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Studies on therapeutic mechanisms of bone marrow-derived mononuclear cell and involvement of hepatocyte growth factor in acute spinal cord injury

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Spinal cord injury is a critical condition that is often encountered in the clinical site. Mild cases are responsive to treatment and allow for functional recovery, but in severe cases, locomotor, sensory and physiological functions do not recover, causing a deterioration of the QOL. In addition, since many spinal cord injury patients live with a deteriorated QOL during their full life span and no effective treatment has yet been established, the development of new treatment is urgently needed. The basis of the treatment of acute spinal cord injury consists of preventing secondary injuries that spread to surrounding normal tissue. On the other hand, in the chronic phase, the purpose of the treatment is to promote axonal re-extension, and to reconstruct neural circuits that have been destroyed. The chronic phase is extremely difficult to treat. However, suppression of secondary damage through treatment in the acute phase allows for a higher possibility of functional recovery during the chronic phase. Cell transplantation therapy has recently been found effective in treating spinal cord injury, and various types of cell for transplantation have been reported. Bone marrow-derived mesenchymal stem cell (BMSC) is a source of transplantation cells commonly used recently. However the problems are that isolation and culture of BMSC are time-consuming, and that they cannot be used in the treatment of the acute phase. Bone marrow-derived mononuclear cell (BM-MNC) is a multicellular population composed of bone marrow cells excluding megakaryocyte and mature erythrocyte, and can easily be conditioned and adjusted by only centrifugation. Therefore, they can be transplanted on the same day as the onset of disease, and have been applied for the acute treatment of various diseases including spinal cord injury. The therapeutic effect of BM-MNC in the treatment of spinal cord injury was first reported in 2001; and since then, therapeutic effects such as their anti-apoptotic effect and angiogenic effect have also been reported.

The therapeutic effects of BM-MNC are believed to be brought about by the paracrine effects of growth factors, but thus far, its detailed mechanisms have elusively uncleared. The therapeutic effect of cell transplantation therapy tends to attract attention, and determination of details regarding the underlying mechanism could potentially lead to an elucidation of the targets, and a proper timing for transplantation. In addition, elucidation of the main therapeutic mechanism may lead to the development of effective therapeutic methods. The purpose of this study was to determine their therapeutic mechanism, and explore the less invasive and effective therapeutic methods.

Chapter 2 Differentiation of BM-MNC into vascular component in the injured spinal cord

When tissue damage occurs, bone marrow cells are known to migrate to the lesion, after which they get involved in angiogenesis by differentiating into vascular component cells such as vascular endothelial cells, pericytes, or perivascular macrophages. Further, the kinetics of their differentiation into various cells can be different depending on the disease, suggesting that the fate of bone marrow cells is determined by microenvironments specific to the injured tissue. On the basis of such a background, the differentiation kinetics exhibited by BM-MNC at the transplantation site

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were analyzed in myocardial infarction and hind-limb ischemia models, and the findings revealed that most BM-MNC differentiated into vascular endothelial cells at the transplantation site. Meanwhile, no previous study has analyzed BM-MNC kinetics after transplantation for spinal cord injury. Prostacyclin derived from newly formed blood vessels has recently been found to promote axonal regeneration, and the importance of angiogenesis in the central nervous system has been re-acknowledged. In Chapter 2, in order to elucidate the underlying mechanism behind the reported angiogenesis-promoting effect of BM-MNC in rat models of spinal cord injury, BM-MNC was tracked with green fluorescent protein (GFP), and their capability to differentiate into spinal cord microvascular component cells was clarified. The findings showed that BM-MNC-derived macrophages were transiently localized around blood vessels, and that some of them exhibited immunoreactivity to perivascular macrophage markers. Some BM-MNCs also differentiated into cells such as vascular endothelial cells and pericytes, but such cells were extremely few in number. Macrophages have been reported to promote angiogenesis through mutual interaction with the vascular endothelial cells, although the detailed mechanism remains unknown. In the treatment of spinal cord injury through BM-MNC transplantation therapy as well, findings have also suggested that BM-MNC-derived macrophages may promote angiogenesis by the interaction with endothelial cells.

Chapter 3 Ability of BM-MNC to produce growth factors in injured spinal cords

Bone marrow cells have been found to be capable of constantly producing high levels of growth factors. A previous report has shown that bone marrow cells migrated to the injured tissue, and produced various growth factors using mice model of myocardial infarction that express GFP only in their bone marrow cells. Similar phenomena have been reported in other disease models e.g. hindlimb ischemia, skin injury and stroke, suggesting that bone marrow cells may be involved in the physiological tissue repair process through the paracrine effects of growth factors. In the same way, BM-MNC that composed of bone marrow cells may also exert therapeutic effects through the paracrine effects of growth factors. In Chapter 3, BM-MNC tracked with GFP was transplanted into a rat model of spinal cord injury to evaluate survivability of BM-MNC at the transplantation site, and to determine what kinds of growth factors they produced. As a result, BM-MNC survived in the injured spinal cord at 7 days after transplantation. However, the number of BM-MNC decreased from 3 to 7 days after transplantation and a few of survived BM-MNCs were immunoreacted with the activated caspase-3 in two time points. The survived BM-MNC was also immunoreacted with hepatocyte growth factor (HGF), vascular endothelial growth factor and monocyte chemotactic protein-1. In particular, the expression rate of HGF was found to be the highest. In addition, the therapeutic effect of BM-MNC was evaluated. As a result, the number of caspase-3-activated cells and demyelinated area were significantly decreased in BM-MNC transplanted group compared with control. In this chapter, the findings revealed that transplanted BM-MNC survives more than one week and produces various growth factors including HGF in the injured spinal cord. In the injured spinal cord of acute phase, expression levels of c-Met, HGF receptor are known to start increasing rapidly immediately after the injury, regard less of the late increase of HGF production. BM-MNC may bring about therapeutic effect such as anti-apoptotic effect by paracrine HGF that is a deficiency state in acute spinal cord injury.

Chapter 4 Neuroprotective effects of BM-MNC mediated by the paracrine of HGF

HGF is known as a growth factor with various physiological activities including angiogenesis and cytoprotection. Thus far, the therapeutic effect of HGF has been widely confirmed in various models such as cirrhosis and renal failure, amyotrophic lateral sclerosis and multiple sclerosis; and its clinical applications are promising. Particularly, HGF has been reported to strongly inhibit cell death through induction of Bcl-2 expression and inhibition of reactive oxygen species (ROS) production mediated by inactivation of Rac-1. On the basis of the results in Chapter 3, we have speculated that the anti-apoptotic effect found in the treatment of acute phase spinal cord injury was due to the paracrine effects of HGF. Therefore, in Chapter 4, the underlying mechanisms behind the neuroprotective effect of BM-MNC were analyzed with a focus on ROS production and c-Met phosphorylation using rat adrenal pheochromocytoma cell line (PC12), a neuronal cell model. PC12 cell was induced cell death with CoCl2, and at the same time, treated with BM-MNC conditioned media. As a result, BM-MNC phosphorylated c-Met expressed in PC12 cells through the paracrine effects of HGF and significantly reduced the production of

endogenous ROS and cell death. These effects were suppressed by SU11274, a c-Met inhibitors, and the latter caused a significant decrease of the cell protective effects of BM-MNC, suggesting that BM-MNC inhibited ROS-induced cell death by c-Met phosphorylation. In this chapter, the findings revealed that BM-MNC, at least partly, suppresses neuronal cell death induced by intracellular ROS production by activating HGF/Met signaling. The concentrations of the c-Met inhibitors used in this study were low enough not to affect the viability of PC 12 cells itself. Then, the influence of autocrine of HGF by PC12 cells was considered small. In spinal cord injury, production of ROS is induced immediately after injury, and returns to normal within 2 or 3 days. Therefore, BM-MNC transplantation therapy is expected to be most effective when transplantation is performed within two days after injury.

Chapter 5 Comparison of the therapeutic effects of the BM-MNC transplantation and HGF single-dose administration into the injured spinal cord parenchyma during the acute phase

The results in Chapter 4 revealed that the cytoprotective effect of BM-MNC was at least partly mediated by the paracrine effects of HGF. This suggested that administration of HGF instead of BM-MNC during the acute phase of a spinal cord injury may provide a comparable or highly effective. HGF is already reported therapeutic effects including anti-apoptotic effect in spinal cord injury, and its continuous administration into the subarachnoid space by using a catheter as well as its administration through gene transfer, have been devised as therapeutic methods. In this study aimed at delivering HGF more efficiently at the site of the injury, a single-dose HGF administration into the spinal cord parenchyma was performed, and its therapeutic efficacy in the treatment of acute spinal cord injury was compared with that of BM-MNC transplantation therapy. As a result, fractional anisotropy value of diffusion tensor imaging in HGF group showed significantly higher than that of control group at 14 and 28 days after administration. Besides, positive area of neuron, axon, and astrocyte markers in HGF group were significantly preserved compared with control at 28 days after administration, but did not have enough effects compared with BM-MNC transplantation. Our result demonstrated that single-dose administration of HGF suppressed tissue degeneration, but did not have enough effects compared with BM-MNC transplantation. Further studies are needed to clarify the causes of inferior effect of single-dose HGF administration.

In conclusion, the findings of our study suggest that at the site of injury, BM-MNC produces various kinds of growth factors, mainly HGF, and that HGF secreted by BM-MNC suppresses neuronal cell death by causing a decrease in the production of ROS through phosphorylation of c-Met. Besides, single-dose administration of HGF showed efficacy of a new therapy, although it is not enough effective compared with BM-MNC transplantation in vivo. In addition, our study revealed that BM-MNC exhibited a characteristic behavior that they adhered to blood vessels in an injured spinal cord, suggesting that they were associated with an angiogenesis promoting effect. In the future, more detailed analyses may potentially lead to the finding of new healing mechanisms and to the development of more effective therapeutic methods.

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Analysis on mechanisms of glucose uptake on high K^+-induced contraction in smooth muscle

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Summary

Glucose is one of the most important energy substrate in organism. Glucose uptake into a cell is mainly achieved by sodium-glucose cotransporter (SGLT) and glucose transporter (GLUT).

Smooth muscles are classified into phasic and tonic muscle by characters of electrophysiological and mechanical reaction. It has been suggested that the differences between phasic and tonic muscle are also related to the dependence of the aerobic metabolism. Moreover, it may be suggested that the relationship between contractile response and mechanisms of glucose uptake in muscles differ from each tissues.

On the other hand, phloridzin, an inhibitor of SGLT, inhibits high K^+-induced contraction, but the inhibitory effect differ from each type. It has been suggested that the relaxing mechanism is inhibition of glucose uptake via SGLT. However, there is no report showing that the effect of phloridzin on glucose uptake in smooth muscles. It has been reported that aorta, a tonic muscle, expressed GLUT4 and that glucose uptake was mediated by insulin and receptor agonist. Activation signals of GLUT4 differ from each organ. However, there are few reports showing the differences between normal and hypoxic condition in the GLUT4 signaling of aorta.

Thus, the present study examined that the relationship between inhibition of high K^+-induced contraction and glucose uptake in phasic muscle, and activation of signals in the presence of insulin and high K^+, inhibition of aerobic metabolism in tonic muscle to provide the new findings contributing to make clear the function of visceral organ in pathophysiological condition such as shock, starved state or diabetes.

The relationship between high K^+-induced muscle contraction and glucose uptake in phasic muscle

In present study, inhibition of aerobic metabolism inhibited high K^+-induced muscle contraction in smooth muscle. The inhibition of muscle contraction was remarkably in phasic muscle such as iris sphincter and ileum, but slightly that in tonic muscle such as aorta. Similarly, phloridzin, SGLT inhibitor, remarkably inhibited high K^+-induced muscle contraction in iris sphincter and ileum, but slightly inhibited that in aorta. SGLT1 mRNA was highly expressed in ileum, but SGLT2 mRNA expression was low. On the other hand, the SGLT1 and 2 mRNA were lowly expressed in aorta. Moreover, application of high K^+ increased glucose uptake in ileum. Furthermore, additional application of phloridzin inhibited high K^+-induced glucose uptake. These results suggest that the high K^+-induced contraction in ileum highly depends on aerobic metabolism and relates to glucose uptake via SGLT1 to maintain the muscle contraction.

The relationship between high K^+-induced muscle contraction and glucose uptake in tonic muscle

GLUT4 is expressed in skeletal muscle and adipocyte, it also does in aorta. The present study showed expression of GLUT4 mRNA in rat aorta. Furthermore, application of insulin increased glucose uptake and GLUT4 translocation to membrane in aorta. The increase was inhibited by application of PI3K and Akt inhibitor, but not by AMPK inhibitor. Theses results suggest that the GLUT4 is activated via PI3K/Akt pathway in aorta, similar to skeletal muscle. However, the application of high K^+ did not affect glucose uptake in aorta. This result suggests that aortic smooth muscle contraction highly depends on exogenous energy

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substrate such as glycogen, but not endogenous energy substrate differ from skeletal muscle. Simultaneous application of high K^+ and NaCN increased glucose uptake and GLUT4 translocation to plasma membrane. The increase of glucose uptake was inhibited by application of AMPK inhibitor, but not PI3K/Akt inhibitor. However, the increase of GLUT4 translocation was inhibited by PI3K/Akt and AMPK inhibitor. These results suggest that the inhibition of aerobic metabolism on muscle contraction activates several glucose transporters which depend on AMPK activation. It remains unclear what kinds of glucose transporter relating to muscle contraction. According to the above reasons, in rat aorta, insulin dependent and independent glucose uptake and signaling are similar to those of skeletal muscle. On the other hand, it may be suggested that the high K^+-induced muscle contraction depends on endogenous energy substrate, but the inhibition of aerobic metabolism activates several glucose transporters in aorta. Specifically, it may be implied that GLUT4 translocation needs stimulation of AMPK in aorta. This study demonstrated GLUT4-related signals and mechanisms of glucose uptake on high K^+-induced muscle contraction and inhibition of aerobic metabolism in aorta at the first time.

Conclusion

According to the above results, it is suggested that high K^+-induced contraction highly depends on aerobic metabolism and increases glucose uptake via SGLT1 in iris sphincter and ileum as phasic muscle.

On the other hand, aorta expressed insulin dependent glucose uptake and signaling via GLUT4, as well as skeletal muscle and adipocyte. However, it was different from skeletal muscle in which the high K^+-induced contraction do not stimulate GLUT4 in aorta. Moreover, NaCN-induced decreases of aerobic metabolism, slightly inhibited high K^+-induced contraction and increased glucose uptake via GLUT4 is simillar as skeletal muscle. Furthermore, the present study shows the mechanisms of glucose uptake of smooth muscle differ from organs at the first time. These knowledges probably provide the data contributing to make clear the function of visceral organ in pathophysiological condition such as shock or starved state.

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Studies on nontuberculous mycobacterium; *Mycobacterium* sp. pathogenic for filefish

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Mycobacterial species except for *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, *M. orygis*, *M. pinnipedii*) and *M. leprae* crade has been called as "nontuberculous mycobacteria (NTM)".

According to the Runyon classification, NTM are classified into four groups by their growth rate (SGM: slowly glowing mycobacteria and RGM: rapidly growing mycobacteria) and their photochromogenicity.

Infectious diseases caused by NTM have been reported in more than 165 fish species in saltwater, brackish water and freshwater, regardless of wild, ornamental or aquaculture species. *M. marinum*, *M. chelonae*, *M. fortuitum*, *M. abscessus*, *M. chesapeaki*, *M. shottsii*, *M. pseudoshottsii* and *M. salmoniphilum* are the most commonly identified NTM species as fish pathogen.

In 2009, high levels of mortality were observed in thread-sail filefish, *Stephanolepis cirrhifer*, at a fish farm in Ehime prefecture, Japan. Acid-fast, non-photochromogenic rapid-growing NTM were isolated from the dead fish. DNA-DNA hybridization tests showed that the representative strain, NJB0901, was closely related to *M. chelonae*.

From 2009 to 2013, similar infectious disease cases have been reported in both farmed and wild thread-sail filefish, and farmed black scraper (*Thamnaconus modestus*) populations in several areas of Japan. Black scraper is a closely related species to thread-sail filefish. Some NTM strains were isolated from the infected fish of these cases.

In Chapter 2, twenty-six NTM isolates from the infected file-fishes (thread-sail filefish and black scraper) were characterized using biological and biochemical analyses. In addition, susceptibility tests for antibiotics were also performed with the strains.

These isolates showed identical biological and biochemical characteristics. Growth occurred at 15-35℃, with the optimum temperature being 30℃. Most colonies appeared rough and white colored without pigmentation after incubation at 30℃ for 5 days.

The filefish isolates and *M. chelonae* JCM6388^T showed positive results for catalase activity at 68℃ and negative for growth on medium containing picric acid, while *M. salmoniphilum* ATCC13758^T showed negative and positive results for the two tests, respectively.

In contrast, the filefish isolates and *M. salmoniphilum* ATCC13758^T showed very weak growth on the media containing 5% NaCl, while *M. chelonae* JCM6388^T showed apparent growth on it. From the results, it was indicated that these biological and biochemical tests could be useful to distinguish NTM species among the file-fish strains, *M. chelonae* and *M. salmoniphilum*.

The filefish isolates showed relatively low MIC values with erythromycin and were susceptible to clarithromycin, doxycycline, and ciprofloxacin.

In Chapter 3, transmission trials were performed to evaluate the invasion route of NTM into thread-sail filefish, and pathogenicity of the strain isolated from thread-sail filefish against black scraper.

Transmission trials were performed by immersion, peroral administration and intraperitoneally injection, however, only intraperitoneally injection could reproduce the typical features of file fish NTM infection. The results suggested that some other factors assisting NTM infection might involve in the spontaneous infection.

In the pathogenicity test for black scraper, the cumulative mortality of the experimental group exceeded 50% at 4 days after inoculation and reached 100% at the end of the experiment. This showed the strain isolated from filefish was also pathogenic to black

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scraper. The dead fish showed similar histopathological features to those in the thread-sail filefish characterized by pyogranulomatous lesions on the surface of serosae of alimentary tract and mesentery.

The most common and prominent pathological features of piscine NTM infection are enlargement of the spleen and kidney associated with greyish-white nodules consisted of typical epithelioid cellular granulomas with NTM on these organs, therefore, these organs have been used for isolation of the pathogens. In contrast, such pathological features could not be detected among the diseased filefish in the present study. From the results, it was likely that spleen and kidney could not be suitable for isolation of the pathogens in filefish NTM infections.

In Chapter 4, multi locus sequence typing and molecular phylogenetic analysis, 65-kDa heat shock protein (*hsp*65) PCR restriction enzyme digestion assay (PRA) analysis and pulsed field gel electrophoresis (PFGE) were performed.

PCR and sequencing analyses were performed targeting the 16S rRNA gene, RNA polymerase β-subunit (*rpo*B) gene, 65-kDa heat shock protein (*hsp*65) gene, recombinase A (*rec*A) gene, and superoxide dismutase A (*sod*A) gene.

The partial sequences of these five genes showed 100% similarity among the tested filefish strains. Representative sequences from designated filefish type strain NJB0901 have been deposited in the GenBank database (16S rRNA: AB971866; *rpoB*: LC008146; *hsp65*: LC008145; *recA*: LC008147; and *sodA*: LC008148).

In the phylogenetic tree based on the 16S rRNA gene sequences, NJB0901 was located in the *M. chelonae*-*M. abscessus* group. As for the other four genes, NJB0901 was classified into the same cluster with *M. chelonae*.

In the tree generated from the concatenated data of 16S rRNA, *hsp65*, *rpoB*, *recA*, and *sodA* genes sequences, NJB0901 was distinctly separated from *M. salmoniphilum* and *M. chelonae*, with high bootstrap values among them.

PRA pattern analyses of hsp65 were performed using NJB0901, *M. chelonae* JCM6388^T, and *M. salmoniphilum* ATCC13758^T. Aliquots of the resulting amplicons of hsp65 gene were then digested with restriction enzymes *Bst*EII or *Hae*III. Digested products were electrophoresed and observed by UV transilluminator.

Additionally, the *Bst*EII and *Hae*III restriction sites within the *hsp*65 sequences of tested strains were investigated virtually using GENETYX ver 11.0 to determine the sizes of the expected fragments. These fragment sizes were compared with those of other mycobacterial species in the PRASITE database.

All tested strains showed an identical pattern followed by digestion with *Bst*EII. These findings were supported by the results of the GENETYX program. However, the NJB0901 was predicted to produce bands of 220, 54, and 58 bp following *Hae*III digestion, while *M. chelonae* and *M. salmoniphilum* were expected to produce bands of 197, 60, 54, and 58 bp in the same reaction, respectively. Therefore, *Hae*III digestion could be used to distinguish NJB0901 from the control type strains examined in this study.

Pulsed field gel electrophoresis analysis was performed with six filefish isolates originated from different fish species, the areas of sea around Japan and the isolation periods. The completely same PFGE patterns were observed in all tested strains treated with two different restriction enzymes (*Xba*I, *Ase*I). This results showed all the strains isolated from different conditions have same genotypic character, and it was also suggested that filefish NTM species has already colonize in considerably wide area of sea around south-western Japan.

In Chapter 5, protein profiling and lipid profiling were performed by MALDI-TOF MS. Protein profiling using MALDI Biotyper 3.1 (Bruker Daltonics, Inc.) was performed with filefish strain NJB0901, *M. chelonae* JCM6388^T, and *M. salmoniphilum* ATCC13758^T.

Mass spectra were acquired by autoflex speed (Bruker Daltonics, Inc.) and the homology of NJB0901 with two type strains was evaluated by the Biotyper score values.

The score values for NJB0901 against two type strains, *M. chelonae* JCM6388^T and *M. salmoniphilum* ATCC13758^T were 1.893 and 1.301 respectively. According to the manufacture's instruction, these results suggested that NJB0901 have unique protein profile.

MALDI-TOF MS analysis of total lipids was performed using filefish strain NJB0901^T, *M. chelonae* JCM6388^T, and *M. salmoniphilum* ATCC13758^T. Total lipids were extracted from the bacteria cultured on Middlebrook 7H11 agar with/without Tween80.

The mass spectrum patterns of NJB0901 and *M. chelonae* showed distinctive 44 amu-interval clusters, which only appeared when cultured with Tween 80. In addition, these two strains did not share the mass range. These results suggested that NJB0901 possessed a unique metabolic mechanism for Tween 80.

In Chapter 6, PCR primers set was designed for

specific detection of *Mycobacterium* sp. isolated from filefish, and evaluated the availability for rapid diagnosis. The primers set was designed by using draft genome sequences acquired by next-generation sequencer. The designed specific primers, M ste-F and M ste-R, were specifically reacted with *Mycobacterium* sp. isolated from filefish and did not react with other NTM type strains. The detection limit was 1pg/uL of genomic DNA extracted from pure cultured NJB0901. The result indicated high availability of the PCR primers set for rapid detection of filefish NTM pathogen.

In addition, detection tests for bacterial cells in the kidney and spleen of black scraper experimentally infected with filefish NTM were performed. PCR primers set could detect 10^3 CFU of mycobacterial cells as the lowest level. From the results, the author concluded that the detecting methods would have room for improvement in efficiency of DNA extraction or choosing the appropriate specimens.

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Analysis on the mechanism of reduced nephron number and the pathological progression of chronic renal failure in Astrin deficient rats

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Chronic kidney disease (CKD) is a common disease exhibiting globally high morbidity rate. About 13.3 million people corresponding to 10-13% of general population is thought to have CKD in Japan. However, at present, there is no effective treatment for end stage CKD except for dialysis and kidney transplantation. Recently, the patients requiring dialysis therapy are increasing every year, because the treatment that can drastically improve quality of life in CKD patients has not been established. CKD ranks in the top 3 of the cause of death in dogs and cats, because the application of dialysis therapy and kidney transplantation is very limited in veterinary medicine. Therefore, the elucidation of pathogenesis in CKD, the identification of surrogate markers, the establishment of effective treatments for CKD is desired. In this situation, many challenging research has been performed to develop renal regeneration therapy. In renal development, however, three-dimensional architecture is intricately constructed with many types of cells, and many developmental events remained unclear. Thus, regeneration technique is delayed in kidney compared to other organs. Although CKD is caused by various congenital and/or acquired factors, there is a common pathway in which reduced number of nephrons causes overload to remaining individual nephron, irreversibly deteriorating renal damage (Brenner's theory). Progressively reduced number of nephrons result in reduced excretive function, renal anemia, and renal fibrosis at end stage of kidney disease. On the other hand, total number of nephrons has been reported to vary in humans and to be closely associated with birth weight. A congenitally reduced number of nephrons is considered to be an important risk factor related with pathogenesis and prognosis of CKD. Therefore elucidating mechanism for determining nephron number during renal development will provide important information useful for developing kidney regeneration therapy and estimating a risk factor for CKD. In addition, elucidating mechanism by which congenitally reduced number of nephrons causes CKD might provide clues to understand pathological process common in different types of CKD caused by various factors. In this thesis, I revealed the cause of reduced nephron number during embryonic development and the pathological progression of CKD in hypoplastic kidney (HPK) rats with congenitally reduced (-80%) number of nephrons resulting from loss-of-function type mutation of Astrin gene which is known to be related with the progression of mitotic metaphase and with the inhibition of hyperactivation of mTOR signaling. Finally, I discussed about possible therapeutic strategies and molecularly targets based on the results obtained in this study.

In chapter 2, I demonstrated that HPK rats show macrocytic erythropeina with the progression of CKD. In general, major source of erythropoietin (EPO) is considered to be fibroblasts located in juxtamedullary interstitium. EPO is secreted from fibroblasts in response to hypoxia and can induce erythropoiesis in bone marrow. Normocytic normochromic anemia accompanied by reduced response of EPO production to hypoxia is often observed in end stage of CKD. Recently, this pathological condition is considered to be caused by the transdifferentiation of EPO-producing fibroblasts into myofibroblasts with the progression of interstitial fibrosis. I found that HPK rats have

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normal level of plasma EPO concentrations under normoxic condition in despite of the appearance of CKD symptoms. Interestingly, EPO mRNA expression was decreased in kidney and increased in liver of HPK rats, indicating that increased hepatic EPO production might compensate decreased renal EPO production. Moreover, EPO mRNA expression were normally induced by hypoxic condition in both kidney and liver of KPK rats at 140 days of age, indicating HPK already affected by fibrosis still has potential to produce EPO in response to hypoxia. On the other hand, we found increased the fragility of erythrocyte membrane, the promotion of splenic hemosiderosis, and decreased serum transferrin concentration, normal level of plasma ion due to significantly increased transferrin saturation in HPK. These results suggested that, although erythropoiesis in HPK is maintained almost normal by hepatic compensative EPO production against renal reduced production, erythropenia is induced by hemolysis of red blood cells.

In chapter 3, I demonstrated that glomerular lesions are prior to interstitial alterations in renal fibrosis of HPK with 80% nephron reduction. Although it is known that a considerably reduced number of nephrons progressively induces renal damage via overload to individual nephron, it is still unknown how many nephrons are required for maintenance of normal renal function through life span. We found glomerular hypertrophy, discontinuous immunostaining of podocin along the glomerular basal membrane, and infiltration of inflammatory macrophages into glomerulus in HPK at 35 and 70 days of age. At 70 days of age and afterwards, accumulation of extracellular matrixes (ECMs), increase in mesangial cells, and glomerular sclerosis were gradually deteriorated in HPK. In accordance with these changes, glomerular PDGF- and TGF-β-positive areas were increased in HPK. Glomerular PDGFr-β-positive area was significantly increased in HPK, whereas α-SMA -positive myofibroblasts were rarely detected in glomerular tufts in spite of the appearance of myofibroblasts in glomerular parietal epithelium along Bowman's capsular walls of HPK. On the other hand, in interstitial tissue, age-related increase in accumulation of ECMs was accompanied by the age-related increase of myofibroblats by transdifferentiation from increased fibroblasts in the intrestitium of HPK after the progression of glomerular injury. In accordance with these alterations, we detected infiltration of macrophages into interstitium at 140 and 210 days of age. PDGF-positive area was also increased in tubulointrestitium with fibrosis. These results indicated that congenital 80% nephron reduction results in progressive CKD resulting in renal fibrosis. In HPK, moreover, glomerular lesions appeared early but progressed slowly without increased myofibroblasts, whereas interstitial fibrosis appeared later but progressed rapidly with increased myofibroblasts. These pathological changes might be mediated by growth factors including PDGF and TGF-β.

In chapter 4, I demonstrated that loss of functional Astrin causes nephron reduction via decreased branching of ureteric bud (UB) associated with increased apoptosis of metanephric mesenchyme (MM). Normal renal development progresses through interaction between UB and MM. UB is the first branch of Wolffian duct as a primordium of collecting duct, advances into MM, and interacts with MM surrounding UB. In HPK metanephros, increased apoptosis and decreased proliferation were observed in MM cell surrounding UB. In addition, population of MM cells expressing upstream interaction signals (Sall1 and Pax2) and mRNA expression of Sall1, Kif26b, and Pax2 were already decreased in HPK metanephros at embryonic day (E) 14.5, whereas decrease in mRNA expression of other interaction signals was not detected. In normal metanephric development, MM cells form Six2-positive cap cluster surrounding UB tips. I found decrease in metanephric size, thinning of Six2-positive cap cluster, and decrease in Six2 positive cap area around individual UB in HPK metanephros at E14.5, whereas reduced branching of UB was initially detected at E15.5 in HPK. Therefore, it was suggested that MM is mainly affected by loss of Astrin, secondarily causing reduced branching of UB. MM clusters are believed to differentiate into most of nephron components, and tubular epithelia and podocytes are differentiated through mesenchymal-epitherial-transition (MET). I observed normal progression of MET in HPK metanephros. It has been reported that the knockdown of Astrin in HeLa cells causes mitotic arrest at metaphase in cell cycle, resulting in apoptosis. In accordance with the phenotype in HeLa cells, abnormal mitotic metaphase and subsequent apoptotic cell death were observed in immature Sertoli cells of testicular dysplasia accompanied in male HPK rats. Therefore I assumed that decrease in MM of HPK metanephros would be caused by loss of Astrin function associated with cell cycle progression. Unexpectedly, increase of metaphase cells positive for phosphor-histion H3 was not detected in HPK metanephros at all embryonic days examined. Alternatively, as another function of Astrin, it has

been reported that Astrin inhibits apoptosis through suppressing hyperactivation of mTOR signaling via recruiting Raptor, a component of mTOR complex 1, into stress granules in HeLa cells under stress condition. In my experiments regarding to mTOR signaling, increased mRNA expression of mTOR and it's downstream S6K1 and increased phosphorylation of S6K1 were detected in HPK metanephros, suggesting that Astrin is associated with the regulation of mTOR signaling in developing metanephros.

In chapter 5, I tried to replicate metanephric phenotype using in vitro organ culture method and analyzed the phenotype of MM cells affected by Astrin deficient. The growth of E14.5 HPK metanephros cultured for 3 days was apparently delayed compared to that in normal metanephros. When metanephros were cultured with low dose (0.05ng/ml) of mTOR inhibitor (Evelorimus), metanephros derived from normal embryos were significantly decreased in size compared to vehicle control (DMSO), whereas metanephros from HPK embryos were almost comparable in size to control. In addition, the increment of metanephric size for 3 days culture was significantly lower in HPK than in normal in vehicle controls, whereas HPK metanephros exhibited significantly larger increment compared to normal in Evelorimus treatment. These results suggested that the regulation of mTOR signaling with Astrin is related with metanephric phenotype of HPK. Furthermore, Six2-positive MM cells were markedly decreased in HPK metanephros cultured for 3 days, indicating early loss of MM cells. In order to analyze the affect of Astrin defect in MM cells, I established culture system of isolated MM cells from E14.5 metanephros in individual rat embryo. Primary-cultured MM cells derived from normal metanephros expressed mesenchymal markers and Astrin but not epithelial and stromal markers. Passage 1 (P1)-MM cells derived from HPK metanephros showed decreased expression of mesenchymal markers and increased expression of a stromal marker. RT-PCR fragment including insertion mutation of Astrin transcript was hardly detected in HPK P1-MM cells. These results indicated altered stemness in HPK MM cells losing Astrin function. Furthermore, I observed increased apoptosis in Six2-positive HPK P1-MM cells, suggesting cultured HPK MM cells replicate similar phenotype as shown in vivo. Although both normal and HPK P1-MM cells formed immature podoplanin-positive clusters through induction with embryonic spinal cord, clusters in HPK MM cells look like immature and small compared to normal MM cells, suggesting it need more time to form glomerulus in HPK compared to normal.

In summary, the present study suggested that the loss of Astrin changes stemness in MM cells and decreases signals related with interaction between MM and UB. It is also suggested that increased apoptosis and decreased proliferation in MM cells induce reduced branching of UB with thinning of nephron formation layer and early reduction of stem cells. These sequential events might finally lead to 80% nephron reduction. Interestingly my experiments also suggested that these defective processes are involved in hyperactivation of mTOR signaling under the loss of Astrin. In HPK rats, resulting 80% nephron reduction causes CKD at adult and leads to renal fibrosis at advanced age. The progression of CKD in HPK rats is characterized by early and slow progression of glomerular sclerosis, later appearance and rapid progression of interstitial fibrosis, and specifically macrocytic erythropenia. In last chapter, I described my collaborating research demonstrating that low dose of Everolimus treatment for long period attenuates renal dysfunction and fibrosis in HPK rats. These drastic effects might be mediated by preventing PDGF signaling by mTOR inhibition. Taken together, through a series of my studies, it was indicated that activation of mTOR signaling is associated with not only fibrosis via increased myofibroblasts but also early reduction of mesenchyme nephrogenic progenitors. I hope in future these evidences will contribute to reveal congenital risk for CKD, to improve renal regeneration therapies, and to develop molecularly targeted drag therapies against renal fibrosis.

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The effects of cardiac dyssynchrony in dogs

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In human medicine, numbers of large clinical trials has shown that the prolonged QRS duration is associated with survival times in heart failure patients. Wide QRS duration indicate ventricular myocardial electrical activation delay (i.e. electrical dyssynchrony). As the electrical dyssynchrony could cause dyscoordinate and inefficient stroke of left ventricular, the wide QRS duration would lead malignant prognosis. This theory has been supported by the marked effects of cardiac resynchronization therapy, which correct only electrical activation timing by pacing device. However, it was reported that the criteria for approving cardiac resynchronization therapy based on QRS duration could cause failure of treatment in 40-50 percent of patients. Therefore, not only electrical dyssynchrony but also mechanical dyssynchrony, which is the delay of myocardial contraction, would be more sensitive criteria for cardiac resynchronization therapy. A large number of indices using echocardiography, cardiac computed tomography, or cardiac magnetic resonance images have been developed for detecting mechanical dyssynchrony.

In veterinary medicine, it was reported that the QRS duration was associated with survival time in dogs with dilated cardiomyopathy, and there is a case report which described left ventricular dysfunction observed in 2 dogs with left bundle branch block (LBBB). As the electrical conduction system of dogs is similar to humans, the impairment of cardiac function by dyssynchrony could also be occurred in dogs with wide QRS duration. However, it has not been sufficiently studied how affected cardiac function by cardiac dyssynchrony and what factor would influence the effects of dyssynchrony. In dogs having higher heart rate, echocardiography would be suitable for detecting mechanical dyssynchrony, as echocardiographic study is non-invasive and have higher time resolution. However, it has not been sufficiently studied whether the echocardiographic indices is useful for identifying mechanical dyssynchrony in dogs.

This study was designed to assess 1) measurements of echocardiographic dyssynchrony indices in normal beagles, 2) the ability of dyssynchrony indices to identifying dyssynchrony in a canine model of left bundle branch block, 3) changes of cardiac function and dyssynchrony indices and effects of exercise in a canine model of left bundle branch block, 4) body size effects of the deterioration of cardiac function in a canine model of left bundle branch block.

This study was conducted as a part of the research development of "optimal medical development using simulator of human heart", which was performed by Professor Toshiaki Hisada affiliated national University of Tokyo, supported by the Japan Society for the Promotion of Science (JSPS) through its "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)."

1. Assessment of Dyssynchrony Indices in Normal Beagles (Chapter II)

The electrical conduction time of myocardium is defined by conduction velocity and conduction distance. The reference range of dyssynchrony indices in each breed should be determined, as body size of dog is varied widely. With regard to echocardiographic dyssynchrony indices, there is only one report which described reference range from dogs of widely variety breeds. Therefore, the dyssynchrony indices using M-mode and two-dimensional speckle-tracking echocardiography (2D-STE) were measured in 53 healthy beagles, and the possibility, repeatability, and reference rage of dyssynchrony indices were assessed. As the septal to posterior wall motion delay (SPWMD) measured as the time difference from the time point of interventricular septal peak inward motion to the

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time point of left ventricular posterior wall peak inward motion using M-mode echocardiography had shown high repeatability and relatively narrow reference range, the SPWMD would be simplified index for identifying dyssynchrony. With regard to the dyssynchrony indices used 2D-STE, maximal difference of time to peak radial strain for 6 segments (MaxD-TpSR), standard deviation of time to peak strain for 6 segments (6SD-TpSR), and percentage of the frame-to-frame changes which differ from the averaged strain change in systolic phase (DysSR) had shown narrow reference range similar to humans. These dyssynchrony indices would be useful to detect mechanical dyssynchrony.

2. Assessment of the ability of the dyssynchrony indices to identifying mechanical dyssynchrony in a canine model of left bundle branch block (Chapter III)

There are several number of reports described dyssynchrony indices in dogs, but there was only one report described dyssynchrony indices in dogs with dyssynchrony, which studied the standard deviations of radial and circumferential strain for 6 segments in a canine model of dyssynchrony induced by atrioventricular node ablation and right ventricular pacing. There is no report which compared ability of dyssynchrony indices to identify mechanical dyssynchrony. Therefore, the ability of the dyssynchrony indices from M-mode and 2D-STE, included in difference between first inward peak of interventricular and left ventricular posterior wall (first SPWMD), SPWMD, MaxD-TpSR, 6SD-TpSR, and DysSR, to detect dyssynchrony were assessed in a canine model of left bundle branch block (LBBB) induced by radio frequently ablation. To assess the ability of dyssynchrony indices, receiver operator characteristic analysis was performed using ten beagles undergone left bundle branch ablation as positive control. The optimal cut-off value, sensitivity and specificity were SPWMD 42.7 ms (sensitivity 1.000, specificity 0.400), first SPWMD 143.3 ms (sensitivity 1.000, specificity 1.000), DysSR 7.32 % (sensitivity 1.000, specificity 0.900), MaxD-TpSR 13.5 ms (sensitivity 0.900, specificity 0.600), and 6SD-TpSR 4.21 ms (sensitivity 1.000, specificity 0.500). Although SPWMD would be difficult to use for identifying mechanical dyssynchrony independently, first SPWMD and indices from radial strain using 2D-STE (especially DysSR) were useful to detect mechanical dyssynchrony in a canine model of LBBB.

3. The effects of exercise on deterioration of cardiac function via the cardiac dyssynchrony in a canine model of left bundle branch block (Chapter IV)

With regard to changes of cardiac function in a canine model of dyssynchrony, one report had shown significant decrease of left ventricular ejection fraction immediately by induction, while the other report had shown no significant change of left ventricular ejection fraction. Therefore, it has not been clear whether the dyssynchrony could induce deterioration of cardiac function independently. Moreover, it was reported that right ventricular high frequent pacing could decrease left ventricular ejection fraction significantly. Therefore, the hypothesis that dyssynchrony with some stress could cause deterioration of cardiac function was studied. Twelve beagles (body weight 10.4±1.0 kg) undergone left bundle branch ablation was divided into 2 groups; non-exercise group (n=6), cage rest during observation period, and exercise group (n=6), added treadmill exercise (13 km/hour, for 15 minutes, once in a day) from 2 week after ablation, and echocardiography and measurements of dyssynchrony indices were performed at before (Pre) and 4 weeks (i.e. 2 weeks after starting exercise stress; Ex2weeks) and 8 weeks (i.e. 6 weeks after starting exercise stress; Ex6weeks) after ablation. In non-exercise group, the left ventricular ejection fraction was not changed significantly (Pre vs. Ex2weeks, p=0.188, Pre vs. Ex6weeks, p=0.087). In exercise group, however, the left ventricular ejection fraction was decreased with time, and significant changes were observed when comparing Pre with Ex2weeks and Pre with Ex6weeks (p=0.030, p=0.005, respectively). In both groups, DysSR measured as an index of mechanical dyssynchrony were increased with time, and significant changes were observed as follow; in non-exercise group, when comparing Pre with Ex2weeks, Pre with Ex6weeks, and Ex2 weeks with Ex6weeks (p=0.004, p<0.001, p=0.024, respectively); in exercise group, when comparing Pre with Ex2weeks and Pre with Ex6weeks (p=0.008, p<0.001, respectively). In both groups, first SPWMD was increased with time, and significant changes were observed when comparing Pre with Ex2weeks and Pre and Ex6weeks (p<0.001, respectively). Dyssynchrony induced by LBBB could not cause left ventricular dysfunction independently during 8 weeks observation period, however, with exercise, it cause significant decrease of left ventricular ejection fraction with time. These findings were not according

to a previous study. It might be caused by difference of body size of dogs. In both groups, echocardiographic dyssynchrony indices were increased with time. It would support the theory of "dyssynchrony begets dyssynchrony".

4. The effects of body size on deterioration of cardiac function via the cardiac dyssynchrony in a canine model of left bundle branch block (Chapter V)

From the results of Chapter IV, deterioration of cardiac function by dyssynchrony would be associated with body size of animals (i.e. heart size). To assess this hypothesis, six mongrel dogs (body weight 21.5±3.4 kg) larger than beagles undergone left bundle branch ablation were divided into 2 group; non-exercise group (n=3) and exercise group (n=3). Echocardiographic study and measurements of dyssynchrony indices were performed before (Pre) and 4 weeks (i.e. 2weeks after starting exercise stress; Ex2weeks) and 8 weeks (i.e. 2 weeks after starting exercise stress; Ex6weeks) after ablation. Different from the results of Chapter IV, the left ventricular ejection fraction was decreased with time and significantly decreased when comparing Pre with Ex6weeks (p=0.014) in non-exercise group. In both groups, DysSR measured as index of mechanical dyssynchrony were increased with time, and significant increase was observed when comparing Pre with Ex2weeks and Pre with Ex6weeks (p=0.032, p=0.013, respectively) in exercise group. In both groups, first SPWMD were increased after ablation, and significant increase were observed when comparing Pre with Ex2weeks and Ex6weeks (p<0.001, respectively). In large breed dog, therefore, dyssynchrony induced by LBBB could cause deterioration of cardiac function without any exercise stress. Deterioration of cardiac function via dyssynchrony would depend on body size. Although the left ventricular ejection fraction decrease has not been emphasized by exercise stress, the dyssynchrony was developed earlier in exercise group. In large dogs, dyssynchrony was more severe than beagles. Thus, the effect of exercise might be more significant in this study.

This study confirmed that the ability of dyssynchrony indices from M-mode and 2D-STE reported in human medicine were assessed in dogs, cardiac function and dyssynchrony indices changes with time were observed in a canine model of LBBB, and the factor, especially exercise and body size, could influence the effect of dyssynchrony. First SPWMD from M-mode and indices from radial strain based on 2D-STE were useful to identify mechanical dyssynchrony in dogs. In these dyssynchrony indices from radial strain, DysSR would have highest reproducibility and ability to detecting mechanical dyssynchrony.

In medium size dogs, such as beagles, or more small size dogs, dyssynchrony from LBBB could not cause significant deterioration of cardiac pump function independently, though dyssynchrony with exercise could cause that. While in large size dogs, dyssynchrony from LBBB could cause left ventricular dysfunction without any exercise stress. In veterinary practice, therefore, the body size of dogs should be considered when assess mechanical dyssynchrony in dogs suspected dyssynchrony, such as dogs with ventricular electrical conduction delay, without evidence of cardiac disease. Assessment of mechanical dyssynchrony would provide beneficial information to estimate the risk of left ventricular dysfunction without primary cardiac disease. Moreover, an exercise could cause deterioration of dyssynchrony and might influence long term survival in large dogs. Even in medium or smaller size dogs suspected electrical activation delay, assessment of dyssynchrony and consideration whether exercise restriction should be imposed would be important.

In veterinary medicine, the prevalence of LBBB is relatively rare. However, the cases of dogs undergone ventricular pacemaker treatment for bradyarrhythmia have been reported frequently, and these patients would show similar electrical conduction behavior to dogs with LBBB. Therefore, evaluating dyssynchrony would be important in dogs undergone pacemaker treatment in practice. Finally, farther investigation intended for clinical cases is expected.

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Studies on preventive medicine for family dogs

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Summary

Preventive medicine means is the onset prevention and the progression prevention of diseases. In the present medical care for family dogs, prevention of infectious disease by vaccination is mainstream, and the periodic medical examination for prevention of diseases begins to spread among a part of dog owners. In this study, I performed an epidemiology investigation using pet insurance data for the purpose of preventive enlightenment, searched for the risk factor associated with foreign body ingestion in family dogs, and searched for the dog breast tumor early checkup marker as the progression prevention. Furthermore, in a past study, adiponectin was proved to be useful as an early checkup marker in a breast tumor. So I examined possibility of the application to the preventive health care of such a biochemistry marker in dogs. Through these, I aim at becoming the clue to realization of the preventive medicine about various diseases of the dog.

1. Disease statistics of the dog by pet insurance data

Insurance payment data are the epidemiology data which had the information "that received payment of the insurance for a certain reason in a certain population at a certain time".

Those information has the possibility that they can realize not only the aggravation prevention but also the onset prevention by sending it to the necessary one by an effective timing and method.

256, 144 0-12-year-old insured dogs were surveyed and performed the disease statistics by pet insurance data. The disease that was frequent in a dog was 23.0% of skin diseases, ear disease 15.4%, digestive organ disease 14.7%, ophthalmopathy 10.0%. The disease that a notable tendency was seen by seasonal variation was a skin disease. A tendency to onset more different than a season was seen in this disease. There was a lot of dogs which contracted a disease from July to September and a few dogs which contracted a disease from January to March. The skin disease, the disease of the ear, the digestive organ disease showed high prevalence at all 0-12-year-old age. The disease of eyes and the tumor disease showed high prevalence of more than 10.0% at after 7 years old. Prevalence tended to increase the circulatory organ disease, the tumor disease with aging. A Japanese dog showed high prevalence to the ear disease, the skin disease, the symptom in comparison with the result of the epidemiology investigation into British royal veterinary college more than 5%. The prevalence of the disease that an owner is easy to notice a symptom rises. It leads to going to the hospital early that an owner checks the health condition of the dog at home.

2. Search of the foreign body ingestion risk factor of the dog

There is much frequency of foreign body ingestion of the Japanese family dog. It becomes the big stress for an owner psychologically economically. In addition, it may lead to a fatal accident when it is the worst. Therefore it is necessary to take prophylaxis of foreign body ingestion. However, it is hard to say that the search of the risk factor to lead to the prophylaxis has been performed so far. Therefore I performed a risk factor search to lead to the foreign body ingestion prevention by pet insurance data and questionary survey.

The factor that much foreign body ingestion occured was 0-1 years old, a Flat-coated retriever, a bernese mountain dog, a beagle, a French bulldog, RETRIEVER-group. The dog which dealt with sterility had high connection with the foreign body ingestion. In 13 temperament to be considered to be action properties of

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the dog, a related thing with foreign body ingestion was suggested for "chasing" and "attachment". In addition, the following led to fatal foreign body ingestion; things that the risk is recognized "chocolate" "ethylene glycol" (nonfreezing fluid) "slug exterminator" "raticide" "weed killer" "bamboo skewer". Things in life space close "Cloth such as socks or the towel", "seed of fruit and the pickled plum", "a stone and sand", "ball" "lily". The thing which pet owner gave for a dog "supplement" "snacks" "gum". For these risk factor candidates, performing preventive enlightenment with a concrete image, carrying out and measures every individual factor, inspection those effects, and carrying out again are important.

We can expect it when connected for the prevention of substantial foreign body ingestion by doing so it.

3. An investigation into tumor prevalence of the dog and search of the breast tumor early checkup marker

For approach to the tumor high-risk group, I investigated the tumor prevalence of the family dog. In addition, for the purpose of groping for simple procedure, I searched for the early detection marker of breast tumor.

The prevalence of the tumor disease of the dog (0-10 years old) was 7.9% in bitchs 6.4% in male dogs. A bitch showed the ratio that was 1.5% higher than a male dog. In addition, increase in age-related prevalence was seen. After 6-7 years old in particular showed a tendency to increase that was more sudden than it past. The ratio of tumor disease among the claim before the death contract accounted for 16.9% in 10 years old. In 16 dog breed and a mixed dog less than 10 kg, the dog breed that showed the prevalence of the highest tumor disease was golden retriever 15.4% the prevalence of breast tumor of the bitch was 0.4% at 4 years old, increased by aging afterwards, and was 3.9% at 11 years old. The dog breed that showed the value that the prevalence of breast tumor was higher in than 0.7% of whole dog was Maltese dog 1.3%.

I measured mRNA expression of blood of the dog which became the operation adaptation of breast tumor and isolated tissue tumor-related gene p21, p53, erbB2, BRCA1 and BRCA2 for the purpose of searching for breast tumor marker. However, all genetic expression had a big individual difference, and the significant difference ($P<0.05$) was not recognized between a malignancy of the illness.

By the prophylaxis of cancer, the making of of the early detection structure and maintenance of the information infrastructure of that purpose are important. The enhancement of the support system when promotion of the examination in an appropriate timing, establishment pro-inspection such as tumor marker or the genetic test of the dog, "the early detection of cancer" were done every instruction and dog species of the home check by the owner is expected.

4. Possibility as the newly early tumor diagnosis marker of adiponectin

To investigate the role of adiponectin (APN) in development of mammary tumor, plasma adiponectin concentrations and expression of mRNA of adiponectin specific receptors, AdipoR1 and AdipoR2, were measured in mammary tumor tissues of dogs. 65% (17/26) of dogs with mammary tumor (low APN group) showed significantly low plasma APN concentrations ($14.3 \pm 1.0 \mu g$/ml, mean ± 95% C.I.) compared to normal control values ($30.9 \pm 10.6 \mu g$/ml). mRNA expression of AdipoR1 and AdipoR2 were detected in mammary tumor tissues of dogs, and mRNA expression of AdipoR1 was 2-4 times higher than that of AdipoR2. In the low APN group, mRNA expression of AdipoR2 in mammary tumor tissues decrease compared to that in high APN group, however the difference was not significant. Decrease in circulating APN concentrations appears to be a risk factor for mammary tumor in dogs as for postmenopausal breast cancer in women.

By the disease statistics of the dog by pet insurance data, a disease to be seen frequently in Japan became clear quantitatively. In sex, age, breed, the thing investigating in detail of the hereditary or environmental factor of each disease become easy to hypothesize. And we come to be able to prevent the outbreak of a basic disease or aggravation. In this study, I groped for measures of the onset prevention of foreign body ingestion and the aggravation prevention of tumor as example. We practice these measures and inspect the effect and can hope that we reduce illness itself by raising precision.

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Comparative biochemistry studies of the energy metabolism in large animals

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Summary

In recent years, the progress of veterinary science has contributed to the longevity of animals. However, along this development, the number of metabolic and age-related disorders in animals has been rapidly increasing. Similarly to humans, many major diseases are related to metabolic conditions, therefore investigating the metabolic biomarkers is beneficial for both animals and humans. In this study, we investigated and compared the metabolic systems in large animals, such as bottlenose dolphins (*Tursiops truncates*), horses, and cows. Comparative studies are helpful because this knowledge can offer critical information, leading to establishing the basic standard data and discovering the distinct features of the animals. It is also advantageous to use this knowledge for other fields, including the human medical field.

1. Cholesterol Lipoprotein Profiles of Large Animals

Cholesterol profile patterns between bottlenose dolphins (*Tursiops truncates*, hereinafter referred to as dolphins), horses, and cows showed that all animal groups were classified into HDL dominant mammals, such as dogs and cats. Dolphins and horses also displayed clear LDL-Cho peak, which was not shown in cows, thus the cholesterol profile of dolphins is a closer resemblance to that of horses, rather than that of cows. Evidence now indicates that understanding the characteristics in the lipid and lipoprotein parameters of each animal group is necessary for maintaining a healthy metabolism in animals. In addition, various factors can often alter the patterns, so investigating how they are involved in lipid metabolism can lead to a more specific evaluation of an animal's metabolic state. Overall, monitoring the plasma lipid concentrations and cholesterol profile in animals is useful to detect abnormal metabolic states in order to prevent various metabolic-related disorders.

2. Comparison of Energy Metabolism and LDH Isoenzyme Patterns in Large Animals

Regarding energy metabolism between these animal groups, dolphins appear to have the greatest level of oxidative energy metabolism amongst horses and cows, due to having the greatest levels of plasma MDH activity. In addition, dolphin energy production/usage efficiency was second (M/L ratio=0.67) behind that of horses (M/L ratio=0.79). Overall, these results suggest that dolphins may possibly generate more energy than horses, especially for increased mobility in the water; however, all of the produced energy is not utilized for various reasons, such as a smaller tank size or other environmental limitations. However, cows demonstrated the highest plasma LDH activity amongst all animal species. This may have been attributed to their lactating state. Although all animal groups displayed a different plasma LDH isoenzyme pattern distribution, dolphins and horses demonstrated a similarity with LDH-3 isoenzyme predominating in plasma, as opposed to LDH-1 in cows, which would reflect "Symmorphosis" of these two species and their aerobic/anaerobic energy metabolism needs.

Overall, plasma MDH and LDH activity levels, M/L ratio, and plasma LDH isoenzyme pattern can all be useful indicators for a better understanding of the oxidative energy metabolism and monitoring of a captive animals' health. As it is not easy to obtain tissue samples from animals, the development of blood

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indicators for evaluating the whole body metabolic state is necessary, and further research is required and should be pursued.

3. The Aging Effect on the Metabolic System in Riding Horses

The study about the aging effect demonstrated that aging might not induce remarkable changes in a metabolic system for horses, possibly due to their continuous daily exercises. As all the horses had constant physical activity on a daily basis, recovery of the ADN level was possibly a confirmation that aged horses could improve their metabolic and immune systems, preventing a negative aging effect. Additionally, they sustained a balanced M/L ratio among all the age groups. Moreover, as elevated SOD activities in middle-aged and aged horses were displayed, their exercise protocol was a beneficial strategy to enhance anti-obesity and anti-aging promotors. In fact, due to their continuous daily exercises, the riding horses maintained their physical condition as they grew older.

Aging is an inevitable event for all living organisms. The destruction of the metabolic or immune systems accompanied with aging can completely change our lives. Although further research is required for the development of blood indicators, blood analysis can serve as a useful index for early detection and protection from metabolic-related and/or age-related diseases. Finding a key to enhance the metabolic system in order to reduce the risks for various age-related diseases can offer new intriguing avenues for a desirable and healthy life.

4. Future Directions: The Aging Effect in Dolphins

This comparative study suggested that each animal species has a distinct metabolism pattern, but there are some similarities with other species as well. From these similarities, it is possible to presume certain effects on the metabolic system in similar animal groups. Investigating and comparing the metabolism in animals can lead to a better understanding of their normal health condition. Since some animal species are often limited to research, it is important to gain helpful insights from the similarities and the differences with other species.

Evidence now indicates that daily physical activity may help improve the metabolic ability and can attenuate the negative aging effects in active animals, such as riding horses. Although dolphins appear to have a unique metabolic system, it is speculated that their regular physical activity, including shows in the daytime, have a similar impact on their health management when they age. Moreover, dolphins and humans share several features, hence examining the unique metabolic system in dolphins can lead to new, impactful, and beneficial health and disease theories for humans as well.

As stated above, comparative studies can allow us to recognize the remarkable features of the animals. The basic information gained from comparative studies can bring us profitable knowledge for establishing the standards of animal basal metabolism. A better understanding of metabolism patterns can lead to more efficient management and disease prevention strategies for these animals.

An abundance of information is hidden in the biology of animals, which we can carefully and ethically exploit for medicine and technology areas. Given that having a balanced metabolic system is necessary for good health, identification of the advantageous metabolic process can bridge a knowledge gap in metabolic-related issues in animals and humans. Generally, the impact of exercise proved to work positively for anti-obesity and anti-aging in animals and humans. In fact, each animal species has a different energy utilization process, thus it is favorable to apply the most appropriate exercise strategy for each animal species. To improve the health management of animals, it is necessary to understand each metabolic system in order to prevent any dysregulation of metabolism, if it occurs. A comparative study can be the first step to more efficient management and disease prevention strategies. It can also offer new pragmatic avenues and therapeutic approaches for animals and humans.

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Study on function of sirtuins in inflammation suppression of feline tissues

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Summary

A quick PubMed search for articles containing the keyword "Sirtuins" generates more than 4,000 papers, and the number has rapidly increased in recent years. The role of sirtuin in biochemistry, physiology and clinical medicine has been noticed as a key factor for metabolic and age-related diseases. However, there is little information about sirtuins in veterinary medicine. On the other hand, increasing in metabolic and age-related diseases is also a major problem in veterinary medicine in recent years, and development of the early diagnosis and prevention method for the above diseases is urgent subject for veterinary medicine. The aim of this study was to reveal the molecular mechanisms of sirtuins in inflammation of cat tissues.

1. Mammalian sirtuins have been identified as homologs of the yeast silent information regulator 2 (Sir2). Mammalian sirtuins included seven family and each has different target proteins. Most tellingly, SIRT1 and SIRT3 are induced by calorie restriction (CR), and related to the metabolic and age-related diseases in human and mouse. However, very little information is available on cat SIRT1 and SIRT3. Therefore, we considered that to obtain the basic knowledge about cats SIRT1 and SIRT3 is necessary. We determined the cat SIRT1 and SIRT3 cDNA sequences and examined their mRNA expression in several tissues. We successfully cloned the cat SIRT1 and SIRT3 cDNAs. Cat SIRT1 and SIRT3 showed high sequence homology with other vertebrate SIRT1 (>61.3%) and SIRT3 (>65.9%), respectively. Cat SIRT1 and SIRT3 were highly conserved, and they showed especially high homology in the catalytic core domain. This core domain included in zinc fingers and NAD^+ binding sites. SIRT1 and SIRT3 were genetically conserved in the phylogenetic tree, and may have functions similar to those of other animals. The results of real time PCR using tissue total RNA revealed that cat SIRT1 and SIRT3 mRNA were expressed in various tissues similar to other animals. High expression were observed in the liver and skeletal muscle for SIRT1 and in the heart for SIRT3. From the above results, cat SIRT1 and SIRT3 are expected to have various physiological activities as well as other animals. Further examinations of thier detail function and relationship with diseases are necessary.

2. Recently, prevalence of obesity has increased also in cats. Lipotoxicity observed in obese animals seems to be fundamental pathogenesis for various metabolic diseases including diabetes mellitus. SIRT1 and SIRT3 have been considered to play important roles in molecular mechanism of obesity onset particularly via inflammation. However, very little information is available on mechanism of lipotoxicity and the role of sirtuins in cats. Therefore, we induced obesity by feeding on high-fat diet (HFD) for 8 weeks in cats, and investigated expressions of inflammatory makers, cytokines, SIRT1 and SIRT3 in peripheral leukocytes. Body weights of cats significantly increased, but other metabolic markers did not change after HFD feeding. Hepatic injury markers, ALT, ALP and AST activities, significantly increased by HFD feeding. Although peripheral leukocyte inflammatory cytokine mRNA expression did not increase, mRNA expression of SIRT1 significantly increased by HFD. From the above results, we consider that inflammation is induced by lipotoxicity in the liver, and inflammatory signals are suppressed by SIRT1 in the peripheral leukocyte after HFD feeding.

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We consider that SIRT1 is an important molecules to suppress the inflammation concerning the onset of metabolic and age-related diseases.

3. Regulation of nuclear factor kappa B (NF-κB) is central role in the anti-inflammatory function by SIRT1. Post translational modification of p65 subunit of NF-κB (p65) is the main route of regulation of NF-κB transcriptional activity by SIRT1. NF-κB contributes to various diseases including metabolic and age-related diseases through chronic inflammation by activating pro-inflammatory cytokine production. NF-κB seems to relate to onset of various diseases, and clinical pathological research targeting cat p65 has been reported. However, very little information is available on the molecular characterization of cat p65. To obtain the basic knowledge of cat p65, we cloned and characterized cat p65, and examined their immune regulatory function. We successfully cloned the cat p65. The deduced amino acid sequence was highly conserved in mammal p65 (>87.5%). In particular, functional domains were conserved very well. The amino acid residues, which undergo post-translational modifications in mammals, were completely conserved in cat p65. The cat p65 mRNAs were expressed in all examined tissues as reported in other animals. In particular, high expression levels of cat p65 were observed in adipose tissue, heart and skeletal muscle. Transiently expressed cat p65 significantly up-regulated NF-κB transcriptional activity and pro-inflammatory cytokine expression in cat fibroblast tissues. Therefore, cat p65 may have important roles in inflammation and SIRT1 may be involved in the regulation of inflammation.

4. Relationship between SIRT1 and NF-κB in chronic inflammation has important effects on onset of metabolic and age-related diseases in animals. Chronic inflammation is occurred by persistent low level of physiological inflammation through the response to the endogenous-exogenous stress. Sustained chronic inflammation causes organ dysfunction by failure of adaptation. Fibroblasts are involved in wound healing by synthesizing of extracellular matrix in tissues. In addition, fibroblasts produce inflammatory cytokines and modify the level of inflammation. For these reasons, fibroblasts are considered an important factor in the formation of chronic inflammation. SIRT1 activity of regulating inflammation through NF-κB in fibroblast is important in various diseases. However, there is little information in cats. Therefore, we analyzed the effect of NF-κB transcriptional activity and inflammatory cytokine production by SIRT1 in cat fibroblast cells. Transiently expression of SIRT1 suppressed the NF-κB transcriptional activity and pro-inflammatory cytokine expression by cat p65 and LPS in cat fibroblast. These result revealed that SIRT1 inhibit the NF-κB signals and suppress the inflammation in cat fibroblast cells. We consider that SIRT1 is concerned in onset of metabolic and age-related diseases through suppression of chronic inflammation.

In conclusion, cat SIRT1 has anti-inflammatory function via NF-κB. Chronic inflammation causes lipotoxicity and subsequently onset of metabolic and age-related diseases. We consider that SIRT1 involving in the occurrence of chronic inflammation relate to onset of metabolic and age-related diseases. Further study is needed to elucidate the detail molecular mechanisms. Sirtuins are applied to early diagnosis and prevention as a biomarker of various diseases in human. Molecules having high activating ability of sirtuins are detected and it has been applied to drug development. We expect that this study contributes a little to the clinical applications of sirtuins in cats.

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Changes in plasma free amino acids concentration in dogs with cancer

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Cancer is the leading cause of death in humans and dogs in Japan. Up to the present date, a great deal of clinical examination and therapy of cancer has been investigated. In particular, early detection and treatment of cancer are recognized as important challenges. Recently, analysis of plasma free amino acids (PFAAs) using liquid chromatography-mass spectrometry (LC/MS) had been developed in human medicine. PFAAs enables risk assessment of multiple types of cancer with only one blood sampling. However, PFAAs have not ever been clinically applied in veterinary medicine. Furthermore, there are few studies investigating the relationship between cancer and PFAAs using exhaustive analysis. Purpose of the current study was to investigate the changes in PFAAs in dogs with cancer and to obtain new findings showing the relationship between cancer and PFAAs.

Chapter 1 Basic research on measurement of canine PFAAs using LC/MS

First, we evaluated accuracy and reproducibility of the PFAAs assay using LC/MS in dogs. We looked for appropriate blood sampling times, since the concentration of PFAAs could be changed by food intake. In the current study, 39 types of PFAAs were measured by LC/MS.

Intra-assay reproducibility of PFAAs was estimated with pooled plasma samples of healthy dogs. Intra-assay coefficients of variation (CV) did not exceed the commonly accepted 15% limit. The samples were used for evaluation of the inter-assay imprecision too, by 8 independent runs over 1 month. The CV of PFAAs besides Cystine did not exceed 15% limit, but the CV of Cystine was 66%. We suggest that it would be better to treat the specimen immediately after blood collection and measure PFAAs within one week, since Cystine did not show stable results during 8 weeks. Dilution linearity was assessed by serially diluting a pooled plasma sample. Excellent linearity was demonstrated in many kinds of PFAAs, up to 16-fold dilution ($P<0.05$). However some kinds of PFAAs did not show good linearity since these PFAAs concentrations were lower than the detection limit value under the dilution condition, but the P value did not exceed 0.05. From these results, excellent accuracy and reproducibility of PFAAs using LC/MS were confirmed.

We also investigated the changes of PFAAs before and after food intake in healthy dogs. Except for α-aminobutyric acid, dietary effects were not observed in PFAAs after postprandial 14 hours. Furthermore, different daily fluctuation between daytime and nighttime was observed in some kinds of PFAAs. As such, we standardize time of taking blood sampling for measuring PFAAs as the morning with fasting over 14 hours.

From the above results, PFAAs in dogs can be assayed by LC/MS. Furthermore, we recommend that dogs should be fasted over 14 hours, and blood sampling for PFAAs measurement should be performed in the morning. And the assay should be made as soon as possible after blood collection.

Chapter 2 The changes in PFAAs concentrations in dogs with different types of cancer

The concentrations of PFAAs in 39 cancer dogs and 20 healthy dogs were compared. Cancer dogs were categorized by the types of cancer such as transitional cell carcinoma (n=8), mammary gland tumor (n=3), hepatocellular cell carcinoma (n=8), malignant melanoma (n=6) and thyroid carcinoma (n=4).

Cancer dogs had significantly higher Threonine,

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α-aminoadipic acid, Cystine, Cystathionine, Phenylalanine, 3-Methylhistidine, 1-Methyhistidine, Tryptophan and Aromatic amino acid concentrations and had significantly lower Glycine, Histidine and Fischer ratio than healthy dogs. The PFAAs that significantly decreased in cancer dogs might be ingested by cancer cells aggressively, and the PFAAs that significantly increased might be produced by protein catabolism, but not used in cancer cells, or produced in cancer cells and secreted into circulation. These PFAAs are possible markers of cancer in dogs. Significant increase in some PFAAs related to skeletal muscle metabolism might reflect catabolism of muscle proteins in cancer dogs. In particular, 3-Methylhistidine is applied as a biomarker of muscle protein catabolism in dogs as the case with human medicine.

Taurine and Arginine were significantly lower in transitional cell carcinoma. Glutamine was significantly lower in malignant melanoma. Glycine was significantly lower and Tyrosine, Tryptophan and Phenylalanine were significantly higher in malignant breast cancer. Many kinds of PFAAs changed in the same way with other types of cancers, but branched chain amino acids and aromatic amino acids reflecting liver function were significantly higher in hepatocellular carcinoma. Threonine and Proline were significantly higher in thyroid cancer. Isoleucine was significantly lower in thyroid cancer and significantly higher in hepatocellular carcinoma. Collectively, specific changes in PFAAs were observed in different types of cancer. In addition, some PFAAs, such as Glycine, Methionine, 3-Methylhistidine and 1-Methylhistidine showed same changes in different types of cancer. These results may reflect altering amino acid metabolism in different type of cancer.

Chapter 3 Concentrations of PFAAs before and after chemotherapy in canine transitional cell carcinoma

PFAAs are reported to change in human patients with cancer before and after chemotherapy. However, it is unclear in cancer dogs. In this chapter, we investigated the change of PFAAs after chemotherapy in dogs, focusing on transitional cell carcinoma (TCC) because chemotherapy is recommended as treatment for TCC. Significant changes were observed in mean plasma Cystathionine concentrations between before and after chemotherapy. Cystathionine gradually decreased 1-3 weeks after chemotherapy, however, it increased again 6 weeks after chemotherapy. Plasma Cystathionine in TCC dogs could be taken up into cancer cells and utilized to synthesize glutathione for drug detoxification. Since the clinical symptoms improved and the tumor size did not enlarge, Cystathionine might not be used for glutathione synthesis after the second administration, so the anticancer drug effectively acted on cancer cells. From these results, we conclude that chemotherapy influenses the PFAAs concentrauions in cancer dogs.

In conclusion, we performed an exhaustive measurement of PFAAs with difference types of cancer and their changes before and after chemotherapy in dogs. We obtained new findings on the relationship between cancer and PFAAs in dogs. The results indicates that the PFAAs assay is useful for early detection and risk assessment of cancer, and the monitoring of treatment in dogs.

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Analysis of cholesterol lipoprotein separations in Holstein dairy cattle by anion-exchange highperformance liquid chromatography

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Lipid metabolism in dairy cows are consistently active for milk production. Especially, lipoprotein metabolism is significantly changed during the transition period in dairy cows for supply of nutrient to the fetus or milk production. Abnormal lipid metabolism during transition period induces fatty liver and its intercurrent illness. As such, we focused on lipoprotein fraction which plays a key role on bovine lipid metabolism. Measurement of serum lipoproteins in dairy cows has been performed using ultracentrifugation methods, which is considered the "gold standard". However, the ultracentrifugation method has disadvantages given that it is too cumbersome and time-consuming. It is reported that there is problem of measurement accuracy, since density region between HDL and LDL is overlapped. Recently, anion-exchange (AEX) high-performance liquid chromatography (HPLC) methods are recently developed and can measure serum lipoproteins such as total cholesterol, HDL-C, LDL-C, IDL-C, and VLDL-C with rapid, high isolation efficiency in humans and rabbits. However, AEX-HPLC analysis has not been applied in dairy cows.

Therefore, the current study investigated the basis examination whether AEX-HPLC methods could measure bovine lipoprotein fraction in chapter 2. Next, we compared the lipoprotein fraction of dairy cows with different lactation stages between fineness daily farmer (superior of milk quality performance and reproduction performance) and poor daily farmer in chapter 3. Finally, we investigated the changes in lipoprotein fraction in healthy daily cows during transition period and compared it to those in dairy cows with perinatal diseasein chapter 4.

The objective of the chapter 2 was to investigate ① the reproducibility, precision and linearity of AEX-HPLC methods and ② the correlation between AEX-HPLC and other analytical methods (ultracentrifugation methods and gel permeation HPLC [GP-HPLC]) using serum samples from healthy dairy cows. GP-HPLC could be useful for the analysis of lipoproteins in humans, dogs and cats. GP-HPLC separates lipoproteins on the basis of size. Chromatogram of lipoprotein fraction by AEX-HPLC method showed apparent wave profile in HDL-C and LDL-C, however, minimal wave profile was observed in IDL-C and VLDL-C. Analytical evaluation of the lipoprotein assay of total cholesterol, HDL-C, LDL-C, IDL-C and VLDL-C using AEXHPLC methods indicated acceptable analytical precision, with intra-assay coefficients of variation (CVs) not exceeding the commonly accepted 10% limit. Furthermore, analytical evaluation of total cholesterol, HDL-C and LDL-C with inter-assay CVs were below 10%, respectively. However, with regard to concentrations of IDL-C and VLDL-C, we could not evaluate the inter-assay CVs because some measurement results were under the detection limit. Excellent linearity was demonstrated with total cholesterol, HDL-C and LDL-C of the dilutions tested. However, some values of IDL-C and VLDL-C were under the detection limit. Therefore, linearity was not assessed. Furthermore, positive correlation coefficients between the values of total cholesterol, HDL-C, and LDL-C were determined between AEX-HPLC and ultracentrifugation methods and between AEX-HPLC and GP-HPLC. However, definite separation between HDL and LDL could not be performed using ultracentrifugation methods and GP-HPLC in dairy cow, since density

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region between HDL and LDL is overlapped. Therefore, AEX-HPLC methods can measure lipoprotein fraction with accuracy and reproduction as regards HDL-C and LDL-C. Furthermore, AEX-HPLC methods ① was not affected overlapping of specific gravity between HDL-C and LDL-C as ultracentrifugation methods and ② was not affected overlapping of grain size as GP-HPLC methods. Therefore, these results suggest that AEX-HPLC would be a useful method for evaluating the lipoprotein fraction in dairy cows.

The lipoprotein fraction in dairy cows with different lactation stages measured by AEX-HPLC methods were compared between fineness S daily farmer (superior of milk quality performance and reproduction performance) and poor I daily farmer in Miyagi prefecture. No significant difference was observed in changes in Total-C, HDL-C, LDL-C, IDL-C and VLDL-C between 2 dairy farmers. Total cholesterol, HDL-C and LDL-C increased from early lactation to mid lactation, and thereafter decreased from late lactation to dry lactation in both dairy farmers. Significant difference was observed in changes in HDL-C/Total-C (%) between 2 daily farmers. HDL-C/Total-C (%) decreased from early lactation to mid lactation, and thereafter increased from mid lactation to late lactation in S dairy farmers. HDL-C/Total-C (%) of I dairy farmer in mid lactation is higher than that of S dairy farmer. Significant difference was also observed in changes in LDL-C/Total-C (%) between 2 daily farmers. LDL-C/Total-C (%) of peak, mid and late lactation in S dairy farmer is significantly higher than that in I dairy farmer. LDL-C/Total-C (%) increased from early lactation to mid lactation, and thereafter decreased from mid lactation to late lactation in S dairy farmers. Meanwhile, LDL-C/Total-C (%) did not show any significant changes during lactation stages in I dairy farmers. These results suggested that metabolic pathway of VLDL → IDL → LDL is activated by transferring the triglyceride to mammary gland for plenty milk production in S dairy farmer. Meanwhile, milk production of I dairy farmer is lower than that of S dairy farmer. As such, LDL-C might be decreased in I dairy farmer, since transportation of VLDL-C from liver to mammary gland was decreased. For the above reason, measurement of LDL-C/Total-C (%) might be able to pick out between superior dairy farmer and poor dairy farmer.

We focused on the lipid metabolism during transition period. Firstly, for creating a reference value, the changes in lipoprotein fraction in 10 healthy daily cows during transition period were evaluated. Secondarily, we examined associations of lipoprotein profile between healthy cows (reference value) and dairy cows with perinatal disease. Lipoprotein profile of Total-C and HDL-C in healthy cows decreased from 4 weeks before from expected date of delivery to calving date, and thereafter increased. LDL-C in healthy cows decreased from 4 weeks before from expected date of delivery to 1 week after from expected date of delivery, and thereafter increased. LDL-C/Total-C (%) in healthy cows decreased from 4 weeks before from expected date of delivery to 2 weeks after from expected date of delivery, and thereafter increased. Next, we compared lipoprotein fraction between healthy dairy cows and 19 dairy cows with perinatal disease. In the current study, lower values of LDL-C and LDL-C/Total-C (%) was observed in fat liver group and milk fever group as compared to healthy dairy cows. It was considered that inactivation of metabolic pathway of VLDL → IDL → LDL was occurred caused by decreased appetite and nutrient malabsorption. Therefore, measurement of LDL-C during transition period might be reflect metabolic status in dairy cows and used for clinical application.

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Individual identification and extracting risk areas for reducing damage by the Japanese black bear (*Ursus thibetanus japonicus*) in the Satochi-Satoyama of Gunma Prefecture, Japan

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The Japanese black bear (*Ursus thibetanus japonicus*) is large wildlife, and distributed on the islands of Honshu and Shikoku in Japan. In recent years, conflict between human and bears has occurred mainly in the Satochi. The damage by bears in the Satochi has been concerned about the involvement of individuals attached to Satochi (hereinafter, [urban bear]). However, the actual condition of urban bear is not clear. Therefore, in this study, we investigated the status of each bear that inhabited the Satochi using individual identification at Numata City, Gumma Prefecture, for the purpose of elucidating the actual condition of urban bear. Furthermore, based on the actual condition of bears that could be clarified by individual identification, we extracted the risk areas (hereinafter, [RAs]) where the danger of intrusion of bears is high, for the purpose of proposing effective measures in the Satochi. From these results, we expected to contribute to reducing the damage by bears in the study area.

In Chapter 2, we understand the status of each bear that inhabited the Satochi using individual identification by camera-trapping method. We identified individual bears by the chest marks from the photographs taken with camera traps from 2012 to 2013. As a result, we identified 21 bears (16 bears in 2012, 9 bears in 2013, the same 4 bears in two years).

We assumed that the population density of bears in autumn of 2013 was relatively low, based on the frequency with which bears were taken, the mean number of identified individuals when the survey period was divided into the former term and the latter term from the beginning of September of each year, and the number of new individual bears in the latter term. That reason was that the hard mast production in autumn of 2013 was good.

Based on the length of period during which inhabitation could be confirmed, we classified individuals into resident bears and transit bears. As a result, there were 6 resident bears and 19 transit bears in two years. Also, 3 resident bears of 4 bears identified for two consecutive years approached the village.

In Chapter 3, we understand the status of each bear that inhabited the Satochi using individual identification by hair-trapping method. The site, the operation period, and the number of units where hair trap was operated were exactly the same as those of camera trap of Chapter 2. We identified individual bears by microsatellite genotyping of bear DNA from hair collected with hair traps. As a result, we identified 41 bears (29 bears in 2012, 12 bears in 2013).

As in Chapter 2, we assumed that the population density of bears in autumn of 2013 was relatively low, based on the mean number of identified individuals, and the number of new individual bears in the latter term.

We classified individuals into resident bears and transit bears using the same definition as in Chapter 2. As a result, there were 4 resident bears and 37 transit bears in two years. Although there were no individuals identified for two consecutive years, we concluded that bears moved in and out of the study area, because 12 bears were identified in 2013, and the ratio of transit bears in each year accounted for more than 80% of the total. 2 resident bears of each year approached the village.

In Chapter 4, we comprehensively analyzed the status of each bear clarified in Chapter 2 and Chapter 3. There

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was a possibility of individual bears of camera traps and hair traps confirmed at the same sampling times and the same trap location could have the same individual. We examined combinations of individuals with the same individual potential. As a result, 12 pairs of bears could have the same bear in two years. If the individuals were consistent among the 12 pairs, the number of individual bears combined with both methods became 50 bears in the two years.

Among the above assumptions, there were 5 resident bears, 10% of the total number of bears (50 bears). We concluded that bears moved in and out of the study area frequently. Furthermore, from the above assumption, we concluded that 90% of individuals inhabited the study area are transit bears and only 10% of resident bears. Also, we considered that the population density of bears in autumn was affected by the hard mast production. However, we concluded that 3 bears of the above resident bears were strongly attached to the study area regardless of the hard mast production. In addition, we considered that two of them were bears dependent on the village, because the 2 bears have approached the village for two consecutive years. In this study, we decided that these 2 bears are urban bears.

As above, 90% of individuals inhabited the study area are transit bears, and we concluded that bears moved in and out of the study area frequently. Therefore, if bears that had been damaged so far were transit bears, even if they were able to kill the damaged bears, we presumed that the damage did not decrease since new bears were transferred one after another. Also, we considered that there was a high possibility that the 2 urban bears were damaged. However, these bears were not captured at least during the study period. Therefore, we considered that measures put emphasis on capturing only at the study area is not realistic, and a method of proceeding in a complex manner including other measures is effective in the study area.

In Chapter 5, we attempted to extract RAs where the danger of intrusion of bears is high, for the purpose of proposing effective measures in the Satochi. First, we analyze the relationships between Satochi environment that is quantified using a geographic information system and bear intrusion sites to extract RAs. The results showed that among the environmental factors we evaluated, forest surrounding, the forest edge with poor management, and land usage as an orchard were associated with bear intrusion. These results suggest that orchards with poorly managed forest edges along the forest boundary are more likely to be damaged by bears. On the basis of these results, we then extracted RAs classified as Low RAs (hereinafter, [LRAs]), Medium RAs (hereinafter, [MRAs]) and High RAs (hereinafter, [HRAs]) in increasing order of risk. As a result of superimposing the intrusion sites (70 sites) reported between 2004 and 2008 on each RA, there were 1 site (1.4%) in LRAs, 19 sites (27.1%) in MRAs and 33 sites (47.1%) in HRAs. We considered that damage control in HRAs is strongly needed to reduce the damage by bears, because the intrusion sites were concentrated in the RAs.

Furthermore, as a result of superimposing the intrusion sites (28 sites) reported between 2009 and 2010 on each RA, there were 3 sites (10.7%) in LRAs, 7 sites (25.0%) in MRAs and 10 sites (35.7%) in HRAs. The intrusion sites were concentrated in the RAs, and there was no significant difference between period from 2004 to 2008 and period from 2009 to 2010 in the ratio of the number of intrusion sites overlapping with RAs. Therefore, we concluded that RAs had a certain level of universality for bear intrusion.

Then, we attempt to extract RAs in Sayama area adjacent to Hocchi area. As a result, we were able to extract RAs, and the intrusion sites were concentrated in the RAs. As a result of superimposing the intrusion sites (76 sites) reported between 2004 and 2010 on each RA, there were 4 sites (5.3%) in LRAs, 14 sites (18.4%) in MRAs and 50 sites (65.8%) in HRAs. Therefore, we concluded that RAs had universality for bear intrusion in fruit growing regions.

Finally, by using the electric fence spread to the study area, we examined whether the protection of RAs in Hocchi area and Sayama area reduced the damage by bears. We defined the period between 2004 and 2008 as before measures, and the period between 2012 and 2013 as after measures. As a result of examining the installation situation of the electric fence after measures, the ratio of the area of RAs effectively protected by the electric fence to the total area of RAs was 70.7% a year on average in Hocchi area, and 41.2% a year on average in Sayama area. Comparing the number of intrusion sites before and after measures, the number of sites after countermeasures was 61% less in Hocchi area and 43% less in Sayama area. Also, the ratio of damage occurred in RAs within the analysis area was decreasing in Hocchi area. Therefore, by protecting approximately 71% of RAs with electric fence in Hocchi area, we concluded that the damage by bear along the forest edge decreased. However, there was no significant difference in the ratio of damage occurred in RAs within the

analysis area before and after measures in Sayama area. Therefore, by protecting approximately 41% of RAs with electric fence in Sayama area, we concluded that the damage by bear along the forest edge decreased. However, the site where the damage by bears occurred remained concentrated along the forest edge. That reason was that orchards were concentrated along the forest edge. We consider that strengthening measures like installation of a long electric fence along the forest edge will be necessary. We were demonstrated that the protection of RAs with electric fence is effective in reducing the damage by bears. Therefore, we concluded that extraction of RAs and installation of electric fence are effective against bear damage.

We considered that at least 2 resident bears inhabited the study area for two consecutive years were urban bear. There was a high possibility that the 2 bears were involved in the damage, because these bears have approached the village. Furthermore, habitation in the Satochi of such individuals can be a great threat to the local population regardless of the number of individuals, from the viewpoint of living damage and spiritual damage of bears. Also, repeating the approach of these bears to the village means that there is a high possibility of encountering a human, and danger of human injury. This study is the first study to elucidate the actual conditions of urban bears. Then, this study suggest that measures put emphasis on capturing only at the study area is not realistic, and measures to control damage in the village are important for solving the bear damage problem. Also, we can demonstrate that it is possible to reduce bear damage by strategically proceeding measures, using extraction of RAs. We expect to be able to reduce the damage by bears in other areas by dealing strategically like the study area. We consider that the result of this study is knowledge that provides important information to strategically proceed measures against bear damage in the study area and other areas.

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Study for establishment of assisted reproductive technologies in Japanese field vole, *Microtus montebelli*

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Rodents are generally used for various studies, because of clear genetic background and their characteristic including reproductive function. However, development of new experimental animals possessing different characteristic from conventional experimental animals, such as mouse and rat, will expand not only research field but also novel biological knowledgement. Sixty-four of *Microtus* species inhabit in the world and each species possess unique characteristic. The major and common characteristic of vole is a herbivous small rodent with multiple stomachs and chromosome number is different among species. Thus, it has been expected as models of digestion-metabolic control system for middle or large herbivory and/or for study of speciation-species profile. Since some of them possess monogamy system that is less than 3% in mammals, they are utilized for experimental or research models of social behavior in human mating system or brain mechanism. Recently, establishment of iPS cells in prairie voles (*Microtus ochrogaster*) were succeeded and then production of transgenic prairie voles was attempted. In fact, such novel studies on vole is developing. When it considers wide utilization of this genus including Japanese filed vole (*Microtus montebelli*), stable supply of animals for experiments is essential. However, there are few reports about their reproductive pattern and assisted reproductive technologies (ARTs) related with preservation of germ cells and regeneration of individual at present. To advance above-mentioned new research field, it is greatly desirable to establish ARTs such as cryopreservation of germ cells, artificial insemination (AI), *in vitro* fertilization (IVF) and embryo transfer (ET). In addition, ten of sixty four species in this genus have been classified into the endangered category and conservation for such species is also important to maintain the biological diversity. The present study aimed establishment of a series of ARTs to enable researchers to supply animals stably and conserve germ cells in Japanese field vole.

In Chapter 1, we described utility and ARTs on vole and also outlined background of related studies.

In Chapter 2, we examined whether mouse sperm cryopreservation system was applicable to spermatozoa of *Microtus montebelli*. The rate of mortility, viability and sperm integrity after frozen-thawed (FT) were lower than fresh. Then, all of mouse oocytes injected resumed meiosis and formed pronucleus when they were injected with a single vole fresh or FT spermatozoa. These results showed that FT spermatozoa of *Microtus montebelli* sufficiently maintained the fertilizing capacity. Furthermore, effect of two freezing containers (tube or straw) on properties of FT spermatozoa was examined. After frozen-thawed, mortility with straw was higher than tube and there was no difference on viability. Finally, we used non-invasive AI with fresh or FT spermatozoa to attempt production of offspring and perform the final evaluation of sperm cryopreservation technology on *Microtus montebelli*. Although litter size derived from AI with FT spermatozoa was low, we demonstrated that pups could be produced from FT spermatozoa. Furthermore, when delayed sperm transfer (at 7-9 hours after copulation) or hypotaurine treatment were performed, the litter size derived from AI with FT was improved and such value was similar to those of fresh spermatozoa and natural mating.

In Chapter 3, we performed to establish novel superovulation procedure on Japanese field vole for reliable supply of oocytes throughout all ages. In general, combination of pregnant mare's serum gonadotropin (PMSG) and human chrorionic gonadotropin (hCG)

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is used for superovulation in mice. Firstly, female Japanese field voles were divided into two groups of voles before vaginal opening (19-29 days old) and after vaginal opening (30-138 days old). Then, voles were administrated with various concentration of PMSG and hCG and number of ovulated oocytes from voles were compared. In results, regardless of hormonal concentration, number of ovulated oocytes from voles after vaginal opening was less than those of voles before vaginal opening. Therefore, we examined a new superovulation procedure to induce more effective ovulation on voles after vaginal opening. Mating stimulus (because Japanese field vole is a copulation ovulation animal) or gonadotrophin releasing hormone (GnRH) that is a up-regulated hormone of luteinizing hormone (LH) were used, since it was estimated that GnRH facilitated endogenous LH release and then induced superovulation. When females were treated with mating or 20% PVP-GnRH, twice number of oocytes could be obtained comparing with PMSG-hCG. Furthermore, in case of applying 20% PVP-GnRH to voles before vaginal opening, number of oocytes similar to PMSG-hCG was obtained.

In Chapter 4, in order to examine the conditions of *in vitro* culture for embryos derived from IVF or intracytoplasmic sperm injection (ICSI), we tested various media with *in vivo* embryo at first. As a result, it revealed that all *in vivo* pronuclear embryos cultured arrested at 2 cell stage. When early stage of *in vivo* 2 cell embryos was examined, it could develop to blastocyst stage. Finally, because it is assumed that vole offspring derived from chimera or transgenic will be produced by embryo-manipulations, *in vivo* blastocysts were non-surgically transferred to uterine and production of offspring was succeeded.

In Chapter 5, recently, it has been known that Somnopentyl has problems of not only safety but also unstable effects as an anesthetic. Alternatively, availability of mixture anesthetic (medetomidine/midazolam/butorphanol; M/M/B: mg/kg) that is known to obtaine more safe and effective surgical operation time was examined in *Microtus montebelli* and such optimum concentration was determined. That results revealed that mortality with Somnopentyl was higher and anesthesia score was lower in male and female, compared with mixture anesthetic. When all concentration tested in male and 0.3/4/5 (M/M/B) and 0.23/3/3.75 of concentrations in female were administrated, more effective surgical operation time and anesthesia score was obtained. Morerover, it was suggested that optimal concentration was 0.23/3/3.75 in *Microtus montebelli*, since disorder of respiratiry rhythm and several time of apneic states were observed in many voles administrated with 0.3/4/5. Next, we examined the effect of mixture anesthetic on number of offspring from non-surgical embryo transfer (NSET) and AI. As results, there was no detrimental effect. Furthermore, with proper interval, it was shown that mixture anesthetic could be used repeatedly (at least three times), suggesting that recipient for NSET or AI is able to use several times and total number of offspring will be increased.

In Chapter 6, to examine optimum condition for IVF, hypotaurine treatment at pre-culture or insemination with fresh spermatozoa was investigated. This result showed that although fertilization rate was not affected by hypotaurine treatment in IVF with fresh spermatozoa, fertilization rate with FT spermatozoa was increased. Next, when we examined media for IVF embryos, development of embryos arrested at 2 cell stage in all media tested. Although EDTA which is known to release the 2 cell block in mice was also examined, there was no effect on vole embryos. From these results, we determined to transfer embryos into oviducts of recipient to produce offspring from IVF embryos. Although litter sizes derived from IVF using fresh and FT spermatozoa were low, we demonstrated that the pups could be produced. Moreover, we compared Somnopentyl with mixture anesthetic on production of offspring from surgical ET. That results showed that mixture anesthetic tended to show higher pregnancy rate and lower abortion rate, although there was no difference on litter size and weight of offspring between Somnopentyl and mixture anesthetic.

In Chapter 7, we performed ICSI with fresh and FT spermatozoa in a manner to mouse procedure and transferred embryos in a manner to IVF. When vole oocytes were microtus-inseminated with fresh or FT spermatozoa, oocytes resumed meiosis and formed pronucleus. Finally, we were successful in production of offspring derived from ICSI embryos.

Taken together, 1) sperm cryopreservation procedure was successfully applicable to mouse system and litter size similar to natural mating could be obtained by non-surgical AI with FT sperm, 2) oocyte could be collected from all weeks-old voles by novel superovulation procedure, 3) medium that was able to develop from early 2 cell stage to blastocyst stage was revealed and using non-surgical ET with these embryos production of offspring was succeeded, 4) optimum anesthetic and

the concentration were examined for surgical operation and then safe and effective anesthetic was determined, 5) offspring derived from IVF and ICSI embryos could be produced. Overall, the results show that ARTs modified for voles could be efficiently contributed to animal supply and conservation of not only *Microtus montebelli* but also other vole species.

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Studies on noninvasive methods to estimate nutritional condition of protein and amino acids for animals

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In animals which have small genetic diversity, like as experimental animals and commercial chickens, same nutritional management is usually carried out for all population in the same age or life period. On the other hand, these had large genetic diversity, like as dairy cow, pets, and wild life animals, require order made nutritional management. For example, in dairy cow, the nutritional status is usually monitored using the lactating quality and quantity as a criterion because the genetic diversity of cows is relatively large and the lactating periods are not the same among individual cows. For appropriate nutritional management, the estimation of nutritional requirements are important. But, especially avian, many of species except poultry were not estimated the nutritional requirements. While energy requirement for maintenance was able to estimate by the body weight using metabolic body size, protein metabolism differs among the feeding habits. Therefore, protein and amino acid requirement have to estimate experimentally. Because nutritional requirements were influenced by genetic diversity, environment, life stage, and stress, it is necessary to analyze these factors. Blood sampling and retention of animals in experiment were stress full for animals. Therefore, it is necessary to develop a new and noninvasive method.

The final amino acid product levels in the urine are noninvasive parameters which can be used to estimate the protein and amino acids requirements. With this criterion, it is also possible to test the same animals repeatedly. Therefore, it is useful for monitoring the nutritional condition. However, the method for protein and amino acid requirements using the taurine excretion as a criterion is not available for carnivorous animals.

Therefore, the present study was conducted to judge whether creatinine excretion will be criterion for estimation of nutritional condition of protein and amino acids or not regardless of feeding habit.

Housing, handling, feeding, and killing procedures were in accordance with policies of Nippon veterinary and life science university committee on laboratory animal care.

1. Study of creatinine excretion as a criterion

Creatinine is one of the final amino acid products in the urine. Creatinine has a precursor, creatine which is synthesized from the following three amino acids: arginine, glycine, and methionine. These amino acids are essential amino acids for poultry. Methionine, in particular, is a primary source of methyl groups, and it is liable to be first limited amino acid. Chamruspollert *et al*. (2002b) reported that dietary arginine and methionine levels influenced muscle creatine levels in broiler chicks. Furthermore, Chamruspollert *et al*. (2002a) suggested that muscle creatine levels can be used as a criterion to assess arginine requirement. Creatinine is non-enzymatically converted from creatine and excreted in the urine without any resorption. It indicated that creatinine excretion might respond to muscle creatine levels or creatine synthesis. Thus, cratinine excretion might reflect dietary it's prescursors levels.

In order to judge whether creatinine excretion will be criterion for estimation of amino acids and protein nutritional condition or not, at first, the responses of creatinine excretion to dietary methionine and arginine were made sure in experiment 1 and 2.

1-1. Effects of dietary methionine and arginine levels on the urinary creatine excretion in broiler chicks

In experiment 1 and 2, 8-day-old Chunky broilers chicks were used. The chicks were assigned to three

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dietary groups, with five chicks each, and were fed an experimental diet for 7 days. The experimental diets mainly consisted of corn and soybean meal, and contained deficient, adequate, or excessive methionine and arginine levels in experiments 1 and 2, respectively. Excreta were collected for the last 3 days of the feeding trial, and chicks were terminated by dislocation of the neck at the end of the feeding trial to collect their livers. Creatinine concentration in the excreta and hepatic L-arginine-glycine amidinotransferase (AGAT) activities were determined.

Urinary creatinine levels increased with increasing both dietary methionine and arginine levels from deficient to adequate recommended by Japanese feeding standard ($P<0.05$), and then remained constant in experiments 1 and 2, respectively. The hepatic AGAT activity decreased when both dietary creatinine precursors levels were increased from deficient to adequate levels ($p<0.05$), and then remained constant. These results suggested that creatinine excretion was changed with both increasing dietary methionine and arginine, dose-dependently.

1-2. Effects of dietary protein levels on the urinary creatinine excretion in broiler chicks

In experiment 1 and 2, 8-day-old Chunky broilers chicks were used. The chicks were assigned to three or four dietary groups, with five chicks each, and were fed an experimental diet for 7 days. The experimental diets contained three levels of protein (deficient, adequate, or excessive) in experiment 3, and two levels of arginine (adequate and excessive) and two levels of methionine (deficient and adequate) in experiment 4. Excreta and collect the livers were collected in the same method as experiment 1.

In experiment 3, urinary creatinine levels decreased with increasing protein levels from deficient to adequate ($P<0.05$), and then turned to increase. The hepatic AGAT activity was same response as creatinine concentration. In experiment 4, urinary creatinine concentration was higher at excess arginine levels and deficient methionine levels than other dietary groups ($p<0.05$). The hepatic AGAT activity was decreased at excess arginine diets ($p<0.05$). In this results, excess dietary arginine levels promoted creatine synthesis, and increased creatinine excretion regardless of methionine deficient. These results suggested that creatinine excretion would be useful parameter for estimating the protein and amino acids requirements.

2. Study of feeding habit

Because the carnivorous birds were restricted on the experiments, little is known about nutritional and metabolic studies in carnivorous birds. Many species of the carnivorous birds are regarded as endangered or near threatened species, and they were restricted by law. In addition, usually carnivorous birds are weak from stress, and have high palatability for foods. Hence the conventional methods to estimate protein and amino acid requirements are not available for carnivorous birds.

Therefore, in order to make sure the response of creatinine excretion to methionine and protein regardless feeding habit, experiment 5 and 6 were conducted using scops owls as carnivorous birds.

In Experiments 5 and 6, 4 adult Eurasian scops owls (*Otus scops*) were allocated to 4 dietary methionine or protein levels x 4 periods recommended by Latin square experimental design, respectively. Each period was consisted of the acclimatizing 3 days and the experimental 4 days, and excreta was collected for last 24 hr. Experimental diets used were neonatal mice (*Mus musculus*) containing capsule of crystalline amino acids mixture (in experiment 5) or casein and lard (in experiment 6) in abdomen. Total dietary methionine levels were 0.22%, 0.35%, 0.60%, and 0.72% in experiment 5. Total protein: energy ratio were 0.113, 0.125, 0.138, 0.15 in experiment 6. These results showed the similar response of broilers ($p<0.05$). The facts suggest that creatinine excretion will be criterion for estimation of protein and amino acid requirement for carnivorous birds.

In conclusion, cretatinine excretion would be useful parameter for estimating the protein and amino acid nutritional condition regardless of feeding habit.

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Factors involved in beef palatability

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Beef cattle raised to supply our country's meat has three types of classification, namely meat-only (Japanese black cattle); Holstein (domestic steers); and crossbred (F1). These types are respectively cattle bred only to produce beef; Holstein steers that are a by-product of dairy management and are fattened to produce beef; and cattle from crosses between Japanese black cattle and Holstein bulls. In this thesis my focus is on improving the meat quality of Japanese-produced beef.

All Japanese cattle are fed for 6 months from age 12 months; they are then fattened from an average of age 20 months to 30 months. Therefore, compared with overseas beef cattle raised for meat, Japanese black cattle, especially, are characterized by marbling, in which fat accumulates in the muscles. However, the situation faced by Japan's dairy farmers in recent years is serious; there was a 6.2% decrease in the number of beef cattle bred in 2014 compared with the previous year, with a 2.8% reduction in the number of breeding farms. In future, many cattle farms will be faced with the predicament of needing to comply with the provisions of the Trans-Pacific Strategic Economic Partnership (TPP) Agreement. If the amount of beef imported increases under a future TPP, it will become important to highlight the features of domestic beef from the perspective of methods of preservation and cooking in order to maintain consumption of the local product.

Although there have been many studies of the palatability of beef, the relevance of the intramuscular fat factor, which greatly influences meat quality, as well as aging after slaughter and flavor, has not been reported in detail.

Here, I examine heating method as a factor affecting the palatability of beef. I look at the fat content and long-term aging treatment of beef. Whereas analytical sensory evaluations have been used previously to determine the influence of each factor on palatability, I measured the physicochemical factors relevant to evaluation criteria. I also discuss the relationship between sensory and instrumental analysis.

Chapter 1 Influence of Heating Method on Palatability of Beef

The Complete Meat Cookbook, one of the most popular cookbooks in the United States, provides information on selecting a heating method. My aim was to clarify the taste and texture characteristics of Holstein loin meat cooked until it reached an internal core temperature of 60° by using different methods, namely grilling, roasting, poaching, vacuum-packed low-temperature (VPLT) cooking, and microwaving.

Cooking loss was lowest in grilled or roasted beef, whereas it was highest in microwaved beef. Moisture content after cooking was highest in beef cooked by the VPLT method and low in beef cooked by poaching or microwaving. The fat content that remained after cooking was lowest in beef cooked by the VPLT method. The breaking energy of microwaved beef was the highest. Beef cooked by using the VPLT method contained the highest quantity of total free amino acids. Sensory analysis showed that grilled or roasted beef was judged to possess greater juiciness, a more desirable odor, and greater *umami* intensity. Beef cooked by the VPLT method was tenderer and had greater *umami* intensity but a less desirable odor. Microwaved beef did not receive a high score for any of the above criteria. These results revealed that the differences in sensory properties of cooked beef loin were caused by differences in cooking loss, water content, *umami* compound content, and breaking energy resulting from cooking by different methods.

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Chapter 2 Influence of Fat Content on Palatability of Beef

The meat of Japanese black cattle has a high fat content, and this has a great influence on palatability. Increasing the crude fat content is especially likely to improve texture.

I analyzed the sensory characteristics of meat samples with crude fat contents between 23.8% and 48.6% that were taken from 34 Japanese black steers. I also analyzed samples with crude fat contents of 8% to 25% taken from 27 crossbred cattle. We grilled the meat and subjected it to analytical sensory evaluation. We also measured the amounts of moisture, protein, nucleic acid, and glutamic acid.

An increase in crude fat content increased the tenderness, juiciness, and fattiness in the meat quality evaluation. An increase in crude fat content reduced the crude protein and moisture contents; it also slightly reduced the nucleic acid and glutamic acid contents, although when the reductions in these *umami* components were assessed relative to the moisture content they changed little. Increasing the fat content up to a certain point greatly enhanced the *umami* intensity and beef flavor intensity in the meat quality evaluation and raised the overall evaluation score; the peak appropriate crude fat content for these purposes was about 36%.

Chapter 3 Influence of Long-term Aging on Palatability of Japanese Black Cattle Beef from Tajima

After slaughter, beef is generally air- or vacuum-packed and refrigerated or preserved by freezing before being passed on to consumers. (These processes are known as wet aging.) However, boutique butcheries are now selling beef kept at low temperature, constant moisture of about 80%, and wind circulation to surface of block meat that has been dry-aged for a fixed period. Although Tajima beef, which is produced from high-quality Japanese black cattle, is aged for 60 days, the meat quality changes during this period are not clear. My analysis of the quality of highly marbled beef during this 60-day dry-aging period after slaughter showed that the changes in some qualities differed from those of conventional meat.

The tenderness of these meats did not change during aging for 50 days, but thereafter it gradually increased until day 60. The juiciness of these meats, as determined by sensory evaluation, did not change during aging for 60 days, except for a decrease on day 20. The *umami* intensity of these meats in the sensory evaluation, and the calculated glutamic acid and inosine monophosphate quantities, were highest on day 40. This high *umami* intensity was induced by the synergistic effect of *umami* compounds such as glutamic acid and inosine monophosphate.

These results for tenderness, juiciness, *umami* intensity, and flavor intensity suggested that the best duration of dry aging for highly marbled beef was 40 days.

As mentioned above, as a result of examining factors affecting the palatability of beef, in heating lean meat it became clear that grilled and roasted beef were judged to possess greater juiciness, a more desirable odor, and greater *umami* intensity. These findings could form the basis of an index of the optimal heating conditions for beef. Moreover, in highly marbled beef from Japanese black cattle, an increase in crude fat content increased the tenderness, juiciness, and fattiness in the meat quality evaluation. An increase in crude fat content reduced the crude protein and moisture contents; it also slightly reduced the nucleic acid and glutamic acid contents. Increasing the fat content up to a certain point greatly enhanced the *umami* intensity and beef flavor intensity in the meat quality evaluation and raised the overall evaluation score; the peak appropriate crude fat content for these purposes was about 36%. Furthermore, analysis of highly marbled beef aged for between 4 and 60 days showed that the *umami* intensity of these meats in the sensory evaluation, and the calculated glutamic acid and inosine monophosphate values, were highest on day 40. These results for tenderness of highly marbled meats did not change during aging, and flavor intensity evaluation suggested that the best duration of dry aging for highly marbled beef was 40 days.

The knowledge acquired in this thesis has helped to elucidate the best methods of preserving and cooking to highlight the features of domestic beef. These data can be used to establish the status of our marbled beef as a specific major livestock export product of which our country can be proud.

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Studies on soybean milk fermented with lactic acid bacteria and yeast

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Summary

Recently, soymilk products fermented by lactobacilli and bifidobacteria have become widely adopted for promoting and maintaining health. Fermented soy milk is known to alleviate intestinal disorders, suppress breast and colon cancer, regulate blood pressure, and improve diabetes. Soybeans contain substances beneficial for human health, including soy protein, peptides, oligosaccharides, phospholipids, isoflavones, saponins, minerals, and vitamins. The fermentation of soybeans by different kinds of micro-organisms results in various compositional and functional changes, such as the production of a large variety of peptides that stimulate immunomodulation and regulation of blood pressure. Isoflavones and saponins exist in glucoside form in the soybean, but are degraded by the β-glucosidase of microorganisms into aglycons that are easily absorbed in the intestine. Soybean milk-fermented product (SFP) used in this study was the product which was fermented with a symbiotic combination of several species of lactic acid bacteria (*Lactobacillus plantarum*, *L. casei*, *Lactococcus lactis*, etc.) and yeast (*Saccharomyces cerevisiae*).

Intestinal microbiota and flora play numerous symbiotic physiological roles in the host, including the production of energy, promotion of intestinal peristaltic motion, digestion and absorption, metabolic regulation of bile acids and cholesterol, protection from infection, immunostimulation, and prevention of cancer. From recent research, intestinal flora has also been implicated in obesity, diabetes, cancer, allergies, autoimmune diseases, and aging in humans. Aging, stress, diet, drugs, and pathogens all influence intestinal flora. Diet in particular greatly impacts the intestinal microenvironment, for which recent advances in flora research have identified functional foods that contribute to human health. Functional foods can be classified into 3 groups based on their mechanism of action: probiotics, prebiotics, and biogenics. Probiotics are viable microorganisms, such as lactobacilli, bifidobacteria, and yeasts, that benefit the host by improving intestinal bacteria balance. Prebiotics are nondigestible food ingredients, including oligosaccharides and dietary fiber, which selectively stimulate the growth or activities of beneficial intestinal bacteria in the colon to improve host health. Biogenics are biologically active peptides, immunopotentiators (i.e., biological response modifiers), and plant flavonoids that ameliorate health directly or indirectly through the modulation of intestinal microbiota. SFP is also considered to be a biogenic, however, the precise function and mechanisms of SFP remain unclear. This study evaluated the effects of SFP on the intestinal environment and immunity in mucous membranes (1) and on the suppression of colon cancer in an animal model (2). *Lactobacillus plantarum* BF-LP284 (LP284) was selected for SFP fermentation based on its high cytokine inducibility properties for investigation of anti-tumor activity and mechanisms (3). Lastly, the impact of soluble fraction SFP (SFP-s) on hypertension (4), liver and kidney dysfunction (5), and arthritis (6) were assessed.

1) Improvement of the intestinal environment and immunological enhancement of mucous membranes in humans by SFP

The effect of SFP (450 mg/day) on human fecal flora was determined by comparing the changes in intestinal flora between human volunteers consuming SFP and those receiving a placebo. An occupation rate

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of *Bifidobacterium* of more than 25% was significantly greater in the SFP group than in the placebo group. The concentration of secretory IgA in the saliva which enhance immunity in mucous membranes was also significantly higher in the SFP group than in the placebo group. The occupation rate of *Clostridium* in the fecal flora of volunteers increased after shifting from a traditional Japanese diet (TJD) to a Western diet (WD), in which a total daily amount of 300 g of meat (900 kcal) was eaten at lunch for 3 days. The occupation rate of *Clostridium* in the WD group was reduced by SFP ingestion (900 mg/day) to a level similar to that in the TJD group, while the occupation rate of *Bifidobacterium* was higher in the WD+SFP ingestion group than in the WD group. Moreover, β-glucuronidase activity in the feces was up to 5 times higher after conversion from the TJD to the WD but returned to normal levels by SFP inclusion in the WD. The above findings suggested that SFP could reduce the risk of colon cancer by improving the intestinal environment, enhancing immunity in mucous membranes, and accelerating the excretion of carcinogenesis-induced agents.

2) Suppressive effect of SFP on colon cancer in an animal model

The incidence of 1, 2-Dimethylhydrazine-induced colon cancer was significantly lower in CF#1 mice given SFP as compared with controls. Winn assays were performed using spleen cells to examine the mechanism of SFP tumor suppression. SFP administered for more than 6 days inhibited tumor growth relative to tumor cells inoculated with Meth-A alone, which indicated an induction of anti-tumor immune cells in SFP-treated spleen cells. Whereas the number of spleen cells increased to a greater degree in gnotobiotic BALB/c mice in association with *Bifidobacterium* than in saline-treated germ-free mice, that in mice given SFP or soybean milk for 4 weeks was comparable. Our results implied that the suppressive effect of SFP on tumorigenesis involved intestinal bacteria modulation of host immunity.

3) Anti-tumor activity of LP284

Heat-killed LP284 (H-Lp) was selected as the most potent immunomodulator from among 7 strains of lactobacilli during fermentation of SFP in terms of TNF-α induction ability in peritoneal macrophages. *In vitro* TNF-α and IFN-γ induction in Peyer's patch (PP) cells was significantly higher when incubated with H-Lp than with live LP284 (L-Lp). Suppression of syngeneic Meth-A tumors in a murine model by oral administration of H-Lp was greater than that of L-Lp and of controls. Thus, H-Lp was considered to contribute to the anti-tumor activity of SFP by stimulating IFN-γ production in spleen cells, which displayed inhibited tumor growth in Winn assays under H-Lp treatment. Moreover, H-Lp increased the ratio of CD3+ cells among peripheral blood mononuclear cells in Meth-A tumor-bearing mice, suggesting an H-Lp-mediated anti-tumor mechanism whereby immune cells that are activated by H-Lp in PP and acquire anti-tumor activity in the spleen migrate to tumor sites through cytotoxic lymphocyte homing to inhibit tumor growth.

4) Anti-hypertensive effects of SFP and SFP-s

The anti-hypertensive effects of orally administered SFP and SFP-s were investigated in spontaneously hypertensive rats. Systolic blood pressure was reduced significantly by a single dose of SFP or SFP-s compared with controls. However, soy milk administration alone did not produce this result. The sugar fraction of SFP-s separated by Sephadex-G25 column chromatography exhibited a lowering effect on systolic blood pressure and inhibited the activity of angiotensin I-converting enzyme. Accordingly, SFP-s may play a role in hypertension management and have utility in maintaining health.

5) Improvement of liver and kidney dysfunction by SFP-s

In rat hepatic disorders induced by oral administration of deoxycholic acid or intraperitoneal injection of D-Galactosamine, the increase in serum L-Aspartate aminotransferase level was significantly inhibited by a diet containing dried SFP-s. Moreover, the SFP-s rat group displayed a lower concentration of blood urea nitrogen and greater urinary output as compared with a control group. Pretreatment of primary rat hepatic and renal cell cultures with SFP-s prior to exposure to dichromate resulted in a marked decrease in dichromate-induced cytotoxicity as evaluated by the leakage of lactate dehydrogenase. The levels of dichromate-induced lipid peroxidation, as evidenced by malondialdehyde formation, were also reduced by SFP-s pretreatment of hepatocytes. These collective findings indicated that the anti-oxidative activity of SFP-s might improve hepatic and renal disorders by suppressing membrane peroxidation.

6) Anti-arthritic effect of SFP-s

The anti-arthritic effects of SFP-s were investigated in bovine type II collagen (bCII)-induced arthritis (CIA) in mice. The disease incidence and mean clinical score of CIA were significantly suppressed in an SFP-s +glucosamine (GM) group, while hind foot pad thickness was significantly reduced. Histopathological scores of the severity of lesions in arthritic hind paw joints were significantly ameliorated in the SFP-s and SFP-s+GM groups. Serum bCII-specific IgG antibody production was markedly down-regulated in SFP-s and SFP-s+GM groups, and IL-6 level in the homogenate supernatant of diseased hind foot pad tissue was significantly lower in the SFP-s+GM group. Taken together, a combination of SFP-s and GM is considered to be additively effective in reducing the severity of CIA, presumably due to the abilities of SFP-s to modulate the immune response to bCII and of GM to reduce CIA-associated inflammation.

In conclusion, there is a considerable body of evidence demonstrating SFP to be a clinically meaningful fermented biogenic that acts directly or indirectly through the modulation of intestinal microflora. SFP may alleviate intestinal disorders, enhance immune function, suppress cancer, regulate blood pressure, and improve hepatic and renal disorders and arthritis, and is thus potentially useful for maintaining health in humans.

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Reconsideration for effective use of thyrotropin (TSH) and thyrotropin-releasing hormone (TRH) stimulation test in dogs

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Hypothyroidism is a common endocrinology disease in dogs. The thyroid gland is responsible for the production of the two active thyroid hormones, Triiodothyronine (T3) and thyroxine (T4). The principal product of the thyroid gland is T4, which is entirely derived from thyroid synthesis. The main physiological action of thyroid hormone is to promote basal metabolic rate including heat production and i activation of glucose metabolism. Primary hypothyroidism has abnormality in the thyroid gland and secondary hypothyroidism has abnormality in the pituitary. Primary hypothyroidism (the most common cause) is induced by idiopathic atrophy or lymphocytic thyroiditis. The loss of thyroid gland function leads to an insufficient production and secretion of thyroid hormones. Thyroid hormone deficiency affects canine metabolic systems. The clinical signs are numerous and variable and often nonspecific for hypothyroidism. There are many diagnostic tests for assessing thyroid function, but no single test has 100% accuracy. Low thyroxine syndrome, named euthyroid sick syndrome is a common finding in dogs. Euthyroid sick syndrome is attribute to nonthyroidal illness such as the severity of the disease and treatment with specific drugs such as phenobarbital. Up to date, the gold standard test for evaluating thyroid function in dogs was the thyrotropin (TSH) stimulation test (TSH-ST). However, the TSH-ST for clinical use is no longer performed by the expensive cost of reagent.

Therefore, the purpose of this study are two-fold. First, we investigated the effects of two diagnostic tests (recombinant human thyroid stimulating hormone (rhTSH) stimulation test and thyrotropin-releasing hormone (TRH) stimulate on test) for assessing thyroid function for serum T4 concentration free thyroxine (FT4) concentration, and TSH concentration in healthy dogs. Second, we wanted to determine whether the low dose of each stimulating tests could be applied in clinical setting.

In Phase I, TSH-ST and TRH stimulation test (TRH-ST) were performed in 6 healthy beagle dogs, using 50 μg/dog (TSH-ST) and 5 μg/kg (TRH-ST), respectively. TSH and TRH administered intravenously. All dogs underwent overnight fasting prior to use. Blood sample were taken before (0 min) and 30, 60, 120, 240, 360, 480 min after administration of each reagent for determining TSH, T4 and FT4 concentrations. Significant ($P<0.05$) increases in T4 and FT4 concentration were observed at 120～480 min after TSH and TRH administration. The peak value of T4 concentration after TSH-ST was at 360 min, whereas the peak value of T4 concentration after TRH-ST was at 240 min. TRH-ST induced shorter times of peak T4 concentration than TSH-ST. We suggested that both stimulation test were valuable as diagnostic tool to assess thyroid function in dogs, since significant increases in T4 concentration are observed after TSH and TRH-ST. Furthermore, TSH concentration measurement besides T4 concentration would be possible to evaluate not only function of thyroid gland but also that of pituitary.

In Phase II, in order to confirm the effect of the lower dose of TSH and TRH-ST as compared to Phase I, each stimulation tests were performed using 9 μg/dog of TSH or 5 μg/dog TRH in 4 healthy dogs. Each test was carried out with similar protocol for Phase I.

In Phase II nevertheless using lower dose of TSH and TRH-ST, stimulated T4 and TSH concentrations was significantly increased, confirming similar effect of stimulation test as Phase I study. Therefore, it was recommended using lower dose of TSH and TRH reagent for stimulating TSH, T4 and FT4 in dogs. Both

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stimulation test with low dose using in the current study are valuable as diagnostic tool to assess thyroid function in dogs. Furthermore, in the TSH-ST, it was suggested that the optimal blood sampling time would be earlier in using lower dose of TSH stimulation test than using traditional dose of TSH stimulation test.

In veterinary practice, diagnosis of hypothyroidism in dogs is performed by measuring TSH, T4 and FT4 concentration. However, as the value of such thyroid hormone is influenced by various factors such as euthyroid sick syndrome, drugs and environmental factors. As such, concurrent disease and receiving drugs complicate the diagnosis of hypothyroidism. For the above reason, this study evaluated the change in T4, FT4 and TSH using TSH and TRH-ST in healthy dogs. Previous studies, the TSH stimulation test was used as the "gold standard" for the diagnosis of hypothyroidism. Another study reported that TRH-ST was a poor sensitivity as evaluating thyroid function in dogs. However, we suggested that both stimulation test were a valuable diagnostic tool to assess thyroid function. Moreover, the TSH and TRH-ST might be recommended using lower dose as compared to past study in dogs. Furthermore, lower dose of TSH stimulation test induce earlier time of peak concentration of T4 than traditional dose of TSH stimulation test.

Future studies involving the administration of TSH and TRH in dogs with hypothyroidism dogs are needed for the use of thyroid function test. Finally, better diagnosis criteria should be established using TSH and TRH stimulation test in dogs.

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Structural and functional analysis of canine prostate cancer related gene *SGTA*

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The incidence of canine prostate cancer is low, but almost all cases are associated with high malignancy and metastasis. The majority of cases present poor clinical outcomes during early stages. Prostate cancer is the most commonly diagnosed cancer in men. In its early stages, prostate cancer progression depends on androgen receptor (AR) signaling. Therefore, hormonal strategies including androgen ablation and/or AR antagonists have been used in prostate cancer therapy. Alterations in AR signaling are responsible for most human cases of prostate cancer, but a proportion of these cases do not respond to androgen ablation therapy. Although androgen ablation has been used for the treatment of canine prostate cancer, almost all tested cases were resistance to this treatment. These pathological conditions are similar both in canine and human prostate cancer, and thus, overcoming resistance to androgen ablation therapy is required to establish novel strategies for both canine and human prostate cancer.

The small glutamine-rich tetratricopeptide repeat-containing protein α (SGTA), an Hsp70/Hsp90 co-chaperone, is overexpressed in human androgen-independent prostate cancer. However, little is known about the structure and function of canine SGTA (cSGTA). In this study, I used cloning and molecular biological analysis to investigate the role of cSGTA in the occurrence and acquisition of resistance to androgen ablation therapy in canine prostate cancer.

I. cDNA cloning and sequencing of canine SGTA

The sequence for canine SGTA is unknown except for a predicted sequence. Therefore, I first cloned the open reading frame (ORF) of a canine SGTA homologue and analyzed cSGTA structure. Using canine EST sequences that are homologous to the human SGTA gene, I cloned the cSGTA homologue. The cSGTA ORF has 942 base pairs (bp), and the canine SGTA protein shows 95% and 91% homology with the human and murine SGTA proteins, respectively. The high level of sequence conservation led me to hypothesise that cSGTA is also functionally homologous to the human SGTA. The presence of a putative SGTA dimerization region at the N-terminus and a tetratricopeptide repeat (TPR) in the middle of the sequence suggests that the cSGTA protein may form a dimer on overexpression, which is responsible for the acquisition of resistance to androgen ablation therapy in canine prostate cancer.

II. Canine SGTA homodimerization and its contributions to AR signaling

Second, I investigated cSGTA expression in prostate tissue, homodimerization and its contributions to AR signaling. The six prostate hyperplasia samples were not stained by an anti-SGTA antibody, but the three primary prostate carcinoma samples were moderately or strongly stained by the same antibody. cSGTA homodimerization was demonstrated in a mammalian two-hybrid assay (MTH) and in vitro pull-down assay. Next, I examined the role of intracellular cSGTA in AR signal transduction. cSGTA and/or replacement AR were expressed in cells and measured using luciferase reporter assay. The addition of cSGTA significantly suppressed AR signaling with dihydrotestosterone (DHT) treatment. These results suggest that cSGTA has a role as a negative regulator of AR signaling in canine prostate cancer, and may trigger the acquisition of androgen-independency.

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III. The control of canine SGTA by REIC/Dkk-3 and FKBP52

Third, I used the MTH and AR signaling assay to investigate molecules that could interfere with the suppression of AR signaling by cSGTA. REIC/Dkk-3 interferes with SGTA dimerization and upregulates AR signaling in humans, and it showed a similar effect on canine SGTA. FKBP52 replaces SGTA at the AR complex after androgen stimulation in humans and transports the AR complex to the nucleus. The canine FKBP52 did not bind to cSGTA but promoted SGTA dimerization. Moreover, canine FKBP52 upregulated AR signaling in an androgen-independent manner, and may play an important role against the occurrence and acquisition of resistance to androgen ablation therapy in early stages of prostate cancer in castrated dogs.

IV. Conclusion

These results contribute to unraveling the mechanism by which resistance to androgen ablation therapy develops in canine prostate cancer. Furthermore, the control of SGTA by REIC/Dkk-3 and FKBP52 in the response to androgen stimulation could lead to a potential novel treatment for canine androgen-independent prostate cancer.

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The evaluation of dietary effect on the fecal microbiome in healthy dogs

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Recently, intestinal microbiomes are thought to be related to health, longevity and disease. Diets are one of the key factor that has a significant influence on intestinal microbiomes. It is suggested that diet affects canine intestinal microbiomes and intestinal diseases. However, there are few studies on intestinal microbiomes in dogs, and there is no report on the influence of commercial diets for intestinal microbiomes.

Commercial diets are the most common diet for pet dogs. It is important to know the influence of commercial diets on intestinal microbiome for good health, life extension, prevention of obesity and risk reduction of disease. Therefore, in the current study, we examined the influence of commercial diets on fecal microbiomes in healthy dog.

In Chapter 1, we examined the effect of diets with different composition of nutrients on fecal microbiomes of experimental dogs. The test diets were selected focusing by the composition of nutritional components in the diets. As a test diet, Satiety Support Special (high dietary fiber and high protein diet), Kidney Support (low protein and high fat diet), Digestive Support Low Fat (low fat and high carbohydrate diet), and Aminopeptide Formula (only amino acids and oligopeptides were used as nitrogen source) was were used. Feces were collected after the feeding of the test diet for 3 weeks and the fecal microbiomes of each diet was compared. Feces were collected after feeding the test diet and DNA was extracted from the feces. Extracted DNA was analyzed by 16S ribosomal RNA analysis using next-generation sequencing. Then, we compared the fecal microbiomes between test diets. Fecal microbiomes of experimental dogs composed of Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria and these results agreed with past reports. Aminopeptide Formula and Satiety Support Special induced significant changes in fecal microbiome in experimental dogs. The phylum Actinobacteria and the phylum Firmicutes showed significant differences between test diets. The phylum Actinobacteria was significantly increased in the Aminopeptide Formula feeding group compared to the Satiety Support Special feeding group. The phylum Firmicutes were significantly decreased in the Satiety Support Special feeding group as compared to the Kidney Support, Digestive Support Low Fat and Aminopeptide Formula feeding group. These changes were thought to be due to the dietary amount of protein. Dietary fat and carbohydrate might not have significant influence on microbiomes. In the diversity index, the Aminopeptide Formula induced lower value than other diets. This change might be also related to dietary amount of protein. From the results of the principal coordinate analysis, similarity of fecal microbiomes was observed in the Aminopeptide Formula feeding group and the Satiety Support Special. From the above results, it was considered that the commercial therapy diet influences the fecal microbiome of the experimental dog. Furthermore, dietary amount of proteins might be related these results.

Intestinal microbiomes are associated with disease. Prebiotics and probiotics are used for the prevention and treatment of intestinal diseases in humans. As prebiotics and probiotics are used for treatment in veterinary medicine, pet dogs are considered to be the subject. However, there are few studies on fecal microbiomes using pet dogs. In addition, intestinal microbiomes are generally affected by genetic background and living environment. As such, fecal microbiome in pet dogs may be diversified. Therefore, in Chapter 2, we examined the influence of the

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commercial diet on fecal microbiomes in pet dogs. After changing the diet from previous diets with each pet dog to test diets, we compared fecal microbiome between feeding previous diet and test diet. From the results of Chapter 1, we used Digestive Support Low Fat (the Digestive Support Low Fat feeding group) and Aminopeptide Formula (Aminopeptide Formula feeding group) as test diets. Fecal microbiomes of all pet dogs with before and after feeding test diets composed of Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria. Tenericutes were also detected in a few dogs in the Digestive Support Low Fat feeding group. This composition was similar to Chapter 1. When compared between two test diets, Aminopeptide Formula showed a significant changes in fecal microbiome. The phylum Firmicutes was significantly increased and the phylum Fusobacteria was significantly decreased in the Aminopeptide Formula feeding group as compared to the diets feeding before study. These changes were also thought to be due to the amount of dietary protein as in Chapter 1. Also, the diversity index also decreased in the Aminopeptide Formula feeding group as compared to the Digestive Support Low Fat feeding group. From the results of the principal coordinate analysis, similarity of fecal microbiomes was observed in the Aminopeptide Formula feeding group. Therefore, it was suggested that feeding amino peptide formula can produce specific fecal microbiomes condition in pet dogs with different breeding environments. From the above results, it was shown that fecal microbiomes could be changed and homogenized by feeding the same diet as for pet dogs. In conclusion, it is possible to product characteristic microbiomes by feeding the same diet both experimental and pet dogs. In the future, we need to perform long-term testing in pet dogs to examine the effects of diets, probiotics and prebiotics on canine fecal microbiomes.

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Population Density and Mode of Transmission in the Epizootic of Sarcoptic Mange of Raccoon Dogs (*Nyctereutes procyonoides*)

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Sarcoptic mange is a parasitic skin disease caused by *Sarcoptes scabiei*. Among wild mammals in Japan, there have been many reports of sarcoptic mange in raccoon dogs (*Nyctereutes procyonoides*). Previous studies have suggested that factors underlying epizootics of sarcoptic mange in raccoon dogs include 1) an increase in raccoon dog population density and 2) direct contact transmission between parents and offspring. The objective of this study was to consider the causal factors for the epizootic of sarcoptic mange in raccoon dogs in the study area of Takasaki City, Gunma Prefecture. We monitored an outbreak of sarcoptic mange in a population of raccoon dogs and estimated the raccoon dog population density by conducting a camera trapping survey. We also examined the mode of transmission of sarcoptic mange by genetic analyses of raccoon dogs and *S. scabiei*.

After Chapter 1, which provides an overview of sarcoptic mange, Chapter 2 describes a preliminary study aimed at characterizing the outbreak of sarcoptic mange in raccoon dogs in the study area and determining whether clinical signs from the camera trapping survey can be used to estimate infestation with *S. scabiei*.

In this study, 12 out of 27 raccoon dogs showed clinical signs of mange-compatible lesions. *S. scabiei* was isolated from 11 of the raccoon dogs that showed clinical signs. Therefore, sarcoptic mange in raccoon dogs spread in the study area. In addition, 91.7% of raccoon dogs with clinical signs were infested with *S. scabiei*. These results suggested that clinical signs observed on recordings in camera traps could be used to diagnose *S. scabiei* infestation.

In Chapter 3, the outbreak of sarcoptic mange was monitored to consider the epizootic of sarcoptic mange with respect to raccoon dog population density and to determine the impact of sarcoptic mange epizootics on population dynamics. We analyzed camera trapping data to identify clinical signs of mange-compatible lesions on raccoon dogs.

The number of raccoon dogs per trap without clinical signs showed a significant increase in the autumn of 2012 and the number of raccoon dogs per trap with clinical signs increased in the summer of the following year. These results suggest that a high relative population density of raccoon dogs was a factor in the epizootic of sarcoptic mange in raccoon dogs.

There was a significant positive relationship between the raccoon dog population density estimated by the random encounter model and the number of records per trap, indicating that the population density estimated by the random encounter model is appropriate. An increase in the raccoon dog population density to more than double the normal population density ($<5/km^2$) may be associated with the epizootic of sarcoptic mange in raccoon dogs.

In addition, after the epizootic of sarcoptic mange, a few raccoon dogs with clinical signs were consistently observed. Thus, the raccoon dog population likely maintained sarcoptic mange over time, and this may have influenced the long-term population dynamics of raccoon dogs.

In Chapter 4, direct contact transmission of *S. scabiei* between parents and offspring and the genetic structure of raccoon dog populations were analyzed. We estimated kinship among raccoon dogs and analyzed the population structure by genetic analyses.

In the kinship analysis of infested individuals, we only detected one pair of individuals that were related as parent–offspring. A genetic population structure

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analysis indicated the raccoon dogs in the area could be divided into two major groups. Additionally, F_{ST} estimates indicated that the two groups were genetically differentiated. Infested individuals were distributed closely within the same genetic population and transmission to the 0-year-old infested individuals probably occurred after dispersion. Therefore, the transmission of *S. scabiei* in the raccoon dog population occurred by direct contact between unrelated individuals in close proximity within the same genetic group. These findings suggested that the mode of transmission is one of the factors explaining the sarcoptic mange epizootic in the raccoon dog population.

In Chapter 5, the objective was to estimate the genetic population structure of *S. scabiei* isolated from the raccoon dogs described in Chapter 4. In an analysis of genetic population structure, we found that *S. scabiei* collected from raccoon dogs belonged to a single group; we detected high genetic relatedness among individuals within the *S. scabiei* population. Accordingly, it is possible that closely related *S. scabiei* spread recently in the study area corresponding to the sarcoptic mange epizootic in raccoon dogs.

These results indicated that when the raccoon dog population densities increased to over double the normal population density, the epizootic of sarcoptic mange in raccoon dogs may have been triggered. Moreover, the main mode of transmission of sarcoptic mange among raccoon dogs was direct contact among unrelated individuals inhabiting adjoining areas within each genetic population. This mode of transmission may be one factor explaining the sarcoptic mange epizootic in the raccoon dog population. In future studies, it is necessary to focus on the behavioral ecology of raccoon dogs to characterize the disease ecology of sarcoptic mange.

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Bovine viral diarrhea virus field strains support the concept of viral quasispecies

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Bovine viral diarrhea virus (BVDV) together with classical swine fever virus and border disease virus in sheep belongs to the family *Flaviviridae*, genus *Pestivirus*. BVDV has global distribution and results in great economic loss in the stock raising industry. BVDV is classified into two biotypes, cytopathogenic (CP) and non-cytopathogenic (NCP). NCP BVDV can be further classified into two biotypes, one that exhibits exaltation of Newcastle disease virus phenomenon (END^+) and one that does not (END^-) but interferes with heterologous viruses. Both biotypes coexist not only in laboratory strains, but also in field strains. Moreover, the frequencies of these biotypes vary among field strains. Thus, BVDV comprises viruses with different characteristics. However, some isolated field strains cannot be classified into a known biotype. Therefore, in this study, the biological properties of these viruses were determined, and examined the likelihood of a new biotype of BVDV and its viral diversity.

Chapter 1 Viral cloning and biological analysis of BVDV isolates with unclear biotype

Three BVDV isolates that had undetermined biotype were cloned by limiting dilution technique three times, and cloning strains YL41cl, YL45cl, and YL47cl were obtained. Each cloning strain was assayed by the END method to detect END^+ virus, an interference method with vesicular stomatitis virus (VSV) to detect END^- virus, and peroxidase-linked assay to measure the total titer of BVDV in each strain. All strains showed the interference phenomenon without END phenomenon, therefore they were classified as END^- viruses. Although the titer of YL45cl was $10^{6.91}$ $TCID_{50}$/mL, the titer of END^- YL45cl was significantly lower at $10^{6.24}$ $TCID_{50}$/mL ($p<0.05$). This result suggests that END^- viruses may have different VSV interference abilities. In addition, these viruses are considered to have properties not found in current viral tests, including viral gene detection.

Chapter 2 Analysis of innate immune responses against BVDV isolates that differ in VSV interference ability

Our initial findings suggested that END^- viruses may exhibit different VSV interference abilities. Therefore, we analyzed the innate immune control ability, which is known to be associated with END phenomenon and VSV interference ability, to evaluate VSV interference. All three cloning strains were evaluated in this section. After analyzing the predicted amino acid sequence of the N^{pro} gene to modulate the host cell innate immune response, we confirmed neither strain had the reported amino acid substitution for END^- virus. Additionally, mRNA levels of host innate immune factors *Mx1* and *bISG15* were analyzed. *Mx1* mRNA levels after infection with all three cloning viruses were 6.73～9.04-fold higher than that after END^+ virus infection. Moreover, *bISG15* mRNA levels after infection with all three cloning viruses were 2.53～4.66-fold higher than that after END^+ virus infection. Thus, the innate immune control ability of END^- viruses though the N^{pro} region was not associated with amino acid substitutions in the END^- biotype as observed in the cloning strains. Because no difference was observed in the mRNA levels of innate immune response factors among all three cloning strains ($p<0.05$), though the strains exhibited different VSV interference abilities, an unknown mechanism of the innate immune system might be involved.

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Chapter 3 Sequence homology analysis of BVDV isolates with various VSV interference abilities

Analysis results of the predicted amino acid sequence of the N^{pro} region involved in VSV interference ability and biotype characterization estimated from mRNA analysis of innate immune response factors suggested differences among the viruses analyzed in this study. Therefore, to identify a new putative gene region responsible for regulation of the innate immune response, whole genome sequencing was conducted. Strain-specific amino acid residues were found in five locations in YL41cl, 42 locations in YL45cl, and two locations in YL47cl. YL45cl and YL41cl or YL47cl demonstrated 97～99% homology at the nucleotide and amino acid levels, and YL41cl and YL47cl demonstrated 99～100% homology at the protein level. In addition, when we compared the amino acid sequences of these three strains with known END^+ and END^- viruses, the amino acid substitution common to END^- viruses was not found. However, a site associated with VSV interference ability not previously reported was considered to be present because many strain-specific amino acid substitutions were found in YL45cl. It was speculated that these amino acid substitutions also resulted in the observed diversity among END^- viruses.

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Molecular genetic study of the *CMAH* gene associated with cat AB blood group systems

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Cat's AB blood group system (blood types A, B, and AB) is of major importance in feline transfusion medicine. Type A and type B antigens are Neu5Gc and Neu5Ac, respectively, and the enzyme CMAH participating in the synthesis of Neu5Gc from Neu5Ac is associated with this cat blood group system. Rare type AB erythrocytes express both Neu5Gc and Neu5Ac. Cat serum contains naturally occurring antibodies against antigens occurring in the other blood types. To understand the molecular genetic basis of this blood group system, we investigated the distribution of AB blood group antigens, *CMAH* gene structure, mutation, diplotypes, and haplotypes of the cat *CMAH* genes. Blood-typing revealed that 734 of the cats analyzed type A (95.1%), 38 cats were type B (4.9%), and none were type AB. A family of three Ragdoll cats including two type AB cats and one type A was also used in this study. *CMAH* sequence analyses showed that the CMAH protein was generated from two mRNA isoforms differing in exon 1. Analyses of the nucleotide sequences of the 16 exons including the coding region of *CMAH* examined in the 34 type B cats and in the family of type AB cats carried the *CMAH* variants, and revealed multiple novel diplotypes comprising several polymorphisms. Haplotype inference, which was focused on non-synonymous SNPs revealed that eight haplotypes carried one to four mutations in *CMAH*, and all cats with type B (n=34) and AB (n=2) blood carried two alleles derived from the mutated *CMAH* gene. These results suggested that double haploids selected from multiple recessive alleles in the cat *CMAH* loci were highly associated with the expression of the Neu5Ac on erythrocyte membrane in types B and AB of the feline AB blood group system. This study was published in PLoS ONE 11(10): e0165000, 2016.

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Study on cytopathogenicity of porcine circovirus type 2

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Porcine circovirus (PCV) is classified into nonpathogenic/avirulent PCV type 1 (PCV1) and pathogenic PCV type 2 (PCV2). PCV2 has been identified as an etiologic factor of post-weaning multisystemic wasting syndrome and considered to be involved in porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex and reproductive difficulties. Porcine diseases induced by PCV2 infection are called porcine circovirus associated disease (PCVAD). Diagnosis of PCVAD in Japan is based on pathological and epidemiological indices, as virological methods are generally inefficient because growth of PCV2 is challenging in *in vitro* cell cultures. Moreover, since PCV2 is usually noncytopathogenic, viruses in cultured cells should be detected by immunological staining or amplification of a viral gene. Although PCV2 does not induce a cytopathic effect (CPE), PCV2 Yamagata strain induced detachment of an adherent porcine kidney cell line, CPK-NS cells, from the bottom of culture dishes. Therefore, the CPK-NS cell line may be a useful tool for diagnosis of PCV2 infections and study of PCV2 replication. However, since animals are usually co-infected with nonpathogenic PCV1 and pathogenic PCV2, purified PCV2 should be prepared to analyze its characteristics. In this study, to explore the development of a diagnostic method, cytopathogenicity of PCV2 in CPK-NS cells was analyzed using PCV2 particles prepared by a reverse genetics system.

Chapter 1 Production of infectious PCV2 particles using a reverse genetics system

Infectious PCV2 particles were produced by a reverse genetics system. Porcine kidney PPK-3f cells were transfected with plasmid pPCV2x2, which contains two copies of the complete genome of PCV2 Yamagata strain. Cells were passaged every 3 days. After the fourth passage, supernatant was harvested (called vPCV2/P4). The titer of infectious particles in vPCV2/P4 was calculated as $10^{4.5}$ focus forming units (FFU) per mL by indirect immunofluorescence assay, and the supernatant contained $10^{9.5}$ copies of PCV2 genome per mL by real-time PCR assay. PCV1-specific real-time PCR and conventional PCR did not amplify PCV1 genome. Moreover, vPCV2/P4 showed 100% homology with PCV2 Yamagata strain (parent strain). These results indicate successful production of purified infectious PCV2 particles without PCV1 contamination by using a reverse genetics system.

Chapter 2 Cytopathogenicity of PCV2 in CPK-NS cells

CPK-NS and PPK-3f cells were inoculated with 10-fold diluted vPCV2/P4, and cell detachment was observed during cell passage. Remarkable cell detachment was confirmed in CPK-NS cells infected with $10^{4.5}$ and $10^{4.0}$ FFU of vPCV2 after first passage and in PPK-3f cells with $10^{4.5}$ FFU of vPCV2 at first and second passages. Cell detachment appeared to be viral dose- and cell passage-dependent. Additionally, cell detachment was inhibited in cells inoculated with vPCV2 neutralized by antiserum. Moreover, the viral copy number in the supernatant and cells increased with each passage. When cell detachment appeared in CPK-NS cells infected with $10^{4.5}$ FFU of vPCV2, the viral copy number peaked in the supernatant but rapidly decreased after the appearance of cell detachment. For PPK-3f cells, the viral copy number slightly increased but was not related to the appearance of cell detachment and appeared to maintain a certain level. These results indicate that replication efficiency of PCV2 is related to cell detachment, and CPK-NS cells may be more suitable than PPK-3f cells for PCV2 replication. Moreover, cell detachment observed in CPK-NS cells is likely CPE induced by PCV2 infection.

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Chapter 3 Analysis of innate immune responses in CPK-NS cells infected by PCV2

CPK-NS cells are not useful to evaluate biological diagnostic methods of PCV2 infection because CPE induced by PCV2 infection does not appear until several passages after inoculation. To reduce the culture time until appearance of CPE induced by PCV2 infections, the relationship between cell detachment and innate immune responses was analyzed. After inoculating CPK-NS and PPK-3f cells with $10^{4.5}$ FFU of vPCV2, mRNA levels of innate immune response factors *Mx1*, *PKR*, and *OAS* were measured 6, 12, 24, 48, and 72 hours (pre-passage); 96 hours (during passage); and 102 and 108 (post-passage) hours after inoculation by real-time RT-PCR. At 12 hours after passage, mRNA levels of *OAS* were 4-fold higher in CPK-NS cells than PPK-3f cells, however, there was no difference in mRNA levels of each innate immune response factor between pre- and post-passages. These results suggest that PCV2 inhibits antiviral activity at the early stage of infection, and the appearance of CPE in CPK-NS cells is not related to these innate immune response factors. Unfortunately, we did not identify an association between PCV2 infection and CPE induction in CPK-NS cells through an innate immunity mechanism. Therefore, further studies focused on innate immunity and apoptosis are necessary before application of CPK-NS cells in the diagnosis of PCV2 infections.

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Effect of dietary medium-chain triglycerides in healthy cats

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Medium-chain triglycerides (MCT) contain 8 to 12 carbon atoms with no double bond. In contrast, long-chain triglycerides (LCT) typically contain ≥14 carbon atoms. MCT is readily hydrolyzed to medium-chain fatty acid (MCFA) by lingual and gastric lipases. The MCFA are absorbed through the portal system without re-synthesis of triacylglycerol in intestinal cells. Therefore, triacylglycerol in the blood does not increase after intake of MCT. Absorbed MCFA is metabolized by β-oxidation in the liver. Because of the unique metabolism of MCT, it can be used as rapid energy source (Absorption of MCT is five to ten times faster than that of LCT), dietary MCT may be beneficial in several respects. For example, MCT is used for ill patients as a good energy source. In addition, MCT may be used for diet of patients with kidney disease as non-protein energy.

However, the effects of dietary MCT on lipid metabolism in cats have not been evaluated. Therefore, the objective of this study is to investigate the effect of dietary MCT on lipid metabolism in healthy cats.

1. Examination of safety of MCT in healthy cats

Coconuts oil, which is natural MCT oil, is composed of 60% MCT and 40% LCT. We fed the control diet (2.8 g of fat content/100 kcal) with 30% calories of coconuts oil (11.1 g of fat content/100 kcal) in 3 healthy cats. Diet was fed for 28 days. We measured body weight and body fat every 7 days (0, 7, 14, 21 and 28 days). On the same day, preprandial blood samples were collected by bleeding 2.5 mL from the jugular vein of cats for evaluating complete blood count (CBC) and serum biochemistry test. Additionally, we evaluated the health condition of cat every day.

No significant difference was observed in body weight, body fat and CBC during the study period. Serum total-cholesterol concentration was significantly increased at 14 and 28 days as compared to 0 day. Lipase concentration was significantly decreased at 21 days as compared to 0 day. Serum TBA concentration was significantly increased at 28 days as compared to 0 day. Total-cholesterol might be increased because of addition of coconut oil into the diet. Serum lipase might be decreased because MCT (contained in coconuts oil) induced decreasing secretion of lipase. Serum TBA might be increased by fasting status of each cat. In the health assessment, two cats had reduction of appetite. This result might be related to higher amount of test diet (contained in coconuts oil) than previous diet. Therefore, in order to use coconut oil for cat diet, it is necessary to reduce the additive amount.

2. Effect of dietary MCT on fat metabolism in healthy cats

MCT induce different lipid metabolism as compared to LCT in humans. Therefore, we compared the lipid metabolism in cats by feeding MCT (using coconuts oil) and LCT (using lard and soybean oil).

We fed the control diet (2.8 g of fat content/100 kcal) and control diet with 3 different fats (coconuts oil, lard, and soybean oil) in 6 cats. Coconuts oil was replaced 5% and 10% for the diet. Lard and soybean oil replaced 10% for the diet. Each diet was fed by the Latin square design for 14 days. Blood samples were collected by bleeding 2.5 mL from the jugular vein of cats preprandial and 0.5, 1, 2, 4, 6, 8 and 10 hours post feeding on the last day of the 14 days. Serum glucose, insulin, triglyceride (TG) and nonnesterified fatty acid (NEFA) concentrations and plasma glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) concentrations were measured.

Gastrointestinal symptoms and appetite decline was not observed during study period. As a result, body weight and body fat did not change in all test food during study period. Therefore, coconut oil is able to

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give for cats. All serum and plasma blood test did not show any significant difference. These results might be related to low content of MCFA in coconuts oil (60% MCFA and 40% LCFA).

The result in Chapter 1 showed that the MCT added to diets induced some detrimental effect (decreased appetite) in healthy cats. Therefore, in order to use coconut oil for the cat diet, it is necessary to reduce the amount of it.

Furthermore, we investigated the effect of MCT on feline fat metabolism by comparing coconut oil, lard and soybean oil in Chapter 2. Additive coconut oil did not induce gastrointestinal symptoms and decreased appetite during the study period. However, there was no significant difference in laboratory results related to lipid metabolism in all test diets. Coconut oil is able to use for cats, however, its effect for lipid metabolism in cats should be further study. In the future, we need to investigate the effective amount of MCT and to perform more long term study for evaluating effect of dietary MCT in cats.

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Antioxidant activity of peptides derived from porcine myofibrillar proteins

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Objectives

We reported previously that peptides (MfP) obtained via the hydrolysis of porcine myofibrillar proteins with papain have antioxidative activities such as suppression of lipid peroxidation and 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity. MfP also suppress the decomposition of ovalbumin (OVA) by various reactive oxygen species (ClO^-, $HO^\bullet$, $ONOO^-$).

In this study, we aimed to isolate antioxidative peptides that suppressed the decomposition of OVA in the Udenfriend system. We also aimed to determine their structures and investigate the antioxidative activity of a peptide synthesized on the basis of one of the sequences determined.

Methods

Preparation of samples: Myofibrillar proteins were extracted from porcine longissimus thoracis muscle and hydrolyzed by incubation with 1/100 (w/w) papain for 48 h at 37 ℃. To 1 volume of protein solution, 4 volumes of ethanol were added to remove the proteins. The peptides contained in the supernatant were considered to be MfP. The MfP were subjected to ultrafiltration and divided into low (molecular weight: 5000 or less) and high (molecular weight: over 5000) molecular weight fractions.

Suppression of OVA decomposition induced by the Udenfriend system containing an MfP fraction: A fraction (final concentration: 1 mg/ml) of hydrolysate of myofibrillar proteins was added to OVA from egg white (final concentration: 2 mg/ml). The mixture was thoroughly stirred and incubated for 10 min at room temperature. Then, a solution (final H_2O_2 concentration: 10 mM) that contained radicals formed from hydrogen peroxide and iron was added. Decomposition of the OVA after incubation for 5, 15, 30, 60, 90 or 120 min at 37 ℃ was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of highly antioxidative peptide fraction and peptide structure: A peptide fraction that showed strong antioxidative activity was fractionated by being passed through an octadecyl-silica (ODS) high-performance liquid chromatography (HPLC) column. Fractions of high-activity peaks were subjected to NanoLC/MS/MS (Ultimate 3000 RSLCnano and LTQ Orbitrap XL; Thermo Scientific) to determine the structure of the peptides.

Measurement of antioxidative activity: A peptide was synthesized on the basis of the sequence determined. The effects of the synthesized peptide in suppressing decomposition of OVA in the Udenfriend system, scavenging DPPH and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals, and chelating copper ions were measured; the antioxidative activity of the peptide was evaluated.

Amino acid analysis: The synthesized peptide, in either 1) a peptide solution or 2) a solution in the Udenfriend system prepared by mixing the peptide and a radical solution and incubating for 60 min at 37 ℃, was hydrolyzed by adding concentrated hydrochloric acid to a final concentration of 6 N and incubating for 24 h at 110 ℃. After removal of the hydrochloric acid, the samples were subjected to amino acid analysis.

Results and Discussion

(1) Antioxidative peptide fractions and determination of peptide structures

The high-molecular-weight fraction of MfP did not suppress the OVA decomposition induced by the

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Udenfriend system. On the other hand, addition of the low-molecular-weight fraction strongly suppressed the OVA decomposition induced by the Udenfriend system. The low-molecular-weight fraction was further fractionated into five fractions by HPLC. Antioxidative activity was observed in Fraction II. Fraction II was then subjected to HPLC using an ODS column to isolate the peptides, and 19 peaks of peptide origin were obtained. Suppression of OVA decomposition by each peak was evaluated, and four peaks (P1, P3, P5, and P13) were found to have antioxidative activity. The peptide structures of the peaks were analyzed by using NanoLC/MS/MS, and three, three, and one amino acid sequences were estimated from P1, P3, and P13, respectively. No MS peak was obtained from P5. Each of these peptides had a sequence of five to seven residues and was 100% identical to partial sequences of myosin and actin in a BLAST search.

(2) Antioxidative activity of a synthesized peptide

A peptide was synthesized from the amino acid sequence (Leu-Asp-Glu-Asn-Ile-Ala-Lys) (LDENIAK) of P13, which was highly likely to be a single peptide; its effect in suppressing the OVA decomposition induced by the Udenfriend system was then investigated. The peptide suppressed this OVA decomposition. The IC_{50} (concentration required to inhibit 50% of OVA decomposition in a 60-min-long radical treatment) of the peptide in suppressing OVA decomposition was 1.79 mM. Similar tests were performed using glutathione (GSH), carnosine (Car), and anserine (Ans), which are known antioxidative peptides, and their antioxidative activity levels were compared with that of LDENIAK. The IC_{50} values of GSH, Car, and Ans were 5.37 mM, 15.68 mM, and 9.19 mM, respectively; LDENIAK was thus the most antioxidative.

Other antioxidative activities of LDENIAK were investigated. No activity was detected in scavenging DPPH or ABTS radicals. However, the peptide was found to chelate copper ions (0.49 mM; EDTA equivalent). Because the carboxyl group of Asp and Glu, which are acidic amino acids, has been reported to be involved in chelating metal ions, the Asp and Glu amino acids in LDENIAK were considered to have contributed to the chelating activity of the peptide.

(3) Elucidation of antioxidative mechanism of LDENIAK

Peptide solution treated in the Udenfriend system for 60 min was hydrolyzed in hydrochloric acid and the amino acid composition was analyzed. The Leu and Ile contents were lower than those before the Udenfriend system treatment. These results suggested that Leu and Ile in the LDENIAK sequence contributed to the antioxidative activity and that their levels were reduced because radicals had oxidatively modified the amino acids.

Here, we revealed the presence of LDENIAK (Leu-Asp-Glu-Asn-Ile-Ala-Lys), a strong antioxidative peptide that suppressed the OVA decomposition induced by the Udenfriend system, in porcine MfP. Our findings suggest that two mechanisms may be involved in manifestation of the antioxidative activity of the peptide, namely 1) Asp and Glu in the sequence of the peptide chelated the iron ions needed for the generation of radicals, and 2) Leu and Ile suppressed the decomposition of OVA by reacting with the radicals while undergoing oxidative modification.

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The effect of Proteinase-activated receptor 2 (PAR-2) agonist on salivary secretion in the mouse and the possibilities as the saliva sample for the enzyme immune assay

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Introduction

Blood derived samples, such as plasma and serum are generally acceptable for detection of various physiological active substances. However, the collected blood volume in mice is confined within approximately 1 ml of whole blood due to their small bodies, even if exsanguination by cardiac puncture or decapitation was performed. In such case, blood sampling for detection of physiological active substances often required sacrifice of animals. In terms of reducing the use of animals, we have been interested in saliva as the sample instead of blood. Compared with blood, saliva can be collected non-invasively, and make it possible to measure the substance at multiple time points. As a result, using saliva samples contributes reduction and refinement in the 3Rs (replacement, reduction, refinement). As one of application examples, we already established stress evaluation method by detecting salivary corticosterone known as a stress marker (Nohara et al., *JVMS* 2016: 78,775-780). Saliva sampling in mice needs salivator injection under anesthesia, and muscarinic agonists such as pilocarpine hydrochloride are generally used to facilitate saliva secretion. On the other hand, recent studies have shown that proteinase-activated receptor 2 (PAR-2) mediate exocrine secretion. Therefore, the PAR-2 agonist is expected to utilize as a new salivator for several muscarinic agonists being contraindicated toward particular diseases (e.g. ischemic heart disease, bronchial asthma, peptic ulcer).

However, there is no report about direct comparison of the effect on salivary secretion between the PAR-2 agonist and pilocarpine generally used as a salivator, and also about utilization of the saliva collected via PAR-2 activation as the sample.

Consequently, we compare the effects of PAR-2 agonist which makes it possible to collect saliva sample in case of muscarinic agonist being contraindicated toward the diseases model animals and the effects of general salivator, pilocarpine in terms of secreted saliva volume. Moreover, we demonstrate stress evaluation using the saliva gathered via PAR-2 activation to estimate its availability as the sample for enzyme immunoassay.

Materials and Methods

Male ICR strain mice aged 8 to 24 weeks were used. Mice were treated according to the provisions for animal welfare of the Nippon Veterinary and Life Science University. Saliva was collected by impregnating a cotton roll with saliva and repeating exchange the cotton with new one on 38 ℃ hotplate. Ten min after intraperitoneal injection of a mixed anesthetic agent of three drugs (medetomidine, midazolam and butorphanol: 0.3, 6.0, 7.5 mg/kg), either of two different salivators was injected intravenously to stimulate salivary secretion. In the study of comparison with the effect of pilocarpine between PAR-2 agonist on the salivary secretion, pilocarpine (0.15～2.4 μmol/kg) or PAR-2 agonist (2-furoyl-LIGRLO amide: 9.4～150 nmol/kg) was used at serial doses. Saliva sampling was conducted for 40 min at 10 min intervals. In the demonstration of stress evaluation, pilocarpine 0.6 μmol/kg or the PAR-2 agonist 75 nmol/kg was administered intravenously for 40 min at 20 min intervals saliva sampling. The saliva sampling was carried out in the pre-stress and post-stress condition,

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and there was a 7 days recovery period from anesthesia between each sampling. Salivary corticosterone levels were measured by enzyme immunoassay.

Results and Discussion

The peak of salivary secretion was at 0～10 min after intravenous injection of both pilocarpine hydrochloride and the PAR-2 agonist. Although pilocarpine induced saliva increase in a dose-dependent manner, the PAR-2 agonist reached a plateau phase at a dose of 75 nmol/kg. This dose of the PAR-2 agonist induced saliva secretion to be equivalent to pilocarpine 0.52 μmol/kg. In the demonstration of stress evaluation, salivary corticosterone levels significantly increased ($p<0.01$) by restraint stress in both group of pilocarpine and the PAR-2 agonist. Moreover, the stress increased salivary corticosterone levels at 3.5-fold in pilocarpine group and at 4.1-fold in PAR-2 agonist group, respectively, and there is no difference between the two groups.

It was confirmed that PAR-2 agonist (2-furoyl-LIGRLO amide) had 6.7-fold titer compared with pilocarpine by molar ratio and induced sufficient salivation for use as the sample. In addition, the either saliva mediated via PAR-2 or via muscarinic mechanism would be available as the sample for stress evaluation by detecting corticosterone levels. This result indicates that saliva collected via PAR-2 activation can be used as the sample for enzyme immunoassay.

To examine the physiological active substances at multiple time points in mice, there are huge advantages of using the saliva sample. It is well known that pilocarpine can occur some kinds of side effects caused by acting systemically including central. Therefore, there are some considerable risks that repeated administration of pilocarpine for saliva sampling might induce several side effects. Since the pharmacological effect of the PAR-2 agonist disappears immediately, this agonist might be expected to avoid these side effects. The results of this study also imply the contribution to the 3Rs in animal experiments, in addition to the effects of PAR-2 agonist on saliva secretion.

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Studies on methods for DNA preparation from recombinant *Pichia Pastoris* and copy-number analyses of their integrated genes

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Introduction

Yeast fungi have been used for fermentation of alcoholic beverages such as beer and wine, bread etc. In the 1960s, yeasts that were capable of producing high-quality protein using petroleum as the sole carbon source were discovered. They were developed to be used as food, "petroleum protein", for practical use in coping with the future food crisis. However, due to inflation of oil price with oil shocks, the application of "petroleum protein" has been discontinued.

Pichia pastoris is one of a series of esculent yeast that can recycle petroleum. *Pichia pastoris* has strong proliferating power and high protein production capacity. It is also reported recently that they could possibly produce proteins of eukaryotic complex structure while maintaining its structure.

This research aims to establish a recombinant protein production system using this *Pichia pastoris* and to make inexpensive and easy production of useful proteins by genetic engineering. Our laboratory already constructed a proprietary expression vector system for recombinant protein production in *Pichia pastoris* and verified the utility of this vector system by using the fluorescent protein (GFP) of the Aequorea vulgaris and its expression analysis. Since GFP protein fluoresces only by excitation light irradiation and does not require an enzymatic reaction to form a transgene because it has low cytotoxicity and easy to distinguish gene expression. In order to increase expression amounts of target protein, GFP expression gene is tandemly arranged, and introduced into *Pichia pastoris*. An increase in GFP protein expression level by polymerization of the expression gene was confirmed, but the number of copies of the expression gene introduced into the cell has not been estimated yet. In this report, we describe a simple method for preparing DNA from *Pichia pastoris* in which GFP protein expression was confirmed, and a method for efficiently measuring intracellular copy number.

Materials

Bacteria: *Pichia pastoris* GS115 stain (purchased by life technology)

A GFP expression vector fragment (containing 1 to 5 tandem GFP expressing gene fragments, 5 in total) was introduced by electroporation, and a strain in which GFP protein expression had been confirmed was used for DNA extraction.

Methods

<DNA Extraction Method/Purification Method and Confirmation>

Total DNA extraction was performed with GFP expressing stains cultured on YPD plates at 30 ℃ for 2 days using ISOPLANT DNA extraction kit (Nippon Gene Co., Ltd.). Further DNA purification was carried out using Fast Gene Gel/PCR Extraction kit (Japan Genetics Co., Ltd.), and purity was confirmed by agarose gel electrophoresis.

<Copy number measurement using quantitative PCR>

Quantitative PCR was performed using Eco-Real-time PCR system (Illumina). The *URA3* gene (one copy per genome) was used for standard genome copy-number. Copy number of the *GFP* gene of the sample DNA sample were measured using a plasmid into which one

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GFP expression gene was introduced was used as a GFP copy-number standard.

<Calculation of genomic DNA concentration and estimation of genome copy number>

DNA amounts were calculated by absorbance measurement (A_{260}) and gel-image analysis of agarose gel electrophoreses by ImageJ, and genome copy numbers were estimated based on them.

For image analysis by ImageJ, analysis was performed form electrophoresis photographs used for purified DNA confirmation. The concentration of purified DNA was estimated using 1 kbp ladder markers of which concentration were cited as controls. The genome copy number per 1 mL sample was obtained by dividing the DNA concentration (μg/ml) by the genome weight. Since the genome size of *Pichia pastoris* is 9.43 Mbp, 9.43×10^{-9} was calculated as the weight (μg) of the genome per cell.

Results and discussion

There was no significant difference between the concentration of DNA measured at A_{260} and that obtained by ImageJ analysis (Table 1).

Table 1 Genomic DNA concentration and copy-number (copies/mL) of the samples

	Sample	GFP(1)	GFP(2)	GFP(3)
A_{260}	Conc. (μg/mL)	58.67	57.16	74.20
	Copy-numbers	6.2×10^9	6.1×10^9	7.9×10^9
ImageJ	Conc. (μg/mL)	57.12	67.59	81.99
	Copy-numbers	6.1×10^9	7.2×10^9	8.7×10^9

GFP(4)	GFP(5)	GS115
69.79	72.15	41.16
7.4×10^9	7.7×10^9	4.4×10^9
67.76	61.04	41.16
7.2×10^9	6.5×10^9	4.4×10^9

In the copy-number analysis by quantitative PCR, the copy-numbers of *GFP* expression genes were lower than that of the *URA3* (one per gene) gene (Table 2). The reason for this result is that amplification of *URA* gene by quantitative PCR was less quantitative, since linearity of the standard curve of the *URA3* gene upon dilution was not enough. The amplification efficiency might be slightly changed between the selected sequences of the PCR amplification. It might be also necessary to test the dilution buffers, since the plastic tubes very well absorb DNA fragments especially in highly diluted samples.

Table 2 Copy-numbers of GFP Gene introduced in the genomes by Quantitative PCR

	GFP(1)	GFP(2)	GFP(3)
URA3	1.4×10^8	1.2×10^8	8.7×10^7
EGFP	1.9×10^7	3.6×10^7	3.0×10^7

GFP(4)	GFP(5)	GS115
3.5×10^8	7.6×10^8	3.3×10^8
6.4×10^7	7.9×10^7	

Although it was not able to measure the exact number of *GFP* genes in the genomes in this report, it is thought that more accurate copy number measurement would be possible by adjusting dilution methods in our quantitative PCR condition.

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Taste properties of butter contributing to *koku* enhancement

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Objectives

Butter is a processed food made from butterfat. As an ingredient of confectionery and dishes, it is well known to add a distinct flavor, provide richness (*koku*), and improve the taste of food. However, it is poorly understood how the taste properties of butter enhance the *koku* of food.

In this study, we conducted a sensory evaluation of the taste properties of butter and analyzed its composition and flavor components. We discuss the relationship between sensory evaluation and composition with the aim of gaining a better understanding of the role of butter in *koku* enhancement.

Materials and methods

1. Preparation of samples: Unsalted butter was melted and separated into aqueous (serum) and fat (AMF: anhydrous milk fat) fractions by centrifugation.
2. Sensory evaluation: Words that expressed the properties of butter were extracted via a quantitative descriptive sensory analysis by trained panelists. The words were then used to perform a sensory evaluation (n=16).
3. Analysis of free amino acids: Proteins were removed from the serum by adding the same quantity (v/v) of 3% sulfosalicylic acid. Free amino acids in the serum were then analyzed.
4. Analysis of fatty acid composition: The fat fraction was saponified and derivatized. Gas chromatography was performed to analyze the fatty acid composition.
5. Analysis of flavor components: Volatile components emitted from the specimens were captured in a MonoTrap (GL Sciences Inc.) and desorbed with diethyl ether or dichloromethane. The components were then condensed and analyzed by using gas chromatography –olfactometry (GC-O) and gas chromatography– mass spectrometry (GC-MS).

Results and discussion

1. Explication of taste properties by sensory evaluation

By using a quantitative descriptive sensory analysis, 12 terms in total were extracted to explain the taste properties of butter. They were "flavor of butter," "sweet smell," "flavor of milk," "smell of oil and fat," "spreading flavor," and "persistent flavor," for scent, and "melting in the mouth," "sticky," "sticking around the tongue," "lingering on the hard palate," "texture of fat," and "hardness of fat" for food texture. We used these terms to perform a sensory evaluation to investigate the taste properties of butter. The unsalted butter used as samples did not have a taste but was shown to have "sweet," "milk-like," and "butter-like" scents, which were persistent and spreading. As a descriptor of the texture of butter, there was a strong feeling of "lingering on the hard palate." The sensations of "spreading flavor," "persistent flavor," and "lingering on the hard palate," which were likely to be *koku* enhancers, decreased when the serum (i.e. the aqueous fraction of butter) was removed; thus serum was important for the taste properties of butter and for *koku* enhancement.

2. Components of butter

1) General composition

The general compositions of butter, AMF, and serum were analyzed. Butter consisted of 16.1% moisture, 82.7% fats, 0.6% proteins, and 0.5% carbohydrates. AMF was 100% fats. On the other hand, serum contained 90.7% moisture, 1.6% fats, 3.0% proteins and 4.1% carbohydrates. When the butter was fractionated, all components besides fats were transferred to the aqueous fraction.

2) Free amino acids

Various kinds of free amino acids were detected in the aqueous fraction (serum) of butter. Taurine, glutamic

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acid, and glycine were especially abundant. All free amino acids were present at concentrations below the threshold values for taste; this was in agreement with the comment that "unsalted butter was tasteless" in the sensory evaluation.

3) Fatty acid composition

Comparison of fatty acid compositions among butter, AMF, and serum showed no great differences.

In all samples, large amounts of palmitic acid, oleic acid, stearic acid, and myristic acid were detected. The samples also contained lower fatty acids and unsaturated fatty acids, suggesting that lower fatty acids were involved in scent formation in butter and unsaturated fatty acids contributed to the generation of aldehydes.

3. Analysis of flavor components contributing to properties of butter

Because scent contributes greatly to the taste properties of butter, the characteristics of butter scents were analyzed by using GC-O. From butter, "grassy," "sweet," "citrus-like," "sour," and "foot sole-like" scents were detected. The frequency of detection of a "sweet" scent was low in AMF. In serum, the detection frequency of "grassy" scent was low, whereas those of "sweet" and "milk-like" scents were high. These results agreed with our findings in the sensory evaluation that the aforementioned scents were weak in AMF, which was prepared by removing serum from the butter.

We conducted GC-MS to identify the substances responsible for the scents. The estimated candidate odorants for the "grassy," "sweet," and "citrus-like" scents were, respectively, 1) n-nonadecanol-1, 2) acetic acid, chloro-, octadecyl ester, and 3) oleyl alcohol, methyl ether. Candidate odorants for the "foot sole-like" scent were butanoic acid and 3-methyl-butanoic acid; and those for the "sour" scent were acetic acid and hexanoic acid.

The results indicated that serum –the aqueous fraction of butter– was important in providing and enhancing *koku*.

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Copy-number analysis of an expression vector for *Lactococcus lactis*; Its relation with cell-growth

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Introduction

Lactic acid bacteria have environmental advantages to other bacteria by producing organic acids and bacteriocin. This anti-microbial ability is traditionally applied for food preservation. Therefore, the lactic acid bacteria are indispensable in broad areas of fermented food production.

Lactic acid bacteria are generally known for their roles as "good bacteria" in small intestine and clone. They are believed to have abilities of improving digestive health by preventing growth of other harmful bacteria in intestine and colon with their antimicrobial action during staying in the organ for a certain period.

In recent years, lactic acid bacteria also induce positive effect on the intestinal immune system. They are supposed to raise immune resistances to virus, and reduce allergies in our body.

Lactococcus lactis is a homo-fermentative bacterium that is used to produce fermented milk products such as buttermilk, fermented butter, many varieties of soft and hard cheeses, the primary function of *Lactococci* is rapid lactic acid production from lactose that leads to the preservation of the otherwise quickly spoiled milk. In secondary processes, the bacterial culture contributes to both flavor and texture of the product. Flavor can be generated by the production of carbon source-derived aroma compounds, like diacetyl, or by the degradation of milk proteins or fats into specific flavor compounds or their precursors. Almost 150 years ago Louis Pasteur was the first to recognize that lactic acid fermentation is caused by bacteria. In terms of microbiology history, it is noteworthy that *Lactococci* were the first bacteria ever that were isolated in pure culture by especially because of their economic importance, and now these bacteria have been intensely investigated and characterized.

In the past 3 decades, progress has been made in the development of genetic engineering tools and the molecular characterization of *Lactococci*. The tools include electroporation, the availability of various vectors, gene targeting, gene knockout, fusion genes, and constitutive or regulated gene expression systems. Furthermore, the availability of an easy-to-operate and strictly controlled gene expression system has been crucial for the development of many of these applications.

This study's final goal is to produce peptide vaccines by *Lactococcus lactis* to induce immunogenic reaction against cancer or Alzheimer's disease etc. via intestinal immune systems, using genetic engineering technology.

There are two methods of recombinant protein production. One is, by integrating a target gene of interest into genome, and the other is using plasmid vectors. The former method can introduce only few genes into cells, hence the target gene products are often produced in small amount. On the other hand, latter method can introduce many copies of the target genes since the plasmid vectors usually exist in multi-copies in the cells. As for quantitative production of target proteins by genetic engineering, using plasmid vectors are great advantages over genomic integration systems.

In this report, analyses of the copy-numbers of the expression vector, pNZ8148, for *L. lactis*, and copy-number difference with cell stage were described. The copy-number differences in cell stages is one of crucial factors when foreign proteins are produced by this vector system.

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Materials and Methods

Bacterial strains: Lactic acid bacteria NZ9000-*pepN::nisRnisK* (NZ9000) as the final plasmid host was purchased from Mo Bi Tec (Germany). *E. coli* MC1061 (MC1061) for preparation of the vector plasmid was from National Institute of Genetic materials.

Plasmid: pNZ8148 (See Fig. 1) was from Mo Bi Tec (Germany).

Culture medium: LB medium (Difco Laboratories) was used for culturing MC1061. MRS medium (Difco laboratories) was for the cultivation of NZ9000.

<Plasmid preparation from both strains>

pNZ8148 was introduced into NZ9000 by electroporation. The plasmid was also into MC1061 by the calcium chloride method.

Selection of transformants was performed on chloramphenicol (cm) (10 mg/mL) –containing medium. The plasmid was prepared from the transformed NZ9000 and MC1061 with the alkaline lysis silica membrane method kit (Nippon Genetics).

<Preparation of total DNA from the cells>

Cultured cells were corrected by centrifugation. The cells were then treated by Isoplant total DNA preparation kit (Nippongene). Then the DNAs were further purified by DNA purification kit using silica membrane (Nippon Genetics). Purity of DNAs were analyzed by multi wave-length scanning with a spectrometer (Nanodorop).

<Copy-number measurement of pNZ8148 of different cell stages>

The Total DNAs were prepared from cells of early, middle, and stationary cell-stages. Their DNAs were analyzed by agarose gel electrophoresis. The electrophoresis patterns were scanned with Image J and analyzed by its software. The copy-number of the plasmid in each stage was calculated from the area sizes of the bacterial genome and plasmid DNA in the graph, and from their DNA molecular weight. The copy numbers were also analyzed by quantitative PCR. The primer sequences were assigned from the genome and plasmid sequences.

Results and Discussion

<Copy number measurement of lactic acid bacteria expression vector pNZ8148>

In the agarose gel analysis, the plasmid copy numbers were calculated to be approximately 100 copies per cell in each stage, and significant differences were not observed.

In the analysis by agarose gel, higher molecular plasmid bands were detected. This strongly suggests that in early stage the plasmid would be highly polymerized, and those polymers were gradually processed to be a monomer. The final plasmid in the stationary phase consequently existed as the monomer plasmid.

Using quantitative PCR, those copy-numbers were also analyzed. In PCR, circular plasmids usually cannot be effectively used as templates. They need to be completely denatured before PCR amplification. The total DNA sample from each stage was intensively heat-denatured before analysis. However, denaturation of the plasmid was not well enough in our condition, since the copy numbers were calculated to be lower than copy numbers of genome. Thus, when using quantitative PCR, our plasmid denaturation condition must be reconsidered, or plasmid should be linearized by restriction enzymes before analysis.

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Mechanisms of obesity in the obese mouse induced by administration of Monosodium Glutamate (MSG) overdose

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Introduction

Glutamic acid is known as a typical "umami" component and monosodium glutamate (MSG) is widely accepted as a seasoning in today's society. It has previously been reported that glutamic acid is a single amino acid which has an important role as an excitatory neurotransmitter *in vivo* and that it also shows excitotoxicity and destroys neural cells when glutamate is overdosed to nerve cells of culture system. Normally, MSG cannot pass through the blood brain barrier, but that of the neonates is defective. MSG is transferred to the central nervous systems and destroys the brain's feeding-related center in cases where an excessive amount of MSG is administered to newborns such as mice. As a result, it has been reported that it is possible to make overeating obese mice. In our laboratory, MSG obese mice were used to examine the differences of mouse strains, differences of sex and also examine relationship between these factors and the degree of obesity. But it has not been studied the minimum dose of MSG for preparing an obesity model by MSG administration. So in this first study, we examined the minimum dose of MSG obesity in male and female. Next, in order to detect the factor of obesity except overeating, we measured locomotor activities and body temperature as parameters of basal metabolism, and studied the relationship between the above factors and obesity. Furthermore, in order to clarify whether obesity by MSG administration is caused by glutamate itself, an antagonist (MK-801) of N-methyl-D-aspartic acid (NMDA) receptor which is one of glutamate receptor subtypes was administered at the same time as MSG, and after that we examined the body weight change.

Materials and Methods

We used ddY mice in all studies and a MSG solution or saline was injected subcutaneously to the neonate mice at birth. Body weight was also measured once a week in all studies. At the experiment of examining the minimum dose, each male and female mouse was classified into 4 groups: 2 mg/g of MSG, 1 mg/g of MSG, 0.5 mg/g of MSG and 50 mM-PBS (control) treated gropus. Only the male mice were measured for food intake for one week at the age of 9 weeks. Sampling was taken at the age of 10 weeks for males and at the age of 16 weeks for females and blood glucose level and fat weight were also measured, respectively. At the experiment of the factor of obesity except overeating, male mice were classified into 3 groups: 2 mg/g of MSG, 1 mg/g of MSG and control. Locomotor activities were measured at 11 to 14 weeks of age, and body temperature was measured after 16 weeks of age. We measured body temperature in two measurements: one for basal value (once a day) and the other for change in body temperature after administration of Medetomidine Hydrochloride (sedative) and Atipamezole Hydrochloride (antagonist of Medetomidine Hydrochloride). At the antagonist simultaneous administration test, a mixed solution with MSG (1 mg/g) was administered using the NMDA receptor antagonist MK-801 (0.25 μg/g, 0.125 μg/g). Experimental groups were classified into 4 groups: high dose of MK-801, low dose of MK-801, obesity control 1 mg/g of MSG and normal control 50 mM-PBS.

Results and Discussion

At the experiment of MSG minimum dose, there was no significant difference between the 0.5 mg/g group and the control group in all examination items of the

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male and female. The 2 mg/g group in males showed significantly higher value than the control group in body weight, food intake, blood glucose level and fat weight. The 2 mg/g group in females showed significantly higher value than the control group in body weight and fat weight but no significant difference was found in blood glucose level. From these results, it was shown that males exhibit obesity accompanied by overeating and diabetes, and females exhibit obesity without diabetes, whose results agree with those reported in our laboratory in the past. On the other hand, the 1 mg/g group showed a significantly higher value than the control group in body weight and fat weight, but no significant difference was found in food intake and blood glucose level in both sexes. From the results, it was shown that the minimum dose of MSG for preparing MSG obesity model is the 1 mg/g treated group in both sexes, and the 1 mg/g group model shows obesity without overeating and diabetes.

In the locomotor activity and body temperature measurement tests, the 2 mg/g group showed a significantly lower value than the control group in all of the following measurements: the number of wheel turns, basal body temperature, and body temperature recovery after the administration of Atipamezole. This revealed that the obesity factors of MSG obesity model are involved in a decrease in locomotor activity and in basal metabolism including temperature regulation, in addition to overeating. And the 1 mg/g model showed a significantly lower value than the control group in the basal body temperature but no significant difference in the number of wheel turns and body temperature changes after the administration of Medetomidine/Atipamezole. Furthermore, the body weight of the 1 mg/g model gradually decreased from the start of measurement of locomotor activity and the significant difference from the control group disappeared, but in the home cage after the measurement, the body weight was significantly increased, again. From this, there is a possibility that it is different from the pure spontaneous locomotor activity in the home cage, since wheel turning activity involves motivation to rotate a wheel. Obesity is improved by adding exercise load in the 1 mg/g model, and it can be concluded that the clinical condition is reversible and therefore the 1 mg/g model is closer to human and thus is useful for studying factors of obesity.

In the antagonist simultaneous administration test, the high dose of MK-801 group showed a significantly lower values of body weight than the MSG 1 mg/g obese group. From this result, it was found that obesity caused by MSG can be prevented by simultaneous administration of MK-801. It was also suggested that the toxicity of MSG in MSG obesity model was due to glutamic acid itself via NMDA receptor, not sodium added to glutamate.