivation and behavior of animal guardian. To address such difficulty in reducing body fat in companion animals, therapeutic advance in the treatment of obesity is desired.

β3-adrenergic receptor is abundantly and almost exclusively present in adipocytes and essential for induction of lipolysis in white adipose tissue (WAT). It is now qualified as a target of anti-obesity drug, and actually treatment of obese rodents with a selective β3-adrenoceptor agonist reduces body weight as well as body fat content. The reduction of body fat is not only due to lipolysis, but also dependent on dissipation of fatty acids by the function of uncoupling protein 1 (UCP1), which is exclusively present in brown adipose tissue (BAT).

Treatment of dogs with a selective β3-adrenoceptor agonist prevents accumulation of body fat, accompanied with induction of UCP1 mRNA in WAT, while BAT is involuted during postnatal development and no UCP1 mRNA is ordinary detected in WAT of adult dogs. Therefore, a selective β3-adrenoceptor agonist could be an anti-obesity (fat-reducing) drug for dogs, and UCP1 in WAT might be functional, although these assumptions have not been proven. In this dissertation, I examined the acute and chronic effects of AJ-9677 (AJ), a newly-developed selective β3-adrenoceptor agonist, on body fat reduction in obese beagles and the role of UCP1 for its anti-obesity effect.

Oral administration of AJ (0.01 or 0.1 mg/kg) to overnight-fasted obese beagles produced a dose-dependent rise in the plasma levels of free fatty acids and insulin in 1hour, followed by a gradual drop of the plasma glucose level. The administration did not cause apparent abnormal behaviors, but did easily detectable cutaneous flushing.

Daily treatment of AJ at a lower dose (0.01 mg/kg) for 3 weeks produced no notable change in body weight, but at a higher dose (0.1 mg/kg) for subsequent 7 weeks reduced the body weight compared to placebo-treatment. Computed tomographic examinations revealed a remarkable reduction of body fat after the AJ-treatment, being consistent with the histological observations that the adipose tissue of AJ-treated dogs consisted of smaller and some multilocular adipocytes. The plasma levels of leptin and adiponectin were decreased and increased, respectively, after the AJ-treatment, reflecting the reduction of adiposity. Moreover, improvement of glucose tolerance occurred after the AJ-treatment. Thus, it was concluded that AJ-9677 is useful for the treatment of obesity in the dog.

The adipose tissue of 10-week AJ-treated dogs

expressed UCP1 mRNA. To evaluate the role of UCP1 in the anti-obesity effect of AJ, I isolated adipocytes from subcutaneous fat pad of beagles before and after a 2-week treatment with AJ (0.1 mg/kg) and examined their thermogenic activity *in vitro*. Histological and protein analyses revealed that adipose tissues before the treatment were composed of unilocular cells filled with a single large lipid droplet, while the tissues after the treatment contained many smaller and some multilocular adipocytes expressing UCP1 and abundant mitochondrial proteins. Before the treatment, oxygen consumption rate was very low and did not change even when the cells were stimulated by AJ, whereas the cells increased a release of fatty acid in response to AJ. Two-week AJ-treatment increased basal oxygen consumption rate by 7-fold, and produced a clear responsiveness to AJ-stimulation. Thus, chronic treatment with AJ induced UCP1 protein in adipocytes, where oxygen consumption increased in response to AJ-stimulation. It was suggested that UCP1-dependent energy expenditure in adipose tissue contributes to the anti-obesity effect of β3-adrenoceptor agonist in dogs.

In summary, chronic treatment with β3-adrenoceptor agonist, AJ-9677, reduces body fat content in obese dogs, which possibly results from enhancement of energy expenditure by induction of mitochondriogenesis and mitochondrial thermogenic UCP1 in adipose tissue.

Original paper of this thesis appeared in *Res. Vet. Sci.,* **83**: 5-11 (2007) and *Res. Vet. Sci.,* (in press) (2008).

Pathophysiological roles of uncoupling protein 1 in thermogenesis and energy metabolism

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There are two types of adipose tissue in mammals. One is white adipose tissue (WAT) that accumulates large amounts of triglyceride (TG) for storage of excess energy and liberates fatty acids into blood when necessary. The other is brown adipose tissue (BAT) specified for metabolic heat production. Fatty acids from BAT are oxidized and used as the major energy source for heat production within the tissue itself through the activation of uncoupling protein 1 (UCP1), which exclusively exists in BAT. UCP1 uncouples oxidative phosphorylation in mitochondria to dissipate the electrochemical gradient as heat. Thus, UCP1 is a critical molecule for BAT thermogenesis.

It is well known that BAT has a pivotal role in cold-and diet-induced thermogenesis under the control of the sympathetic nerve system, mainly through the β3-adrenergic receptor. In addition, it has been suggested that BAT theromgenesis is activated by cytokines such as leptin, an adpocyte-derived anorexigenic peptide, and interleukin-1β (IL-1β), an inflammatory cytokine causing fever. However, the effects of leptin on the energy expenditure are still in debate and the contribution of BAT theromgenesis to fever remains obscure. To address these issues, in this dissertation, I examined the effects of leptin and IL-1β on UCP1-dependent BAT theromgenesis and energy expenditure, using wild-type (WT) and UCP1-deficient mice.

In Chapter I, I investigated febrile and thermogenic responses to IL-1β *in vivo*. In WT mice, an intraperitoneal injection of IL-1β increased body temperature, decreased physical activity, and

produced an insignificant rise in oxygen consumption. Oxygen consumption dependent on metabolic thermogenesis calculated by correcting the effect of physical activity was increased significantly after IL-1β injection. Almost the same responses were observed in UCP1-deficient mice. In contrast, CL316,243, a β3-adrenergic agonist, increased body temperature, decreased physical activity, and produced a significant rise in oxygen consumption in WT mice. These changes were not observed in UCP1-deficient mice. These results, conflicting with a previously proposed idea of a role of BAT in fever, indicate a minor contribution of BAT thermogenesis to IL-1β-induced fever. In support of this, CL316,243, but not IL-1β, affected the triglyceride content and UCP1 mRNA level in BAT.

In Chapter II, I investigated the acute and chronic effects of leptin on feeding behaviors, whole body energy expenditure, and BAT functions. Single injection of leptin to WT mice reduced food intake, but had no effect on oxygen consumption and the level of UCP1 expression in BAT for up to 17 hours. In contrast, chronic hyperleptinemia induced by adenovirus gene transfer reduced food intake in both WT and UCP1-deficient mice. WT mice with hyperleptinemia, compared to pair-fed controls, showed increased oxygen consumption, elevated UCP1 expression in BAT, ectopic UCP1 induction in WAT, and reduced body fat content. These effects of chronic hyperleptinemia were not observed in UCP1-deficient mice. It was thus concluded that UCP1-dependent energy expenditure contributes to the fat-reducing effect of leptin.

I also found that repeated leptin injection or chronic hyperleptinemia showed a more apparent anorexigenic effect in WT mice than UCP1-deficient mice, while single leptin injection did not produce such a difference. As chronic hyperleptinemia induced ectopic UCP1 expression in WAT, I further investigated the effect of UCP1 expression in WAT on the anorexigenic action of leptin, focusing on the so-called "leptin sensitivity". Chronic CL injection to WT mice induced UCP1 expression in WAT and increased the leptin sensitivity. Moreover, UCP1 expression in WAT of UCP1-deficient mice, induced by adenovirus gene transfer, also increased the leptin sensitivity. These results indicate that ectopic UCP1 in WAT increases the leptin sensitivity to suppress food intake more profoundly.

In summary, I have demonstrated that BAT thermogenesis has a minor role in IL-1β-induced fever, but contributes to the fat-reducing effect of leptin through increasing energy expenditure. I also found that ectopic UCP1 expression in WAT increases the anorexigenic action of leptin, suggesting a new physiological role of UCP1 in the control of food intake. I assure that these new findings contribute significantly to a better understanding of the control mechanisms of thermogenesis and energy metabolism.

Original papers of this thesis appeared in *Obes. Res. Clin. Prac.,* **1**: 61-67 (2007), *ibid,* **1**: 233-241 (2007) and *Am. J. Physiol. Endocrinol. Metab.,* **292**: E1135-E1139 (2007).

Studies on the mechanisms of T-cell lymphomagenesis caused by Marek's disease virus

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Marek's disease (MD) is a malignant T-cell lymphoma of chickens caused by Marek's disease virus (MDV), a highly cell-associated avian herpesvirus. In recent years, MDV has been increasing its virulence, and bringing economic loss in poultry industries despite the vaccination. Thus, studies for understanding the mechanisms of T-cell lymphomagenesis caused by MDV and developing a novel vaccine strategy are required.

In this study, to elucidate the molecular pathogenesis of MD, the expression dynamics and the functions of an MDV oncogene, *meq,* its variant L-*meq,* and their spliced transcript Δ*meq,* were analysed. The results obtained in this study were outlined below.

1) In tumor cell lines derived from MD lymphomas and chicken enbryo fibrobalsts (CEF) infected with MDV, a novel spliced transcript of *meq* and L-*meq,* was identified. This transcript, termed Δ*meq,* encodes 98 N-terminal amino acids of the Meq protein and lacks some parts of the basic leucine zipper and transactivation domains. The expression of ΔMeq was significantly up-regulated in MD cell lines during apoptosis induced by chemotherapeutic compounds. ΔMeq could interact with both L-Meq and Meq and suppressed their transactivation functions. These results suggest that ΔMeq could be involved in apoptosis as working as a negative regulator of L-Meq and Meq by its direct interaction.

2) The up-regulation of the Δ*meq* expression and the down-regulation of the L-*meq* expression along with increased expression of viral antigens were observed in MD cell lines during the induction of lytic replication of MDV. Consistent expression of Δ*meq* and its up-regulation over time were also observed in CEF infected with MDV. In peripheral blood mononuclear cells of very virulent MDV-infected chickens, both *meq* and Δ*meq* were constantly detected durig the experimental period. Thus, ΔMeq could also be associated with lytic replication of MDV as well as cellular apoptosis by functioning as a negative regulator of L-Meq and Meq.

3) It has been suggested that Meq is capable of interacting with p53, a well-known tumor suppressor protein, and inhibit p53-mediated transactivation. In this study, the down-regulation of the p53 expression in the presence of L-Meq and Meq was observed. MG132, a proteasome inhibitor, could restore the down-regulation of the p53 expression caused by L-Meq and Meq, suggesting that L-Meq and Meq could be involved in the proteasome-mediated degradation of p53, and as a result, contribute to the initiation of tumorigenesis. On the other hand, ΔMeq did not affect either the p53-mediated transactivation or the p53 expression.

In summary, the results described above indicate that Meq and L-Meq would contribute to the MDV latency and lymphomagenesis by inactivating p53 as well as modulating the expressions of various target genes. The ΔMeq could nagatively regulate the functions of L-Meq and Meq, and as a result, contribute to the MDV reactivation and replication. This study provided novel insights into the molecular pathogenesis of MD and valuable information into the control of MD and other viral oncogenicity.

Original paper of this thesis appeared in *J. Gen. Virol.* **88**: 2111-2120 (2007).

Studies on the prevalence of virulent Marek's disease virus in wild geese and a factor related to the increased virulence.

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Recent field isolates of Marek's disease (MD) virus (MDV) tend to increase their virulence, and currently, the risk of future outbreaks is pointed out. In addition, an MD case was reported in a white-fronted goose migrating from Russia in Hokkaido in 2001. Therefore, wild geese could play some important roles as reservoirs or carriers of MD to domestic poultry, and future outbreaks may also occur because of the increased virulence of MDV in wild geese. In this study, the prevalence of MDV in wild geese was surveyed. In addition, since the correlation between diversity or insertion in amino acid residues in MDV-*Eco*RI-Q (Meq) and the pathogenecities of serotype1 MDV (MDV1) was reported, the functional analysis of Meq carrying the diversity or insertion was performed.

A method for the detection of oncogenic MDV1 using feather tips has been developed because these sites are suitable for the collection of samples in the field. To distinguish oncogenic from non-oncogenic MDV1, the *meq* gene was analyzed, since a structural difference in the *meq* open reading frame (ORF) between oncogenic (*meq*) and non -oncogenic (L-*meq,* including a 180-bp sequence in the *meq* ORF) MDV1 had been reported. When chickens were infected with Md5, an oncogenic MDV1, the *meq* gene was detected in total DNA extracted from feather tips throughout the experimental period for 10 weeks post inoculation (pi), whereas, in chickens infected with CVI988, an attenuated MDV1, the L-*meq* gene was detected at 2 to 10 weeks pi. Furthermore, the *meq* gene was dominantly detected from feather tips in most of the chickens co-infected with Md5 and CVI988 at 2 to 10 weeks pi. These results suggest that the detection of the *meq* gene using feather tip-derived samples is an effective and reliable method for the diagnosis of oncogenic MDV1. Therefore, this method was applied in the field to survey the prevalence of MDV1 in wild geese. The *meq* gene was detected in white-fronted geese, canada geese and bean geese, suggesting that oncogenic MDV1 is widely spread among goose populations.

Currently, MDV strains are classified into four categories based on their virulence (mild (m), virulent (v), very virulent (vv) and very virulent +(vv+) MDV groups). Distinct diversity and point mutations have been reported in Meq with highly virulent MDV strains. It was suggested that the diversity in Meq may correlate with the difference in transcriptional activities by each Meq and is considered as the most important factor related to the virulence. Since Meq could regulate the expressions of viral and cellular genes as a transcriptional factor, the correlation between the transcriptional activities and the diversity in Meq was analyzed. The enhanced activities were observed dependent on the amino acid substitutions in the transactivation domain, especially the substituion in proline-rich repeat. Thus, the diversity in Meq was considered as one of the factors related to the increased virulence of MDV.

L-Meq, carrying 60 amino acid insertions in the transactivation domain in Meq, has been identified in four MDV strains which are classified into the low virulent categories (m or vMDV). Therefore, the insertion found in L-Meq could be correlated to the low virulence of these MDV strains. In this study, the function of L-Meq was investigated in terms of its transcriptional activity and transforming potential. Reporter assay targeting the Meq promoter region revealed that L-Meq had a modest effect compared to Meq. In addition, evaluation of transforming potentials based on

anchorage-independent cell growth using the DF-1 chicken embryo fibroblast transformation system resulted in significant differences of the colony numbers between Meq-and L-Meq-expressing clones. These results suggest that the tumorigenesis of L-Meq is weaker than that of Meq, and therefore, the MDV strains encoding L-Meq can be categorized into low virulent groups.

The present study suggests that the diversity or insertion in Meq affects the virulence of MDV1. In addition, the deduced amino acid sequences of the *meq* gene detected from white-fronted geese matched those with highly virulent MDV strains. Hence, MDV1 strains, prevalent in wild geese, seem to be highly virulent. Therefore, further characterization of recent isolates from chickens and wild geese is necessary, and continuous monitoring of MD outbreaks in the field would be required.

Original papers of this thesis appeared in *J. Vet. Diagn. Invest.,* **19**: 471-478 (2007) and *Arch. Virol.,* **152**: 1523-1526 (2007).

Studies on controlling *Echinococcus multilocularis* infection in red foxes (*Vulpes vulpes*) in Hokkaido, Japan

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Alveolar echinococcosis caused by larval *Echinococcus multilocularis* is one of the most serious helminthic zoonoses. In Hokkaido, the northern island of Japan, the prevalence of *E. multilocularis* in red foxes, *Vulpes vulpes,* has dramatically increased during the past two decades. In this study, focusing on controlling *E. multilocularis* infection in the red fox population in Hokkaido, control efficacy of anthelminthic baiting to foxes and genetic structure of the red fox population in Hokkaido were investigated as follows.

Chapter I: The use of tetracycline in anthelmintic baits to assess baiting rate and drug efficacy against *Echinococcus multilocularis* in foxes

Since 1990s, deworming trials against *E. multilocularis* infection in wild red foxes have been conducted by distributing baits containing anthelminthic (praziquantel) in Germany, Switzerland and Hokkaido. These studies showed reduction of infection prevalence in foxes, however, bait acceptance by individual foxes has not been evaluated. In this study, baits containing tetracycline (TC) were used for checking bait acceptance in order to investigate deworming effect at individual fox level.

At a 110 km^2 suburban area in Otaru, Hokkaido, bait distributions were conducted twice to seven times between May and November in 2001-2004. Baits were distributed along roads at 20 baits/km by car. The prevalence of infection in foxes before baiting (1999-2000) was 58% (88/153), whereas in the final year (2004), it decreased to 11% (5/45). Analysis of TC marking in the teeth of foxes showed that 39% (77/195) of those captured after baiting were estimated to have consumed baits in the year of capture. Importantly, juvenile foxes (56%, 49/87) were more marked with TC than adult foxes (26%, 28/108), indicating efficient baiting of juveniles which tended to have a higher worm burden of *E. multilocularis.* Of 77 marked foxes, *E. multilocularis* and *Alaria alata* (monitored as the second indicator species of deworming) were not detected in 70 (90%) and 76 (99%) foxes, respectively. The results suggest effective

deworming by bait consumption. However, it was also demonstrated that 9% of the marked foxes were infected or re-infected after bait consumption, suggesting the high infection pressure and the importance of frequent baiting.

Chapter II: Genetic structure of the red fox, *Vulpes vulpes,* populations in northern Japan inferred from mitochondrial DNA sequences.

To plan an effective deworming program, it is important to understand the population structure of red foxes in Hokkaido, such as the distribution of subpopulations and the dispersion pattern of individuals. On the other hand, to monitor introduction of infected foxes from Hokkaido to Honshu, distinction between the two populations is necessary. In this study, to investigate genetic structure of the red fox populations in Hokkaido and northern Honshu, mitochondrial DNA sequence variation in the cytochrome *b* (cyt *b*) gene and the control region was examined and phylogenetically analyzed.

A total of 88 fox samples mainly from Hokkaido and northern Honshu containing a few samples from central Honshu and Kyushu, Japan, and Primorye, Far East Russia, were used. Resultant haplotypes from Hokkaido were subdivided into two distinct groups (I and II), with an average genetic distance of 0.027 for entire cyt *b* gene (1140 bp). Group II was only found in Hokkaido, whereas Group I comprised haplotypes from Honshu, Kyushu (Japan), eastern Russia and Europe, as indicated by a comparison of our own data to the literature. The haplotypes of Group I from Hokkaido were further divided into two subgroups.

However, geographical distribution of mtDNA haplotype groups/subgroups of foxes in Hokkaido revealed no visible genetic structuring within Hokkaido. On the other hand, within Group I, haplotypes from Honshu and Kyushu were clustered with one another and were distinct from all haplotypes originating from Hokkaido and the continent in the constructed trees. The mean sequence divergence between the haplotypes of Honshu/Kyushu and the other regions is around 1% in the entire cyt *b* gene. This suggests no evidence of fox movements between the two islands and its usefulness as diagnostic marker to distinguish the two populations for monitoring spread of *E. multilocularis*.

Original papers of this thesis appeared in *Zool. Sci.,* **24**: 1178-1186 (2007) and *Vet. Parasitol.,* **150**: 88-96 (2007).

Phylogenetic analysis of neuropathogenic avian retroviruses

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Fowl glioma is characterized by multiple astrocytic growth, and is caused by fowl glioma-inducing virus (FGV), belonging to subgroup A of avian leukosis virus (ALV). The elucidation of the neuropathogenic factors of FGV will contribute to understanding the molecular mechanism of neural disorders by retroviruses. Recently, fowl gliomas were observed in layer chickens with subcutaneous neoplasms. To clarify the prevalence of FGV and pathogens of fowl glioma in layers, establishment of a polymerase chain reaction (PCR) assay to detect FGV, surveillance of FGV in domestic zoological gardens, and histopathological and molecular biological analyses of glioma in layers were performed.

In Chapter I, a nested PCR-protocol using

FGV-specific primers was established, and the prevalence of FGV was evaluated in 131 Japanese fowls of zoological garden A in Japan. The FGV proviruses were detected in feather pulps of 52 birds by the nested PCR. Later, 9 dead birds among the 52 birds were diagnosed as fowl glioma and had the FGV proviruses in their brains. By nucleotide sequencing, the PCR-amplified region of 4 birds among the 52 birds showed high identity with that of FGV. These results demonstrated that the PCR-based detection of FGV in feather pulp is useful for the molecular diagnosis of FGV.

In Chapter II, the prevalence of FGV was examined in a total of 129 chickens in three zoological gardens by the nested PCR. Twenty-six to 56 percents of the fowls in each of the examined gardens were positive by the nested PCR. Sequence analysis revealed that the PCR-amplified region of the 14 isolated ALVs showed high identity with that of FGV. In addition, the *env* genes of the 3 isolates frequently showed mutations of nucleotides. These results suggested that FGV mutants were prevalent among chickens in zoological gardens in Japan.

In Chapter III, histopathology of 240 layers was performed. Gliomas concurred in 11 layers with subcutaneous neoplasms and occurred independently in 3 layers. In addition, locally extensive proliferation of small round cells was found in the cerebrum of 2 layers. The FGV genome, however, was not detected in the affected brains by the nested PCR. By nucleotide sequencing, the entire genome of TymS_90, an isolate from the affected brain, included a large part of endogenous viral loci and several parts of other avian leukosis/sarcoma viruses. These results showed that a recombinant ALV, not FGV, cause fowl glioma and have spread among layers in Japan.

The present studies demonstrated that FGV and FGV mutants are prevalent in chickens in domestic zoological gardens. Because the brain lesions caused by the mutants were the same as fowl glioma, it was suggested that common genomic regions between FGV and the mutants could determine the neuropathogenicity. In addition, it was revealed that in layers a recombinant ALV is involved in fowl glioma accompanied by the proliferation of small round cells. Thus, it appears that several strains of ALVs can induce fowl glioma.

Original paper of this thesis appeared in *Avian Pathol.,* **34**: 473-479 (2005) and *Avian Pathol.* (in press).

In vitro production of porcine embryos using chemically defined media

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The original papers of this thesis appeared in *Biol. Reprod.,* **69**: 2092-2099 (2003), *J. Reprod. Dev.,* **50**: 487-491 (2004), *Theriogenology,* **64**: 1287-1296 (2005), *Reprod. Fertil, Dev.,* **18**: 789-795 (2006), and *Zygote,* **15**: 317-324 (2007).

Embryo sexing by loop-mediated isothermal amplification of trace target DNA sequences in cattle and water buffaloes

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The original papers of this thesis appeared in *Theriogenology,* **62**: 887-896 (2004), *Theriogenology,* **66**: 1249-1256 (2006) and *Anim. Reprod. Sci.,* **101**: 38-44 (2007).

The enhancement of radiation-induced apoptosis by TRAIL in hypoxic solid tumor cells.

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Ionizing radiation has been widely used as a tool for tumor treatment. When tumors are irradiated, induction of apoptosis is generally considered to occur in tumor cells through a mitochondria-dependent signaling pathway and radiation-induced double-strand breaks of DNA are known to induce loss of clonogenic ability. However, in solid tumors, hypoxic regions are known to exist as a result of excessive tumor growth with a deficiency of blood vessels. It is well known that the low oxygen concentrations in these regions reduce the cell killing efficacy induced by ionizing radiation. Moreover, several studies have shown the resistance of hypoxic regions in solid tumors to antitumor drugs. Thus, it is very important to develop a cancer therapy technique to conquer the resistance in the hypoxic regions in solid tumors to genotoxic agents. In previous study in this laboratory, it has shown that ionizing radiation induced the expression of DR5 on the cell surface in tumor cell lines and that TRAIL enhanced the apoptotic pathway. In this research, the effectiveness of the combination of TRAIL and X irradiation in tumor cells under hypoxia *in vitro* and solid tumors with hypoxic regions *in vivo* were examined.

The first experiments were performed to examine whether treatment with TRAIL enhanced the cell killing in tumor cells exposed to ionizing radiation under hypoxia. When human lung carcinoma A549 cells were irradiated under normoxia and hypoxia, respectively, radiation-induced enhancement of expression of DR5 was observed in both conditions. It is suggested that incubation in the presence of TRAIL enhanced the death receptor-mediated apoptotic pathway in A549 cells exposed to X rays. Furthermore, it is suggested that XIAP, one of IAP family proteins, has a relationship with the apoptotic pathway induced by the combination of TRAIL and X irradiation in A549 cells. Moreover, it was shown that treatment with TRAIL enhanced apoptotic cell death and loss of clonogenic ability in A549 cells exposed to X rays not only under normoxia but also under hypoxia, suggesting that combination treatment with TRAIL and X irradiation is effective for hypoxic tumor cells.

In the second experiments, based on the results of the first experiment, it was examined that *in vivo* antitumor efficacy of X irradiation combined with TRAIL treatment in tumor xenograft

models derived from human gastric adenocarcinoma MKN45 and MKN28 cells in SCID mice. X irradiation combined with TRAIL synergistically suppressed the tumor growth rates in the xenograft models derived from MKN45 and MKN28 cells, which have wild type Tp53 and mutated Tp53, respectively, indicating that the antitumor effects occurred in a Tp53-independent manner. Histological analysis showed that the combination of X irradiation and TRAIL induced caspase-3-dependent apoptotic cell death in MKN45. Moreover, the immunohistochemical detection of hypoxic regions using the hypoxic marker pimonidazole revealed that caspase-3-dependent apoptosis occurred in the hypoxic regions in the tumors. These results indicated that X irradiation combined with TRAIL may be a useful treatment to reduce tumor growth in not only normoxic but also hypoxic regions.

In conclusion, the combination of TRAIL and X irradiation effectively induced apoptosis in tumor cells under hypoxic condition both *in vitro* and *in vivo*. Clonogenic assay in A549 also revealed that the combination of TRAIL and X irradiation has effectiveness to increasing of reproductive cell death in A549. The combination of TRAIL and X irradiation induced apoptosis in solid tumor cells in Tp53-independent manner, suggesting that the treatment might be effective to several tumor cells with mutated p53. Moreover, the immunohistochemical detection revealed that caspase-3-dependent apoptosis occurred in the hypoxic regions in the tumors. X irradiation combined with TRAIL might be a useful treatment to reduce tumor growth in not only normoxic but also hypoxic regions in radiotherapy.

The original papers of this thesis appeared in *J. Radiat. Res.,* **48**: 461-468 (2007) and *J. Radiat. Res.* **49**: 153-161 (2008).

Treatment combining X-irradiation and a novel ribonucleoside anticancer drug,1-(3-C-ethynyl-β-D-*ribo*-pentofuranosyl) cytosine, ECyd suppresses the growth of implanted solid tumor

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A method combining a low dose of radiation and a low dose amount of anticancer drug administration has been a potent strategy for cancer therapy. ECyd,1-(3-C-ethynyl-β-D-*ribo*-pentofuranosyl) cytosine has been newly developed as a anticancer drug having the cytotoxicity by inhibiting RNA synthesis. Previous studies in this laboratory have demonstrated that a low dose of ECyd can amplify X-ray-induced apoptosis in a variety of tumor cells *in vitro*. The purpose of this research was to examine the *in vivo* antitumor efficacy of X irradiation combined with administration of ECyd in transplanted tumors with various TP53 status. To investigate the mechanism of antitumor efficacy of ECyd, the effects of ECyd on irradiated-tumor cells were also examined under hypoxic condition both *in vitro* and *in vivo*.

In the first experiment, murine rectum adenocarcinoma cells (Colon26 [TP53 unknown]) and human gastric adenocarcinoma cells (MKN45 [TP 53 wild-type] and MKN28 [TP53 mutation]) were implanted into the footpad in BALB/c mice and SCID mice, respectively. They were treated with a relatively low dose of X irradiation (less than 2 Gy) and low amount of ECyd (less than 1.0 mg/kg). The tumor growth was monitored by measuring

the tumor volume from 5 day or 7 day to 16 day or 20 day after tumor implantation. When X irradiation and ECyd treatment were combined, significant inhibition of tumor growth was observed in all types of tumors compared to that of mice treated with X irradiation or ECyd alone. To examine the optimal time sequencing for combining X irradiation and ECyd, Colon26-tumor-bearing mice were administered ECyd at different times before or after X irradiation. The administration at 3 h before X irradiation was an optimal condition for the enhancement of the tumor radioresponse. Histological analyses for apoptotic and proliferative cells in the tumors were performed using terminal deoxytransferase nick end-labeling (TUNEL) method and Ki-67 immunohistochemical staining. ECyd treatment increased the sensitivity of tumor cells to apoptosis and inhibited tumor cell proliferation in X-irradiated tumors, resulting in the enhancement of the suppressive effect of X irradiation on tumor growth. The expression of survivin, a key molecule relating to tumor survival, was assessed using quantitative PCR and immunohistochemical analysis. Parallel to the inhibition of tumor growth, ECyd suppressed survivin expression of MKN45 tumor cells in both mRNA and protein levels. Thus, the inhibition of survivin expression by ECyd is thought to mainly contribute to the suppression of the tumor growth.

In the second experiment, hypoxia (oxygen concentration < 20 mmHg) was achieved by passing 95% N_2 and 5% CO_2 gas continuously in a gas-exchangeable chamber. It was shown that a low dose of ECyd induced radiosensitization of apoptosis to MKN45 and MKN28 cells under hypoxia similarly to normoxia *in vitro.* At the same time, the accumulation of hypoxia inducible factor 1α (HIF-1α) observed under hypoxia was shown to be decreased to the level of normoxia in the presence of 0.1 μM ECyd. To study the function of HIF-1α protein for apoptosis in hypoxic cells, HIF-1α reductive approach was performed using its specific antisense oligodeoxynucleotide. The reduction of HIF-1α gene expression dramatically enhanced X-ray-induced apoptosis in hypoxic cells. In *in vivo* experiments, MKN45 xenografts transplanted in severe combined immunodeficient (SCID) mice were treated with 2 Gy of X-irradiation and/or 0.5 mg/kg ECyd administration. Hypoxic regions in tumors were stained using anti-pimonidazole immunohistochemistry. ECyd significantly suppressed HIF-1α expression and subsequently reduced the area of the hypoxic region in the tumor, and enhanced the induction of apoptosis in the hypoxic region when combined with 2 Gy of X-irradiation. These results suggest the possibility that ECyd acts as a potent radiosensitiser *via* the inhibition of HIF-1α expression and can be a useful agent against radiotherapy-resistant hypoxic cells in solid tumors.

In conclusion, a new anticancer drug having an inhibition of RNA synthesis, ECyd, could potentiate the antitumor activity of X irradiation with less than 2 Gy for solid tumors regardless of their TP53 status. The inhibition of survivin expression by ECyd was probably related to the suppression of tumor growth. Additionally, ECyd could radiosensitize apoptotic induction in not only normoxic but also hypoxic cells *in vitro* and *in vivo,* which might be mainly related to the inhibition of HIF-1α expression. Importantly, ECyd significantly reduced the hypoxic regions in X-irradiated MKN45 xenograft, probably due to this apoptosis induction. To overcome the resistance of apoptosis in hypoxic cells of solid tumors, the use of radiation sensitizers is necessary in combination with radiotherapy. Based on this research, the novel anticancer drug ECyd, which was effective for hypoxic cells *via* survivin and HIF-1α inhibition, is a candidate sensitizer for radiotherapy.

The original paper of this thesis appeared in *Int. J. Radiat. Oncol. Biol. Phys.,* **68**: 218-228 (2007).

Identification and characterization of CYP2C76 in cynomolgus monkeys

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Original papers of this thesis appeared in *Mol. Pharmacol.,* **70**: 477-486, *Xenobiotica,* **37**: 30-43 and *Arch. Biochem. Biophys.,* **466**: 98-105.