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The effects of type VI collagen on the bone formation

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Chapter 1: Introduction

Type VI collagen (Col VI) is a component of the extracellular matrix (ECM) in the periosteum and thought to regulate osteoblast behaviors. Several *in vitro* studies indicate that osteoblast-lineage cells required attachment to Col VI at early stages of differentiation. In addition, Col6a1-deficient mice displayed a reduction in bone mineral density and cancellous bone mass, and an aberrance of the collagen arrangement of the cortical bone. Therefore, Col VI is suggested to play an important role in normal bone formation during fetal and postnatal development. In several cell types, Col VI interacts with Neural/Glial Antigen 2 (NG2) on the cytoplasmic membrane to promote cell proliferation, spreading, and motility. However, the detailed functions of Col VI on the bone formation are still remained unclear.

The aim of this entire study is to clarify the functions of Col VI on behaviors of the osteoblast lineages and the bone formation. First of all, I propose to elucidate the spatiotemporal relationship between Col VI and osteoblast lineages expressing NG2 in the ossifying region, such as the periosteum and the groove of Ranvier (GOR) in the rat long bones during postnatal growing periods. I next investigated the effects of Col VI-NG2 interaction on the cellular behaviors of osteoblast lineages using cultured osteoblast lineages isolated from rat calvariae.

Chapter 2: Accumulation of type VI collagen in the primary osteon of the rat femur during postnatal development

In rodents, the long bone diaphysis is expanded by formation of primary osteons at the periosteal surface of the cortical bone. This ossification process is thought to be regulated by the microenvironment in the periosteum. Col VI is a component of the ECM in the periosteum and involved in osteoblast differentiation at early stages. However, the detailed functions of Col VI and NG2 in the ossification process in the periosteum are still under investigation. In this chapter, to clarify the spatiotemporal relationship between Col VI-NG2 interaction and formation of the primary osteon, I examined the distribution of Col VI and osteoblast lineages expressing NG2 in the periosteum of rat femoral diaphysis during postnatal growing periods by immunohistochemistry. Primary osteons enclosing the osteonal cavity were clearly identified in the cortical bone from 2 weeks of age. The size of the osteonal cavities decreased from the outer to the inner region of the cortical bone. In addition, the osteonal cavities of newly formed primary osteons at the outermost region started to decrease in size after rats reached the age of 4 weeks. Immunohistochemistry revealed concentrated localization of Col VI in the ECM in the osteonal cavity, but not in the osteogenic layer of the periosteum. Col VI-immunoreactive areas were reduced and disappeared as the osteonal cavities became smaller from the outer to the inner region.

In the osteonal cavities of the outer cortical regions, Runt-related transcription factor 2 (RUNX2) -immunoreactive spindle-shaped cells and mature osteoblasts were detected in Col VI-immunoreactive areas. The numbers of RUNX2-immunoreactive cells were significantly higher in the osteonal cavities than in the osteogenic layers of the periosteum from 2 to 4 weeks. Most of these RUNX2-immunoreactive cells showed NG2-immunoreactivity. Furthermore, PCNA-immunoreactivity was detected in the RUNX2-immunoreactive spindle cells in the osteonal cavities. These results indicate that differentiation and proliferation of the osteoblast lineage occur in the Col VI-immunoreactive area. Thus, Col VI may provide a

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characteristic microenvironment for regulation of the osteoblast lineage behavior in the osteonal cavity of the primary osteon. Interaction of Col VI and NG2 may be involved in the structural organization of the primary osteon by regulating osteoblast lineages.

Chapter 3: Distribution of type VI collagen in the Groove of Ranvier during rat postnatal development

The Groove of Ranvier (GOR) is thought to be the ossification area situated around the growth plate cartilage. The inner layer of the GOR consists of mature osteoblasts on the surface of the bone bark, thin trabecular bone of the epiphyseal tip of the cortical bone, around the epiphyseal growth plate. In addition, the middle layer containing undifferentiated mesenchymal cells provides mesenchymal stem cell niche. These lines of evidence indicate that early-stage osteoblast lineages differentiate to mature osteoblasts in the GOR, which form new bone tissues at the epiphyseal region of the cortical bone, resulting in longitudinal growth of the cortical bone. In this chapter, to clarify the spatiotemporal association of Col VI with osteoblast differentiation in the GOR, I examined the distribution of Col VI and osteoblast lineages expressing NG2 in the rat tibia proximal end during postnatal growing periods by immunohistochemistry. Col VI-immunoreactivity was detected in the upper middle layer, but not in the inner and lower middle layer. RUNX2 + / Osterix (OSX) − osteoblast lineages were detected in Col VI-immunopositive areas. However, RUNX2 + / OSX + mature osteoblasts were only found in the Col VI-immunonegative area. Most of the RUNX2 + cells showed NG2-immunoreactivity. These findings indicate that Col VI provided a characteristic microenvironment for differentiation of the osteoblast lineages prior to terminal differentiation in the GOR. Col VI may regulate the differentiation by interaction with NG2 expressed on the osteoblast lineages.

Chapter 4: The effects of type VI collagen on the osteoblastic behavior

The results of Chapter 2 and 3 raised the possibility that Col VI interaction regulates proliferation and differentiation of the osteoblast lineages prior to terminal maturation to the mature osteoblast producing bone matrix. In this chapter, to address this hypothesis, I investigated effects of Col VI on the behaviors of osteoblast lineages using osteoblasts isolated from rat calvariae cultured on Col VI-coated dish. The proliferation of the osteoblasts was significantly decreased on Col VI-coated dish compared to cells on non-coated dish. In the migration assay, Col VI enhanced haptotaxis and motility of the osteoblasts. In the examinations of expression of differentiation markers by quantitative real time RT-PCR, OSX mRNA was decreased in the osteoblasts on Col VI-coated dish at 10 and 15 days after differentiation induction (Day 10 and 15), whereas RUNX2 mRNA expression was not affected during the entire culture period. Expressions of osteocyte markers, such as Dentin matrix protein 1, Sclerostin, and Receptor activator of nuclear factor kappa-B ligand, were significantly decreased in the osteoblasts on Col VI at Day 15. As for bone matrix production, Osteocalcin mRNA expression and mineralization were significantly inhibited in the osteoblast on Col VI at Day 10 and 15.

However, Osteopontin (OPN) mRNA was significantly increased in the osteoblast on Col VI at Day 5 and 10. In the differentiation process of osteoblast lineages, OSX promotes differentiation of the osteoblast lineage at the later phases, and OPN is a negative-regulator of osteoblast proliferation, differentiation, and mineralization. Thus, the results of this study indicate that Col VI suppresses differentiation of the osteoblast lineage and mineralized bone matrix production especially at later phases via inhibition of OSX expression and increase of OPN expression.

Chapter 5: Interactions between Notch1 and DLL1 in the rat femur primary osteon during postnatal development

Notch signaling is one of major negative regulators in osteoblast differentiation and bone formation by inhibition of RUNX2 transcriptional activity. The results of Chapter 4 demonstrated inhibition of differentiation of the osteoblast lineage by Col VI, indicating that Notch signaling is involved in the inhibition pathway induced by Col VI. However, the detailed functions of Notch signaling in the Col VI-associated regulation of the osteoblast lineage are still under investigation. Furthermore, it is also unclear whether Notch signaling regulates the primary osteon formation. In this chapter, to clarify the spatiotemporal relationship between Notch signaling and formation of the primary osteon, I examined the distribution of osteoblast lineages expressing Notch1, activated Notch1 (NICD) and Delta-like ligand 1 (DLL1) in the periosteum of rat femoral diaphysis during postnatal growing periods by immunohistochemistry. I also examined the expressions

of Notch1 and DLL1 mRNA in osteoblasts on Col VI-coated dish to determine whether Col VI regulates Notch signaling in the osteoblast lineage using primary culture of osteoblasts. Immunohistochemistries of the primary osteon revealed that Notch1, NICD, and DLL1 were restricted to the RUNX2-positive osteoblast lineages in the osteonal cavity. Thus, Notch signaling may associates with formation of the primary osteon via down-regulation of the osteoblast differentiation. In the cultured osteoblast lineages on Col VI, Notch1 and DLL1 mRNAs are significantly increased at Day 5 compared with control culture. These findings indicate that Col VI stimulates Notch1 and DLL1 expression in the immature osteoblast, causing the inhibition of osteoblast differentiation.

In conclusion, this study revealed that Col VI provide characteristic microenvironment for the ossification of the cortical bone in the primary osteon and the GOR. Immature osteoblast lineages were detected in the Col VI positive area, while mature osteoblasts in the Col VI negative area, indicating that Col VI regulates differentiation of the osteoblast lineages prior to terminal differentiation. These osteoblast lineages express NG2, indicating that the interaction between NG2 and Col VI may regulate the differentiation of osteoblast lineages. Additionally, *in vitro* study indicates that Col VI inhibits osteoblast maturation/differentiation and bone matrix production via inhibition of OSX expression, and increase of OPN expression and Notch signaling. These findings indicate that Col VI inhibits osteoblast differentiation, leading to regulation of cortical bone formation in the primary osteon and the GOR during postnatal growing periods.

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Studies on the development of resistance to imatinib in canine mast cell tumor

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Canine mast cell tumor (MCT) is one of the common skin tumors and accounts for 20 % of all canine cutaneous tumors. Chemotherapy, using vinblastine and/or lomustine is sometimes needed in the treatment of MCT, especially in the cases of advanced clinical stage and/or high grad of tumor. Recently, in addition of these chemotherapeutic agents, KIT-targeted kinase inhibitor imatinib came to be used for the treatment of MCT in dogs.

KIT is a type III receptor tyrosine kinase encoded by *KIT*. By binding with its ligand stem cell factor (SCF), it is phosphorylated and activates downstream signal transduction, leading proliferation, migration, maturation and survival of cells. Approximately 26 % of canine MCT have *KIT* mutations. These mutations cause a constitutive auto-phosphorylation of KIT, resulting in the neoplastic growth of mast cells.

Imatinib binds to the ATP-binding site within KIT and suppresses phosphorylation of this receptor. Therefore, imatinib shows therapeutic activity in canine cases of MCT with *KIT* mutation. However, they eventually develop resistance to imatinib in the course of treatment. Although this is a crucial issue in the treatment of MCT in dogs, the molecular mechanisms of acquiring of resistance to imatinib have not been clarified.

The purpose of this study is to clarify the molecular mechanisms of imatinib resistance in canine MCT. Firstly, nucleotide sequences of *KIT* were analyzed using tumors collected from the MCT cases that acquired resistance to imatinib. Secondly, imatinib resistant MCT sub-lines were established from the imatinib sensitive MCT cell lines and characterized their biological changes. Finally, using these sub-lines, molecular mechanisms of imatinib resistance were investigated.

1. Analysis of nucleotide sequences of *KIT* using tumors collected from the cases of MCT that acquired resistance to imatinib

Nucleotide sequences of *KIT* were analyzed using tumors collected from six cases of MCT that acquired resistance to imatinib. All dogs had a primary mutation in *KIT*. A second mutation (c.2463T＞A, p.Asp815His) in *KIT* was found in a tumor in one dog. In contrast, other five cases had no second mutation in *KIT*. A same substitution mutation at the corresponding residue (p.Asp816His) has been found in imatinib resistant human GIST. Moreover, phosphorylation of the human mutant KIT (p.Asp816His) has been shown not to be suppressed by imatinib. Therefore, the second mutation (c.2463T＞A, p.Asp815His) could be associated with imatinib resistance in this dog. In the five dogs that did not have second mutation in *KIT*, it could be possible that molecular mechanisms other than second mutation in *KIT* play a crucial role in the acquiring of resistance to imatinib.

2. Establishment and characterization of imatinib resistant mast cell tumor sub-lines

Using imatinib-sensitive canine MCT cell lines CoMS (IC50; 0.04 μM) and VI-MC (IC50; 0.27 μM), imatinib resistant sub-lines rCoMS1 (IC50; 9.0 μM, from CoMS), rVI-MC1 (IC50; 1.86 μM, from VI-MC), and rVI-MC10 (IC50; 12.2 μM, from VI-MC) were established by culturing in increasing concentrations of imatinib.

In rCoMS1, overexpression of KIT and its phosphorylation was observed. Phosphorylation of the overexpressed KIT was not suppressed by imatinib, suggesting association of overexpression of KIT and

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imatinib resistance. Both in rVI-MC1 and rVI-MC10, a second mutation of *KIT* (c.2443G > C) that located in exon 17 was identified. The phosphorylation of KIT was not suppressed by 1 μM but suppressed by 10 μM of imatinib in both rVI-MC1 and rVI-MC10. From these findings, it was suggested that the second mutation in *KIT* contributed to the resistance to 1 μM of imatinib. However, it was considered that other molecular mechanisms underlie the imatinib resistance in rVIMC10.

3. Molecular mechanisms of acquiring resistance to imatinib in CoMS

Association between overexpression of KIT and imatinib resistance was investigated using rCoMS1. From the protein synthesis inhibiting study, it was shown that increase of KIT expression on rCoMS1 was caused by reduced turnover of KIT with prolonged half-life. Moreover, this decrease of KIT turnover was caused by decrease of ubiquitination of KIT triggered by imatinib.

KIT overexpressed on rCoMS1was decreased by culturing in the absence of imatinib. This down-regulated KIT was re-upregulated by re-culturing in the presence of imatinib. In consistent with KIT expression status, rCoMS1 represented changes of imatinib sensitivity; namely, rCoMS1 with decreased expression of KIT was sensitive to imatinib but that re-upregulated expression of KIT was insensitive to imatinib.

From these findings, overexpression of KIT on rCoMS1 was considered to be caused by retardation of KIT degradation via inhibition KIT ubiquitination by imatinib and to play a crucial role in resistance to imatinib in this cell line.

4. Molecular mechanisms of acquiring resistance to imatinib in VI-MC

The effects of the second mutation (c.2443G > C) in *KIT* on the phosphorylation status of KIT and its sensitivity against imatinib were examined using recombinant mutant KIT expressed 293 cells. Regardless of the presence or absence of primary mutation, the mutant KIT harboring the second mutation showed ligand-independent phosphorylation that was not suppressed by imatinib. This indicates that the second mutation in *KIT* is responsible for the imatinib resistance in rVI-MC1 and rVI-MC10.

Despite of carrying a same second mutation, tolerability against imatinib was different between rVI-MC1 and rVI-MC10. Therefore, phosphorylation status of KIT downstream signaling proteins ERK, AKT and STAT3 was then examined. ERK was constitutively phosphorylated in both cell lines. This phosphorylation was suppressed by 10 μM of imatinib in rVI-MC1 but not in rVI-MC10. ERK phosphorylation in rVI-MC10 was also not suppressed by KIT/SFK inhibitor. KIT/SFK-independent activation of ERK would be involved in imatinib resistance in rVI-MC10.

In summary, the frequency of second mutation in *KIT* could be low in imatinib resistant canine MCT. Overexpression of KIT is one of a cause of acquiring resistance to imatinib in the cases without the second mutation in *KIT*. In the cases that have a second mutation in *KIT*, the second mutation was suggested to be a cause of the resistance to imatinib. Moreover, KIT/SFK-independent activation of ERK would be involved in imatinib resistance when the neoplastic cells are exposed to higher concentrations of imatinib.

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Studies on the diagnosis and treatment of canine Cushing's disease

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Cushing's disease is a common endocrine disorder in dogs. Approximately 80 ～ 85 % of Cushing's disease cases in dogs are due to Cushing's disease resulting from an adrenocorticotropic hormone (ACTH) -secreting pituitary adenoma. However, if the pituitary tumor grows, it becomes to have a detrimental phase as an intracranial space-occupying lesion. Therefore, magnetic resonance imaging (MRI) should be performed upon diagnosis and the treatment should be selected accordingly. For dogs, medication to control excessive cortisol secretion is the current treatment of choice, whereas surgery is the first-line treatment for humans with Cushing's disease. Radiation therapy is also available in veterinary medical science. Among these treatments, transsphenoidal hypophysectomy is the only option for radical cure. However, the indications for transsphenoidal hypophysectomy in dogs have not yet been clarified, and the selection of this technique is mainly at the surgeon's discretion. Therefore, it is important to determine the indications for transsphenoidal hypophysectomy so veterinarians can provide owners with a surgical prognosis. Furthermore, if immunohistological examinations of somatostatin receptor (SSTR) and dopamine D2 receptor (DA2R) in ACTH-secreting pituitary adenomas were available, somatostatin analogs and dopamine agonists, which are reportedly effective against ACTH-secreting pituitary adenomas in humans, might be available when incomplete resection or recurrence would occur in dogs treated with transsphenoidal hypophysectomy.

This study clarified the indications for transsphenoidal hypophysectomy by devising a new classification system reflective of the morphological characteristics of ACTH-secreting pituitary adenomas. Additionally, we determined the SSTR and DA2R expressions in ACTH-secreting pituitary adenomas. Furthermore, bone morphogenetic protein 4 (BMP4) and bone morphogenetic protein receptor (BMPR) expression in ACTH-secreting pituitary adenomas, which is reported to be involved in the mechanism of somatostatin analogs, was also determined.

1. An MRI-based classification system for determining indications for transsphenoidal hypophysectomy in canine pituitary-dependent hypercortisolism

This study aimed to establish a new MRI-based classification system for canine Cushing's disease according to pituitary tumor extent to determine the indications for transsphenoidal hypophysectomy and clarify the prognosis at each disease grade.

We developed a five-point classification system (Grades 1 ～ 5) based on tumor extension in the dorsal and craniocaudal directions. Grade 1, no tumor extension beyond the dorsum sellae; Grade 2, tumor extension beyond the dorsum sellae up to the third ventricle but no contact with the optic chiasm or mammillary body; Grade 3, tumor extension beyond the dorsum sellae up to the third ventricle plus contact with the optic chiasm and/or mammillary body but not the interthalamic adhesion; Grade 4, tumor extension beyond the dorsum sellae and contact with the optic chiasm, mammillary body, and interthalamic adhesion; and Grade 5, tumor occupation of the third ventricle. Furthermore, to evaluate blood vessel involvement, tumors of all grades were classified as either Type A, no involvement of the arterial circle of Willis or the cavernous sinus, or Type B, involvement of the arterial circle of Willis or the cavernous sinus.

Complete resection was achieved in three of three

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Grade 1A cases, three of three Grade 2A cases, 22 of 23 Grade 3A cases, and one of two Grade 3B cases. Resection was incomplete in two of two Grade 4B cases. Grade 5 cases were not indicated for surgery; other therapies were used instead. Recurrence was possible after the first remission or complete resection was achieved and was thus evaluated in 29 rather than 33 cases; recurrence was observed in four of these cases, all of which were classified as Grade 3.

Dogs with Type A, Grade 1 ～ 3 Cushing's disease had a good prognosis following transsphenoidal hypophysectomy. However, Type B, Grade 3 ～ 5 cases might not be suitable for transsphenoidal surgery.

2. BMP4 and BMPR expression in the pituitary gland of adult dogs in healthy condition and with ACTH-secreting pituitary adenoma

BMP4 reportedly suppresses ACTH secretion, cell differentiation, and tumorigenesis. Furthermore, it is reportedly associated with the function of the somatostatin analogs retinoic acid and ramelteon, which may be effective for treating ACTH-secreting pituitary adenoma. BMP4 is also reportedly expressed in corticotropic cells in the human pituitary gland, although the number of BMP4-positive corticotropic cells was reduced in ACTH-secreting pituitary adenomas.

This study aimed to investigate the expression of BMP4 and its receptors, BMPRI and BMPRII, in the pituitary glands of healthy adult dogs and those with ACTH-secreting pituitary adenomas.

A quantitative polymerase chain reaction analysis showed that the *BMP4* mRNA expression level in the ACTH-secreting pituitary adenoma samples was significantly lower than that in the normal pituitary gland samples ($P = 0.03$). However, there were no statistically significant differences between samples with respect to the mRNA expression levels of the *BMPRIA*, *BMPRIB*, and *BMPRII*. Double immunofluorescence analysis of the normal canine pituitary showed that BMP4 was localized in the thyrotropic (51.3 ± 7.3%) and not the corticotropic cells. In contrast, BMPRII was widely expressed in the thyrotropic (19.9 ± 5.2%) and somatotropic cells (94.7 ± 3.6%), but not in the corticotropic cells. BMP4 and BMPRII were not expressed in the corticotropic cells of ACTH-secreting pituitary adenomas. Moreover, the percentage of BMP4-positive cells was also significantly reduced in the thyrotropic cells of the surrounding normal pituitary tissue obtained from the resected ACTH-secreting pituitary adenoma (8.3 ± 7.9%) compared to that in normal canine pituitary tissues ($P < 0.001$).

Our study's results revealed a difference in the cellular pattern of BMP4-positive staining in the pituitary gland between humans and dogs and further revealed a BMPRII-positive staining pattern in the normal canine pituitary gland. These species-specific differences in BMP4 should be considered when dogs are used as an animal model for Cushing's disease. Furthermore, somatostatin analogs are reportedly effective for treating Cushing's disease in dogs. However, considering the current results, BMP4 may not be an important factor in terms of somatostatin analog action.

3. Immunohistological analysis of SSTR-2, SSTR-5, and DA2R in the pituitary glands of healthy adult dogs and those with ACTH-secreting pituitary adenomas

In Europe and the USA, the usage of pasireotide, a somatostatin analog, was recently approved for the treatment of adult patients with Cushing's disease for whom pituitary surgery is not a therapeutic option or has not been curative. The dopamine agonists cabergoline and bromocriptine have also been reported as effective for the treatment of Cushing's disease in humans.

This study aimed to clarify the expressions of SSTR2 and SSTR5, which have been reported to suppress hormonal secretion and arrest the cell cycle, as well as DA2R in ACTH-secreting pituitary adenomas.

SSTR2, SSTR5, and DA2R were expressed in the anterior and intermediate lobes of normal canine pituitary glands. However, the positive staining patterns were stronger in the intermediate lobes than the anterior lobes. In the anterior pituitary lobes, SSTR2-, SSTR5-, and DA2R-positive cell ratios in the ACTH-positive cells were 27.0 ± 8.6%, 27.9 ± 5.9%, and 34.0 ± 9.4%, respectively. In contrast, those positive cell ratios in ACTH-positive cells were 97.8 ± 1.5%, 94.1 ± 4.4%, and 96.1 ± 6.6% in the intermediate pituitary lobes, respectively.

Of the 14 Cushing's disease cases, 11, 12, and six cases expressed SSTR2, SSTR5, and DA2R, respectively. Among these positive-staining cases, four of 11 cases expressing SSTR2 showed strong positive staining in which > 80% of ACTH-positive staining cells co-expressed SSTR2. In addition to SSTR2, seven of 12 cases expressing SSTR5 showed strong positive staining. However, no cases showed DA2R-strong positive staining. Furthermore, four cases showed strong positive staining for both SSTR2 and SSTR5.

Two of these cases showed α-melanocyte stimulating hormone-positive staining. This result indicated that these ACTH-secreting pituitary adenomas were derived from the intermediate pituitary lobe.

This study's finding suggested that somatostatin analogs and dopamine agonists may be useful for the treatment of Cushing's disease in dogs according to immunohistological examinations of SSTR2, SSTR5, and DA2R in cases of incomplete resection or recurrence.

This study clarified that dogs with Type A, Grade 1～3 Cushing's disease according to our new classification system were suitable candidates for transsphenoidal hypophysectomy. In these cases, radical cure of Cushing's disease was expected and the dogs would have a good prognosis. Furthermore, these results suggest that the immunohistological staining of SSTR2, SSTR5, and DA2R as well as the use of somatostatin analogs and dopamine agonists would be available for dogs in which incomplete resection or recurrence occurred. However, considering our study findings, BMP4 signaling may not be an important factor with regard to the actions of somatostatin analogs in dogs with Cushing's disease.

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Studies on ganglion cell-like (GL) cells in the skin of Djungarian hamsters (*Phodopus sungorus*)

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There are specific cells in the dermis of the abdominal and thoracic skin of Djungarian hamsters (*Phodopus sungorus*), which have been called ganglion cell-like (GL) cells. Microscopically GL cells have one or two ovoid nuclei and abundant basophilic, foamy cytoplasm and mimic ganglion cells. GL cells more often appear in males than in females and the foci increase in size and number after sexual maturation. Immunohistochemically the nuclei of GL cells are consistently positive for androgen receptor (AR), and the cytoplasms are always positive for vimentin. In addition, the stroma of the foci includes fibers positive for type I and II collagen. These results suggest that the cells may have an androgen-dependent biological behavior and an ability of collagen fiber production. However, the detailed nature and function of GL cells remain unclear.

In this study, the author tried to confirm the *in vivo* reactivity of GL cells to androgen, paying attention to the fact that GL cells express androgen receptor (AR) and find out a lectin specific to GL cells by lectin histochemistry and thereafter identify the core protein modified with its lectin-binding glycan by MALDI-TOF MS analysis and western blotting.

1. Androgen-dependent biological behavior on ganglion cell-like cells in the skin of Djungarian hamsters (*Phodopus sungorus*) following gonadectomy (Chapter II)

To confirm whether GL cells have androgen-dependent biological behavior, the changes of GL cells in the skin of Djungarian hamsters following gonadectomy were evaluated histologically. Both sexes were gonadectomized at the age of 4 weeks, and then necropsied at the age of 18 weeks. The growth grade, distribution, and proliferative activity of GL cells in the thoracoabdominal and dorsal skins of gonadectomized and intact animals were evaluated. The castrated males showed more lowered growth grade and proliferative activity, and smaller distribution than the intact males. Regarding these 3 parameters similar trends were seen between ovariectomized and intact females, and between intact males and intact females.

These results suggest that GL cells of Djungarian hamster have sex difference in its distribution and proliferative activity, and that androgen is involved in the development of GL cells.

2. Morphologic changes of GL cells in the skin due to the *in vivo* long-term testosterone stimulation in gonadectomized Djungarian hamsters (Chapter III)

To access the effect of androgen on GL cell proliferation, after being gonadectomized, both sexes were given low-dose (5mg/kg) or high-dose (20mg/kg) of testosterone propionate (TP) subcutaneously once every week for short-term (12wks) or long-term (24wks) period and were evaluated similarly in Chapter II. In both sexes of short-term and long-term TP treated groups, an increase in growth rate and proliferative activity of GL cells was found in a dose-dependent manner. Moreover, in the low-dose groups the thickness of foci and proliferative activity of GL cells were higher in long-term treated animals than in short-term treated ones. On the other hand, there was no apparent term-related difference in both sexes in the high-dose groups.

The growth inhibition of GL cells occurred following gonadectomy similarly in the Chapter II, but dose-dependent proliferation of GL cells was induced by TP administration. From these results, GL cells have high sensitivity for androgen such as testosterone and

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androgen is strongly involved in proliferation of GL cells.

3. Lectin binding of GL cells in the skin of Djungarian hamsters (Chapter IV)

To detect a new marker of GL cells besides AR and vimentin, lectin histochemistry using 8 lectins: Con A, DBA, PNA, RCA_{120}, SBA, UEA-I, WGA and succinylated WGA (sWGA) was performed. WGA and sWGA specifically reacted to GL cells; Con A reacted to GL cells as well as adjacent stratified muscle fibers. GL cells were consistently negative for remaining 4 lectins.

This indicates that GL cells contain certain proteins glycosylated with *N*-acetylglucosamine (GlcNAc) and/or sialic acid. Thus, WGA lectin is thought to be available as a marker of GL cell.

4. Identification of WGA lectin-conjugated protein in GL cells in the skin of Djungarian hamsters (Chapter V)

Since WGA lectin specifically reacted to the cytoplasm of GL cells in the skin as described in Chapter IV, the author speculated that the protein modified with WGA lectin-binding sugar chain may be associated with the function of GL cells. Therefore, the glycosylated proteins eluted from the abdominal skin tissue of Djungarian hamsters were purified using WGA lectin affinity column and subjected to MALDI-TOF MS analysis and western blotting. The results suggested the presence of MGAT2 (mannosyl (alpha-1,6-)-glycoprotein beta-1,2-*N*-acetylglucosaminyltransfe- rase) and β-actin.

Recent studies have revealed that β-actin functions not only as cytoskeleton for the maintenance of cell shape, but also as signal transduction molecules associated with the transcriptional regulation. Although the significance of glycosylated β-actin remains obscure at present, the possibility exists that this specific glycosylation may take a functional role of GL cells.

In conclusions, it became evident that androgen had a significant influence on the growth of GL cells of Djungarian hamsters. Furthermore, it became clear that glycosylation with GlcNAc by MGAT2 and/or silalic acid took place in the cytoplasm of GL cells and that β-actin was one of those glycosylated proteins. In the future, the specific reactivity with WGA lectin may be available as a new marker of GL cells to elucidate the functional role of the cells. Although the author speculated about their secretion of a pheromone-like material, it was impossible to be demonstrated.

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Metabolome study on canine insulin resistance and diabetes onset

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Canine diabetes is classified as type 1 diabetes in human needing the insulin dosage of the life for treatment. Type 2 diabetes by the insulin resistance caused by the obesity is common in human, and it is thought that diabetes onset mechanism is greatly different from a dog in human. The aims of this study are to analyse a characteristic of the diabetes onset in the dog, and discover the differences between the hyperadrenocorticism (HAC) dog and the obesity dog. In this study, the metabolome analysis mainly performed on the above characterisation.

Chapter 1 Analysis of the insulin signaling gene expression of in peripheral blood neutrophil from hyperadrenocorticism dog

Insulin signaling gene (IRS-1, IRS-2, PI3-K, Akt2, PKC-λ) of the peripheral blood leukocyte of the HAC dog were analysed as preliminary investigation to consider whether the impact statement of glucocorticoid can use a peripheral blood leukocyte.

In HAC dog, the gene expression of IRS-1 slightly decreased, and the gene expression of IRS-2, PI3-K and Akt2 decreased to half of the Control group. From these results, it was clear that the insulin signaling gene expression of peripheral blood neutrophils of the HAC group changed, and it was thought that it was proper to use a peripheral blood leukocyte to evaluate influence of the glucocorticoid. In chapter 2, the metabolites in the cells treated ex vivo with glucocorticoid was analysed using an isolated peripheral blood leukocyte.

Chapter 2 Metabolites analysis of canine peripheral blood mononuclear cells with addition of dexamethasone

Using the established peripheral blood mononuclear cells of the culture method, the influence of glucocorticoid to metabolites of *in vitro* cultured cells was examined. Addition of 0 (control), 1 μmol/L dexamethasone in canine peripheral blood mononuclear cells were cultured and extracted metabolites from cells. For the analysis of the metabolites capillary electrophoresis time-of-flight mass spectrometer was used.

As a result of analysis, 96 metabolites were identified, and a change was accepted as a result of pathway analysis mainly on TCA cycle and glycolysis/gluconeogenesis in the group of treated dexamethasone. In addition, it was suggested by tendency to increase in metabolites of the gluconeogenesis pathway upper reaches and tendency to decrease in intermediate of TCA cycle and pyruvic acid that the addition of the dexamethasone decreased a catabolic reaction of glucose in the culture canine peripheral blood mononuclear cells. Because the non-change of the glucose uptake ability and the decreasing glucose catabolic reaction in the culture canine peripheral blood mononuclear cells by dexamethasone, an intracellular glucose concentration is maintained, and the glucose uptake to a cell is unnecessary, and it is thought that it leads to hyperglycosemia.

Chapter 3 The influence of dexamethasone and TNF-α to cultured canine skeletal muscle cells

The glucocorticoid increasing in blood of HAC and

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the TNF-α increasing in blood of obesity brings about insulin resistance. Therefore dexamethasone and TNF-α were added in the culture medium of the normal skeletal muscle cell for the purpose of examining the influence that HAC and obesity gave to a skeletal muscle. Using a myotube-like cells provided by the differentiation instruction of the normal canine skeletal muscle cells, metabolites were measured gas chromatograph mass spectrometer (GC-MS) and liquid chromatograph tandem mass spectrometer (LC-MS/MS). The glucose uptake ability was evaluated by measuring quantity of intracellular 2-deoxyglucose-6-phosphate by LC-MS/MS and IRS-1, PI3-K and Akt2 gene expression were measured by quantitative PCR method.

The addition of the dexamethasone showed the decrease in much metabolites and a tendency to decrease of the glucose uptake ability, and it was suggested that a catabolic reaction of glucose in the cell decreased like the experiment using the culture canine peripheral blood mononuclear cells which I described in chapter 2. In addition, the decrease in branched-chain amino acid (BCAA) in particular was remarkable, and, as for this, it was thought with a thing by two action with glucocorticoid; 1) resolution promotion of BCAA in the cell, 2) BCAA transportation decrease in the cell. Because the decrease of the quantity of BCAA in the cell inhibits protein translation system, the atrophy of the skeletal muscle is known to happen. Because the skeletal muscle is a maximum glucose uptake organ in the living body, the atrophy of the skeletal muscle to occur because of dexamethasone is connected for decrease of the glucose uptake quantity, and it is thought that it is with one of the factors to cause hyperglycosemia in HAC.

The addition of the TNF-α showed the tendency to decrease of IRS-1 gene expression and the non-change of the quantity of sugar uptake. Glucose uptake is inhibited in rodent myotube cells with TNF-α, but even if TNF-α was added, as for the canine myotube-like cells which I used in this study, the glucose uptake ability was not restrained. Furthermore, β-amino-isobutyric acid markedly increased in canine myotube-like cells with addition of TNF-α. Glucose metabolism abnormality is known to be improved when β-aminoisobutyric acid was added in the culture medium of rodent myotube cells. Therefore, it is thought that the intracellular β-amino-isobutyric acid increase in this experiment by the TNF-α addition is one of the factors that is hard to cause glucose metabolism abnormality in the obese dogs.

Chapter 4 The influence on serum metabolite by serum insulin and glucose levels in dogs

Serum amino acid were analysed by GC-MS while an insulin secretion promoted by intravenous glucose tolerance test to healthy dogs.

Leucine, isoleucine and valine (three amino acid is BCAA) and phenylalanine were significantly decreased in 0 ～ 60 minutes that insulin concentration showed a peak, and it was thought that it was the amino acid which reflected an insulin change precisely. These amino acids are useful for the risk evaluation of diabetes in human, in addition BCAA is taken in depending on insulin to skeletal muscle. Thus, possibility to become a useful marker to evaluate the decrease of the insulin action in the dog was shown.

Chapter 5 Comparison of serum metabolites between hyperadrenocorticism and obese affected dogs

Serum metabolites of the HAC of the dog (HAC group) which reported in that diabetes develops following insulin resistance and the obesity of the dog (Obesity group) which the onset of diabetes of the thing which insulin resistance set up is not reported in were analysed. In this way, the difference of the metabolites in the insulin-resistant from the both obesity and HAC were analysed.

In the HAC group ALP and ALT significantly increased in comparison with the Obesity group, and these changes are in agreement with published data. It is reported that a strong positive correlation between blood cystine concentration and body-mass index in humans. In this study, cystine significantly increased in the HAC group in comparison with Control group and, on the other hand, was a tendency to decrease in the Obesity group. Thus, it was shown that there was the change that was different from human. Besides, it is reported that excessive cystine leads to insulin secretion inhibition and insulin signaling downregulation in human. Additionally, In HAC group, serum glutamine significantly decreased comparison with Obesity group, and it is thought that the decrease of glutamine is connected for the decrease of the insulin sensitivity in human and rodent. Furthermore, in HAC group, stearoyl-Coenzyme A desaturase 1 (SCD-1) activity significantly increased comparison with Obesity group. SCD-1 activity was an index indicating what the gluconeogenesis in the liver increased in human,

and gluconeogenesis sthenia was suggested in HAC group. Valine and isoleucine which are BCAA were significantly increased in both HAC group and Obesity group in comparison with the Control group. Blood BCAA increases in a type 2 diabetes patient of human. It is explained that this is because the BCAA uptake in the cell is inhibited when insulin resistance generated. Therefore, it is thought that insulin resistance generated in both HAC group and the Obesity group together in this study.

From these, insulin resistance generated in comparison with Control group in both groups of HAC group and Obesity group, but in the HAC group, the decreased of the insulin sensitivity and gluconeogenesis sthenia were suggested in comparison with Obesity group, and it is thought that hyperadrenocorticism is in condition that diabetes is easy to on set.

In conclusion, serum BCAA decreased with serum insulin increase in the normal dogs, and the decrease of BCAA showed what was taken in a cell depending on insulin. In canine myotube-like cells which added dexamethasone, because intracellular BCAA in comparison with control group significantly decreased, it is suggested that insulin resistance increased. Furthermore, in canine myotube-like cell which added dexamethasone, glucose uptake ability was inhibited, and a catabolic reaction of the glucose tended to decrease. Also the decrease of glucose catabolic reaction was shown by the analysis result of the metabolites of canine peripheral blood mononuclear cells which added dexamethasone. On the other hand, with canine myotube-like cells which added TNF-α, β-amino-isobutyric acid reinforcing insulin sensitivity significantly increase, and it is thought that it is action to compensate insulin resistance. In addition, serum metabolites of HAC group and the Obesity group compared it with the Control group, and serum BCAA showed significantly high value. This suggests that insulin resistance generated in comparison with Control group in both groups of HAC group and Obesity group, but in the HAC group, the decreased of serum glutamine which is an index of the insulin hyposensitivity and gluconeogenesis sthenia were showed in comparison with Obesity group. Difference of these metabolites which it is recognized for HAC, but are not recognized for the obesity shows the cause that the HAC of the dogs leads to the diabetes onset, and the cause that the obesity of the dogs does not lead to diabetes. Thus, these results may be useful for further study of the diabetes onset mechanism in dogs. In addition, it is thought that the metabolome study using the cultured canine skeletal muscle cells is useful means to parse diabetes onset mechanism peculiar to the dogs.

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Studies on the adjacent segment disease of the cervical spine in dogs

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Cervical intervertebral disc herniation (C-IVDH) and caudal cervical spondylotic myelopathy (CSM) are commonly diagnosed neurosurgical disorders in the cervical region of dogs.

Clinically, as a treatment for C-IVDH and CSM, either ventral slot decompression (VS) or vertebral fixation (VF) is applied, based on the nature of the spinal cord compression (dynamic or static) of each individual case. Although postoperative instability and subluxation are serious complications with VS, these are considered preventable by combining VF. Typically, in caudal cervical lesions, when the slot width after VS is close to 50% of the vertebral body width, consideration of applying VF is necessary. However, after VF, the risk of similar lesions in the adjacent segment (domino lesions) has been reported. In domino lesions, abnormal mechanical environment occurs in the adjacent segment by vertebral fixation, promoting potential instability, and leading to extrusion of nucleus pulposus or hypertrophy of annulus fibrosus. Clinical symptoms secondary to domino lesions have been reported to occur in approximately 20 per cent of cases during the first 6 months to 4 years after surgery. In human medicine, similar lesions in adjacent intervertebral space after vertebral fixation of the cervical and lumbar spine has been reported (adjacent segment disease). In human cervical spines, it was demonstrated that the pressure or mobility of the adjacent segment increased after VF. Additionally, an increase in movement has also been observed at the upper and lower intervertebral spaces of the fixated segment in clinical studies using X-ray observations. However, there are only limited reports on the pathology of the adjacent segment disease and biomechanical and molecular mechanisms are still unknown in dogs. In order to clarify this pathology of the adjacent biomechanical disease, it is essential to study these mechanisms. Furthermore, it is necessary to consider the epidemiological features in order to make more detailed studies.

The purpose of this study is to clarify the pathology of the adjacent segment disease of the cervical spine in dogs. In chapter 2, in order to know the occurrence of adjacent segment disease after surgical treatment with C-IVDH and CSM, we studied retrospectively with respect to various data that were collected from medical records of C-IVDH and CSM cases. In chapter 3, a cervical spine model was created using specimens obtained from healthy beagles, and the effect on the adjacent vertebral space after the vertebral fixation was examined using a 6-axis material tester for. In chapter 4, since the increase in range of motion (ROM) was observed in the adjacent segment in chapter 3, we reproduced the mechanical environment by creating an in vivo vertebral fixation model in dogs, and examined the effects of changes in the biomechanical environment in adjacent segment.

1. Occurrence of adjacent segment disease after surgical treatment of cervical spine in dogs

In order to know the occurrence of adjacent segment disease after surgical treatment with cervical spinal disease in dogs, we studied retrospectively about C-IVDH and CSM cases. In this study, we difined the cases that recurrence of clinical symptoms such as neck pain or paresis and plegia of four limbs was observed as adjacent segment disease. As a result, the occurrence of adjacent segment disease was likely to occur after VF group(15.6%)compared to the after decompression group (5.2%), and significant association of adjacent segment disease was observed with respect to the combination of

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VF. Therefore, it was suggested that adjacent segment disease possibly associated with the stabilization surgery.

2. Changes in the biomechanical environment in the treated site and adjacent segment after the vertebral fixation

A cervical spine model was created using specimens obtained from healthy beagles, and the effect on the adjacent vertebral space after VF was examined using a 6-axis material tester. As a result, the ROMs at the treated site (C5 ～ 6) was significantly decreased compared with the intact model in both the PMMA and Plate models. Our results also showed that the ROM at the adjacent segment (C4 ～ 5) increased significantly in the PMMA and Plate models compared with the intact model. From these results, it was suggested that VF can change the mechanical environment at the adjacent segment and may cause adjacent segment disease. When ROM at C5 ～ 6 during the bending test in the PMMA and Plate models was compared, no significant difference was observed in flexion and extension movement. However, during lateral bending, ROM was significantly lower in the Plate model than in the PMMA model. In the rotational test, the ROM at C5 ～ 6 was significantly lower in the PMMA model than in the Plate Model. Since higher fixation strength was achieved in the Plate model in lateral bending and in the PMMA model in axial rotation, it was suggested that the effects on the adjacent segment differ according to the fixation method used.

3. Effect of changes in the biomechanical environment in adjacent segment after vertebral fixation

Since the increase in range of motion was observed in adjacent segment after vertebral fixation in Chapter 3, we reproduced the mechanical environment of the adjacent segments by creating an in vivo vertebral fixation model in dogs, have examined the changes in the biomechanical environment of adjacent segments. As a result, in the adjacent segments of the vertebral fixation group, increase of chondrocyte-like cells and cell clusters were observed and histological scores increased in the nucleus pulposus. Also with respect to changes in the extracellular matrix of the nucleus pulposus, increase of Col1A1 and MMP13-positive cells and reduction of Col2A1-positive cells were observed in the vertebral fixed group. Therefore, it was suggested that degeneration progressed by the influence of the vertebral fixing. On the other hand, in the annulus fibrosus, a tendency for cell density to decrease and rounded cells to increase was observed, in the vertebral fixation group. In addition, since some parts of the outer layer structure become unclear progressive degeneration due to vertebral fixation was suggested.

With the examination of changes in the composition of the collagen fibers in the annulus fibrosus, reduction of Col1A1 positive region and regional replacement by cartilage matrix was also observed under the influence of the vertebral fixation. Therefore, the degradation of collagen fibers and the progress of cartilage metaplasia were suggested. Although there were no Col2A1 positive regions, positive cell rate was higher in the vertebral fixation group as compared to the control group. From these results, it suggested that due to vertebral fixation, degeneration of the intervertebral disc nucleus pulposus and annulus fibrosus progressed, leading to the adjacent segment disease to develop.

In summary, our findings suggested that the occurrence of adjacent segment disease possibly associated with the stabilization surgery. In addition, the range of motion at the adjacent segment increased, suggesting that VF can change the mechanical environment at the adjacent segment and may cause adjacent segment disease. Also, the results suggested that due to vertebral fixation, degeneration of not only the intervertebral disc nucleus pulposus but also the annulus fibrosus progressed, leading to the development of adjacent segment disease.

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Basic study on serum fatty acid compositions in dogs with mitral insufficiency

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Mitral insufficiency (MI) is caused by myxomatous transformation degeneration, and is the most common chronic heart disease in dogs. A variety of supplements for the MI, have become popular, in addition to the general heart disease therapeutic agents. Products mainly composed of fatty acids are also used for MI patients, but studies on the pathogenesis of MI with blood fatty acid composition are small. Fatty acids are a major energy source for normal myocardium, which occupies 60～90% of the ATP production in the myocardium. However, if the heart muscle is subjected to a load, the energy is known to change to utilization of glucose from fatty acids. Changes in the fatty acid metabolism in heart failure caused by the aberrance of the regulation of calcium ion in myocardial cell construction and of the cardiac muscle cell membrane, by the accumulation of fat in the myocardium are considered as further worsen the heart failure.

The gas chromatograph (GC) and high-performance liquid chromatography are commonly used for the measurement of canine serum fatty acid. The GC method is suited with can be measured by small sample and the capacity of separation is high. However become this method requires the processes of extraction and methylation of the fatty acids from serum. These processes require specialized equipment and high-temperature heating. Because of compensation, serum fatty acid measurement has not become common in the veterinary field. This is one of the reasons, the relationship between serum fatty acid composition and pathophysiology in dogs have not been investigated.

In this study we 1) examined the method with the fatty acid methylation kit and the methylated fatty acid purification kit, to measure serum fatty acids in dogs, 2) examined the circadian variation in serum fatty acids to determine the optimal blood sampling time point for measuring in healthy dogs, 3) set a criterion range for serum fatty acid compositions in healthy dogs and 4) compared serum fatty acid compositions in dogs with MI by the stage of the classification and then correlations between fatty acids and echocardiographic parameters were confirmed.

1. Methods for measuring canine serum fatty acids (Chapter 2)

We examined the practicality of fatty acid measurements by gas chromatography (GC) method using the kits for the methylated fatty acid and purification of methylated fatty acid. First, the results of this method were compared with the conventional method. Secondly, reproducibility of the within-run and between-run, and the inter class reliability was confirmed. As a result, a total of 13 kinds of fatty acids were quantifiable; the saturated fatty acids (SFA) 2 types, monounsaturated fatty acids (MUFA) 2 kinds and polyunsaturated fatty acids (PUFA) 9 types.

This method demonstrated a high correlation with all kinds of fatty acids between the conventional method (correlation coefficient: 0.875 to 1.000). Range of coefficient of variance (CV) in within-run reproducibility was 2.0% to 7.4%. In addition, CV in the between-run reproducibility was 0.4% to 2.8%. Further, no significant differences were observed between examiners. The current method can be used safely and conveniently with high accuracy as compared with the conventional method.

2. Blood sampling point for measuring the serum fatty acid level of dogs (Chapter 3)

We examined the circadian variation in serum fatty

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acids to determine the optimal blood sampling time point for measuring in healthy dogs. Six healthy male beagles were fed the same food that meets the criteria of the Association of American Feed Control Officials for more than 2 months. They were fed twice daily at 7：00 and 19：00. The blood samples collected immediately before food provision at 7：00 am (Pre) and every 3rd hour for 24 hours. The results indicate that the total MUFA of 3 hours after the Pre, the level of total n-9 fatty acid and oleic acid increased significantly ($P < 0.05$) than the Pre. There were no significant differences from Pre, however. In the n-3 fatty acid, the levels of α-linoleic acid (ALA) at 3 hours after the meal in the morning were significantly higher than the corresponding Pre levels ($P < 0.05$), and there were no significant difference of 6 hours or more after. In the serum fatty acid weight ratios, 3h and 6h eicosapentaenoic acid (EPA) and 3h docosapentaenoic acid (DPA) decreased significantly ($P < 0.05$) than the Pre. There were no significant changes at 9 hours or more. These results indicate that the optimal timing of blood sampling is when the animals are hungriest, i.e., before breakfast, and that it is desirable to interpose a 9-hour or more interval when sampling is performed after morning meal.

3. Setting a criterion range for serum fatty acid compositions in healthy dogs (Chapter 4)

The level, weight ratio and proportion of each serum fatty acids of 105 clinically healthy dogs were examined for setting criterion range. The dogs were divided into groups of puppy, young adults and mature adults. Further, these groups were subdivided into an uncastrated male group, a castrated male group, an unsterilized female group, and a sterilized female group. The variable factors of fatty acids were the technical, the intraindivisual and interindivisual. By excluding factor of the blood sampling point, sexual cycle and dietary habits no significant differences by age or sex were observed in the level, weight ratio and the ratio of serum fatty acids. Therefore, the criterion range of the fatty acid level was considered to be fixed regardless of age or sex. However, in order to compare the serum fatty acids of individuals, it is necessary to confirm the effects of various fluctuation factors. Because of the small number of dogs used in this study, further data needs to be accumulated to determine the effect of food and breed. Therefore, these 95% intervals were to be used as a reference range in the present study.

4. Serum fatty acid compositions in dogs with MI (Chapter 5)

We divided 30 dogs with MI into groups I, II, and III, based on MI severity, based guideline with the International Small Animal Cardiac Heart Council and compared levels, weight ratios and the ratios of serum fatty acid among these groups and a healthy control group. The changes in serum fatty acid composition of MI dogs were examined and compared. In addition, correlations of serum fatty acid compositions with echocardiographic parameters in dogs with MI were analyzed. In order to differentiate healthy dogs and dogs with MI, we analyzed and determined the cutoff values, EPA and the ratio of EPA and arachidonic acid (EPA/AA) by the ROC. As a result, arachidonic acid (AA) level in groups I and II were significantly lower than that in the healthy group ($P<0.01$, $P<0.05$, respectively). Serum level and weight ratio of EPA in groups II and III were significantly lower than that in the healthy group ($P < 0.05$, $P < 0.01$, respectively). In addition, the EPA/AA ratio in groups II and III were significantly lower than that in healthy group ($P < 0.05$, $P < 0.01$, respectively). In group I, with low AA, the structural change of myocardium activated AA metabolism and in group II, the decrescence of serum EPA and AA levels is caused by the metabolic activation of EPA following AA. These changes might be concerned in the cytokines as tumor necrosis factor-alpha and interleukin 1 that increased in chronic heart failure. Furthermore, the decrease of AA in group III is thought to be caused by the activation of EPA metabolism by these cytokines.

Significant positive correlations of serum AA and docosatetraenoic acid levels by the left ventricular end-diastolic diameter index were noted in the MI group. A significant negative correlation was noted between the DPA level and fractional shortening (FS). The DPA weight ratio had a significant negative correlation with both left atrial to aortic root ratio (LA/Ao) and FS. Furthermore, a significant negative correlation was noted between EPA/AA and LA/Ao. From these results, the expansion of the left ventricle increases the n-6 fatty acids in the serum fatty acid that are substrates of pro-inflammatory eicosanoids. Additionally, increase of FS and LA/Ao that suggested to develop of mitral regurgitation, decrease n-3 fatty acids and EPA/AA which is a substrate of anti-inflammatory eicosanoids. Therefore, serum fatty acids suggested that reflect changes in myocardial energy metabolism

associated with the progress of the heart failure.

Then, the cutoff value of EPA level, determined to timing of administration of the fatty acid supplements used as a therapeutic adjunct to MI, was 47.5 μg/mL, that sensitivity and specificity were both 83.33%. On the other hand, the cutoff value of EPA/AA was 0.029, with sensitivity and specificity of 83.33% and 66.67%, respectively. Therefore, these values were likely to be the indicative of the timing of treatment of EPA.

The GC method with kits for the purification of methylated fatty acid and methylated fatty acids proved to be a stable and convenient method, with comparable accuracy of the conventional method, for measuring the fatty acid composition in the blood that is to be associated with the pathology of MI. The recommended timing of blood sampling for measuring the serum fatty acids is in the morning feeding before or at least 9 hours thereafter. The reference value of serum fatty acids of healthy dogs, age or sex, showed no significant difference. Further studies with more subjects and different breeds are recommended. The serum fatty acid composition in dogs with MI was different from the healthy dogs, and it the reflected the grade of cardiac function. The correlation between fatty acid ratio and the echocardiographic parameters were observed. It was suggested that changes in serum fatty acid value reflect the abnormal form of the myocardium. The cutoff values of EPA level and EPA/AA were likely to be the indicative of the timing of treatment of EPA. We need to find out the association of clinical effect and serum fatty acid composition by the administration of EPA based on these indicative.

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Studies on pathogenesis of acute thrombosis in Phenylhydrazine treated rat

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Phenylhydrazine (PHZ) is used worldwide, mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries. PHZ is well known for its ability to induce hemolysis via auto-oxidation of erythrocyte in animals including human. Several toxicities of PHZ have been reported in hematology and histopathology, e.g. decreased erythrocyte and hemoglobin, splenomegaly, erythrophagia and blown pigment deposition in various organs. On the other hand, thrombosis caused by PHZ has not been reported in the animal experiments. We could see a few cases of thrombosis in the patients treated with PHZ for polycythemia; however, the mechanism is unknown.

We found that a short-term administration of PHZ caused acute thrombosis in rat lung. In this study, we determined the hematological and histological changes of PHZ-treated rats to reveal the pathogenesis of the pulmonary thrombosis. In addition, we used gene expression profiling to provide a better understanding of biofunctional changes in lungs of PHZ-treated rats.

1. Acute pulmonary thrombosis and other histopathological findings in major organs of PHZ-treated rats (Second chapter)

To obtain the pathological reference data for blood or hematopoietic toxicity, we conducted a short-term repeated administration study of PHZ in young male SD rats. Macroscopically, PHZ-treated rats showed red or dark discoloration in all lobes of the lungs and small white lesions are scattered. Microscopically, fibrinous thrombi were formed in alveolar capillaries coincided with the white lesions macroscopically observed. In the other organs, hemolysis-related changes such as erythrophagia, extramedullary hematopoiesis and brown pigment deposition were observed in the liver, kidney, heart and spleen. Thrombi were observed in the left cardiac auricle mainly in dead animals; however, no microthrombi were observed in the organs except for the lung. Therefore, it is considered that PHZ caused acute thrombosis specific to the alveolar capillaries in rats.

2. The hematological and histopathological time course of change in PHZ-treated rats (Third chapter)

To investigate the pathogenesis of the acute pulmonary thrombosis in PHZ-treated rats, we evaluated the hematological and histopathological time course. As a result, the earliest change except anemia that preceded thrombus formation was congestion (i.e. accumulation of deformed erythrocyte). In addition, abnormality in coagulation parameters and limited endothelial injury in the alveolar capillaries were also observed accompanied by diffuse thrombus formation. Applying these changes to the three major causes of thrombus formation as follows: 1) endothelial injury, 2) stasis or turbulence of blood flow, and 3) blood hypercoagulability; the trigger for acute pulmonary thrombosis in PHZ-treated rats was considered to be regional stasis (Factor 2). Endothelial injury (Factor 1) and blood hypercoagulability (Factor 3) was considered to accelerate thrombus formation.

3. The time course change of mRNA in the lungs of PHZ-treated rats (Forth chapter)

As mentioned above, it was revealed that endothelial injury contributed to acceleration of thrombosis; however, the potential of endothelial dysfunction remains unclear. Therefore, we evaluated the expression changes of well-known thrombosis-related genes in endothelium in the lungs of PHZ-treated rats. In

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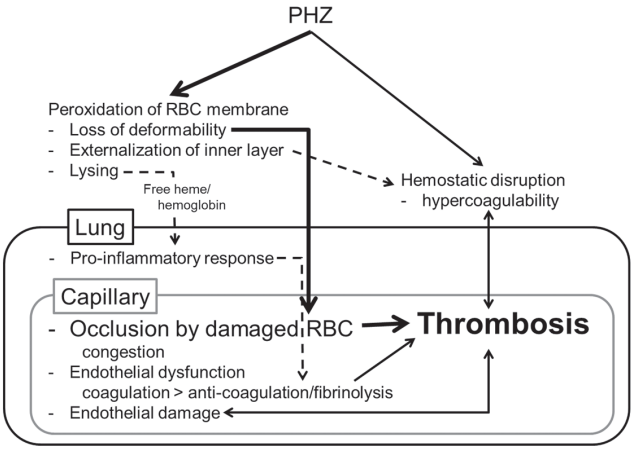
addition, we used gene expression profiling and Gene ontology analysis to provide a better understanding of the pathogenesis of the thrombosis. As a result, some of the thrombosis-related genes we examined were significantly changed mainly during thrombus formation and the balance between coagulation and fibrinolysis was considered to be inclined to pro-coagulant force. This change would contribute to the development of the pulmonary thrombosis in PHZ-treated rats. The gene expression analysis showed that inflammation/immune response was significantly and continuously induced in the lungs of PHZ-treated rats from the early phase of treatment. It has been reported that inflammatory response causes endothelial dysfunction and interact with blood coagulation. Therefore, inflammatory condition in lungs of PHZ-treated rats would be play a role in acute thrombosis.

To summarize the findings of our studies and other relevant reports, we propose a putative mechanism for acute pulmonary thrombosis in PHZ-treated rats (figure below). PHZ affected erythrocytes and might cause various types of disruption, including loss of deformability and morphological alteration, which are attributable to regional stasis, endothelial dysfunction and systemic hemostatic disruption (i.e. blood hypercoagulability). Inflammatory condition in the lung provoked from early phase might induce the endothelial dysfunction. Considering the impact and the onset of the events observed in our study, regional stasis could serve as a trigger, and subsequent endothelial dysfunction in the lungs and blood hypercoagulability would be important contributors to acute thrombosis in the lungs of PHZ-treated rats.



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Changes in diagnostic markers and their clinical application to obese cats

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Summary

As the frequency of feline obesity (25～50%) is considerably high, cats have characteristics to tend to be obese accompanying with insulin resistance. Glucose availability and insulin signaling in feline liver are lower, whereas hepatic gluconeogenesis ability from amino acids is higher in cats than that in dogs. Plasma adiponectin concentration, which improves insulin resistance, is lower in cat than that in dog. Prevention of obesity is most important for cats to avoid onset of serious metabolic disorders. Body condition score(BCS), as subjective parameter by each veterinarian, is a major criterion for obesity in clinical practice. Development of a reliable method for finding early stage of obesity is urgent for cats. The aim of this thesis is development of early diagnosis of obesity for cats. To make objective index for obesity of cat, plasma metabolite concentrations and some parts of body (measured anatomic sites) were measured.

1. Metabolic characteristics and obesity in cats

To investigate metabolic characteristics, 243 cats were collected from some veterinary clinics in Tokyo, Kanagawa, Saitama, Ibaraki prefecture and the laboratory in Nippon Pet Food Co., Ltd. They were divided into some groups based on sex, age, castration and obesity stages. Their body weights (BW), BCS, and plasma biomarkers were investigated.

BW, plasma total cholesterol (T-Cho) and insulin concentrations in the males were higher, and plasma adiponectin concentrations were lower than those in the females. These results suggest that the male cats showed higher tendency to become obese and insulin resistance than the female cats.

Activities of hepatic injury markers (asparate aminotransferase, AST alanine aminotransferase, ALT) and concentrations of chronic kidney disease (CKD) markers (blood urea nitrogen, BUN and creatinine) also increased in cats with aging. Ectopic lipid accumulation in liver and CKD are often found in aged cat.

In cats with BCS2&3, BW, plasma glucose, triglyceride (TG), T-Cho concentrations in the castrated group were higher than those in the intact group. Castration seems to be one of risk factors for obesity. And in the obese (BCS4&5 group) cats, 4-fold higher plasma TG concentrations were found in the castrated group than those in the intact group. These suggest that castration induce the risk for hyperlipidemia in cats.

2. Effect of obesity induced by over-feeding on the biomarkers in cats

To investigate the effect of weight gain by over-feeding on plasma biomarkers in cats, 8 male neutered cats were divided into two groups; overfed (4) and control group (4). To induce weight gain, overfed group cats were fed on commercial diet with 2-fold amount of daily energy requirement (DER) for 4weeks. Changes in BW, BCS, food consumption and plasma biomarkers in the overfed group were compared to those in the control group. The overfed group maintained higher amount of food consumption than the control group during the experimented period. The overfed group cats increased 24% of their BW and become moderate obesity showing slight metabolic abnormality. Total protein concentrations increased in the overfed group after the experiment, which was thought to be an effect of over-feeding. There is strong positive correlation between plasma non-esterified fatty acid (NEFA) and BW. Increased plasma NEFAs are caused by accumulated adipose tissues and ectopic accumulated fat in liver. Increasing in plasma ALT

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activities in the obese group seemed to be resulted from the ectopic hepatic fat accumulation. It is known that insulin resistance is caused by increased plasma TG, NEFA concentrations and sequenced ectopic hepatic fat accumulation. Increased plasma TG and NEFA concentrations induced by ectopic hepatic fat accumulation may induce insulin resistance.

3. Establishment of the index for obese of cat

To diagnose early stage obesity in cats, weight gain was induced by over-feeding for 4weeks. BW, BCS, head and body length (HBL), length from top of patella to end of calcaneus (PCL), neck girth (NG), chest girth, abdominal girth, and hip girth in the overfed group cats were compared to those in the control group cats. HBL and PCL were not affected by weight gain (99.8% and 98.3 % homology, respectively). A new index for cat obesity (feline body mass index (fBMI)) was settled as BW/PCL (kg/m). PCL is measured easily without sedative treatment for cats. fBMI increased significantly at the early stage obesity in cats, and correlated positively to BW, BCS, neck girth, and plasma NEFA concentrations. fBMI is suggested to be available as diagnostic index for early stage obesity. fBMI $\geqq$ 28.0 is decided as a criterion for overweight in cats.

4. Clinical application of feline body mass index to obese cats

The availability of fBMI for obese cats was investigated. 15 cats (overfed group) were fed on high-fat diet with overfeeding for 6weeks. Then, they fed on low-calorie diet for 4weeks to reduce their BW artificially. Control group cats (n = 5) were fed on normal diet for 10weeks. BW, BCS, fBMI, plasma biomarkers were measured at 6weeks and 10weeks of experiment period.

fBMI changed sharply reflecting changes in BW, plasma TG, NEFA concentrations. And fBMI was confirmed as useful index for feline obesity. fBMI 28.0 was decided as overweight accompanying with plasma lipids increase. Measurement of fBMI does not need blood sampling from animals and specific tools except for simple tape measure. fBMI is very suitable index for veterinary medicine and can be available for early diagnosis of obesity before onset of metabolic syndrome.

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The search for canine obesity-related genes and the effects of genetic mutation on metabolism in dogs

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Introduction

Obesity is a condition that the energy intake exceed energy expenditure, and excessive fat is accumulated to white adipose tissues. Two kinds of factors are related to obesity, such as environmental and genetic factors. Environmental factors includes daily lifestyle such as dietary habits and exercise quantity. Genetic factors depend on genetic polymorphism which influences the metabolism. As obesity-related genes in human beings, uncoupling proteins (UCPs), leptin, leptin receptors, beta-2 and beta-3 adrenergic receptors (ADRB3) genes have been reported. Recent years, G protein-coupling receptor 120 (GPR120) has been added to the list.

GPR120 is a fatty acid receptor relating to hormonal secretory functions such as glucagon-like peptide-1 and cholecystokinin. Gene polymorphisms are reported in human GPR120, especially the His270Arg mutant is functionally related to obesity. GPR120 is expressed in the lung, jejunum, ileum, colon, hypothalamus, hippocampus, spinal cord, bone marrow, skin and white adipose tissue in rodents and humans.

ADRB3 is a subtype of adrenergic receptors, which is expressed mainly in adipocytes. When adipocytes were stimulated with adrenergic agonists, acyl-glycerol would be broken down into glycerol and fatty acids, then causes heat production. In humans with Trp64Arg mutated ADRB3, the resting metabolism decrease by 200 ～ 220 kcal compared to wildtype people, and that increases risks of obesity and diabetes.

In veterinary medicine, obesity is the most common nutritional disorder as in human medicine. Epidemiological studies have revealed that one third or fourth of dogs are overweight or obese in developed countries. Furthermore, Obesity is a risk factor of pancreatitis, hyperlipidemia and arthritis in dogs. However, there is no report investigating obesity-related genes in dogs. In the present study, we focused on two candidate genes, such as GPR120 and ADRB3, and analyzed the relationship between their genetic polymorphism and body condition scores in dogs.

We have searched for SNPs of GPR120 (chapter 1) and ADRB3 (chapter 2) in client-owned dogs, and investigated the relationship between their gene frequency and body condition scores. In Chapter 3, we have developed a cell expression system which expresses ADRB3 mutants, and compared their molecular functions.

Chapter 1: Analyses of canine GPR120, a possible obesity-related gene in dogs

GPR120 is a member of the free fatty acid receptor family, which assumes long chain and unsaturated fatty acids as ligands. Fatty acids are not only energy source as substrates of beta-oxidation, but also signaling molecules in various cellular functions. It is reported that GPR120-deficient mice have developed obesity with fatty liver and insulin intolerance following a high fat diet feeding. In human GPR120 studies, higher gene frequency of the Arg270His mutant is detected in obese people, and the signaling function is attenuated, so this mutant is thought to be a risk factor of obesity.

In this chapter, we have cloned canine GPR120 cDNA and revealed the molecular nature. We have explored single nucleotide polymorphisms (SNPs) of GPR120 in 141 patient dogs' genome DNA, and investigated the relationship with obesity. Focus is on finding the candidate obesity-related genetic variations of cGPR120.

Cloned canine GPR120 consisted of 1,086 bases including ORF. Furthermore, canine GPR120 was 84～95

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% identical to those of the human, mouse, rat, cat, horse, pig, and white bear. They were comprised of 361 amino acids, and the homology of the amino acid sequences were 78 ～ 96 %. The highest identity was found to cats, and the lowest to rats. Tissue distribution analysis revealed that canine GPR120 was expressed in the lung, jejunum, ileum, large intestine, hypothalamus, ippocampus, spinal cord, bone marrow, skin, and adipose tissue.

We have analyzed GPR120 genomic sequences of 141 dogs, and found 5 synonymous and 4 non-synonymous SNPs. Gene frequencies of c.287T＞G (Leu96Arg) variant was 0.125 in all dogs (n＝141) and 0.500 in beagle dogs(n=36). The variant c.595C＞A(Pro199Thr) was detected in 40 of 141 dogs tested, and the gene frequency was significantly higher in overweight and obese dogs than that in normal dogs (p＝0.022).

The purpose of this chapter was to discover the genetic variants of canine GPR120 as candidate obesity-related genes. We have cloned canine GPR120 cDNA, and revealed the tissue distribution according to rodents and human studies. We have found 4 non-synonymous SNPs, especially the gene frequency of c.595C＞A (Pro199Thr) was significantly higher in overweight and obese dogs suggestive of that the variant is a candidate obesity-related gene in dogs.

Chapter 2: Analyses of canine ADRB3, a possible obesity-related gene in dogs

Beta 3-adrenergic receptor (ADRB3) is a subtype of adrenergic receptors, which has a seven times transmembrane structure and G protein-coupling structure expressed mainly in the white adipose tissue. When adipocytes were stimulated with adrenergic agonists, acyl-glycerol would be broken down into glycerol and fatty acids, then causes heat production. In human beings, when the 189th base of the ADRB3 gene mutates from thymine to cytosine, the 64th amino acid constituting ADRB3 mutates from tryptophan to arginine. In this mutant, the resting metabolism decrease by 200～220 kcal compared to wildtype people, and that increases risks of obesity and diabetes.

In this chapter, we have explored single nucleotide polymorphisms (SNPs) of ADRB3 in 160 patient dogs' genome DNA, and investigated the relationship with obesity. Focus is on finding the candidate obesity-related genetic variations of ADRB3.

We have analyzed ADRB3 genomic sequences of 160 dogs, and found 5 synonymous and 7 non-synonymous SNPs. Gene frequency of c.749C＞T (Ser150Phe) was 0.194, detected in 13 dog breeds including Yorkshire terrier and Miniature dachshund which are reported easy to grow fat, and it was significantly higher in overweight and obese dogs than that in normal dogs (p＝0.0001). Gene frequency of c.1121C＞G (Pro374Arg) was 0.053, detected in 7 dog breeds including Yorkshire terrier and Miniature dachshund, and it was significantly higher in underweight dogs than that in normal dogs (p＝0.0001). Gene frequency of c.1121C＞G (Pro374Arg) and c.1184A ＞ C (Pro395Gln) were 0.053 and 0.697, detected in 7 and 17 dog breeds, respectively.

The purpose of this chapter was to discover the genetic variants of canine cADRB3 as candidate obesity-related genes. We hve found 7 non-synonymous SNPs, especially the gene frequency of c.749C＞T (Ser150Phe) was significantly higher in overweight and obese dogs, and that of c.1121C ＞ G (Pro374Arg) was significantly higher in underweight dogs, respectively. It was suggested that these variants are candidate obesity-related genes in dogs.

Chapter 3: Development of a cell expression system of canine ADRB3 variants, and their functional analyses

In chapter 2 and 3, we have discovered candidate obesity-related variants in canine GPR120 and ADRB3 genes, but the data depend on epidemiological research and functions of the receptors have not ever been demonstrated. In human ADRB3, *in vitro* studies using a cell expression system of Trp64Arg variants have been performed and it was revealed that this mutation induces attenuated intracellular cyclic AMP production. Moreover, metabolic rates in humans with this mutation decreased by 200 kcal compared to those with wild type (WT) variants based on *in vivo* studies. Also in the present study, it is the next step to analyze functions of these mutants if they cause changes in metabolism.

In this chapter, we have developed a cell expression system of ADRB3 variants (WT, Ser150Phe, Pro374Arg, Pro395Gln and empty) with HA-tags and demonstrated functional analyses by reference to human studies. Western blotting analyses using anti HA tag antibody detected ADRB3 proteins in 4 mutants (WT, Ser150Phe, Pro374Arg, Pro395Gln), but not in the mock one.

Subsequently, we have stimulated these cells with adrenergic agonists (adrenaline, noradrenaline, CL316,234, IBMX and RO20-1724) for 30 minutes and measured intracellular cAMP concentrations. Adrenaline, noradrenaline and CL316,234 induced cAMP production in the cells expressing ADRB3, but not in

the mock mutants. Amounts of produced cAMP were tended to be lower in the Ser150Phe and Pro395Gln mutants compared to WT, but not in the Pro374Arg mutant.

The purpose of this chapter was to develop a cell expression system of ADRB3 variants, and analyze the difference in their functions. The Ser150Phe and Pro395Gln variants showed attenuated intracellular cAMP production when they were stimulated with adrenergic agonists, suggestive of that these mutations may decrease metabolic rates and increase the risk of obesity in dogs.

Conclusion

We have analyzed GPR120 and ADRB3 as candidate obesity-related genes in dogs. Gene frequency of c.595C＞A（Pro199Thr）variant of GPR120 was significantly higher in overweight and obese dogs than that in normal dogs. Gene frequency of c.749C＞T（Ser150Phe）variant of ADRB3 was significantly higher in overweight and obese dogs, and that of c.1121C＞G（Pro374Arg）was significantly higher in underweight dogs than that in normal dogs. Cell expression studies of ADRB3 variants have revealed that Ser150Phe and Pro395Gln mutants caused decrease in adrenergic agonists-induced intracellular cAMP production. These variants may be obesity-related mutations. Considering these variants as gene test items and utilizing the results for nutritional management, it, may be helpful for treatment and prevention of obesity in dogs.

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Evaluation of stress response using saliva sample in adult male mouse

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Plasma and serum samples are generally acceptable for detection of various physiologically active substances, while drawing blood could be stress for human and animals. In case of collecting repeated blood samples from mouse, using saphenous and lateral tail vein are recommended, and these sites yield 5% of circulating blood volume from saphenous veins and 0.1 ～ 0.15 ml from lateral tail vein, and the detection of the substances by any assay in this amount of blood sample is limited. Blood sampling by decapitation or cardiac puncture is employed as collection to whole blood, and it obtained approximately 1 ml of whole blood and modestly 0.5 ml of plasma. This volume is enough to measure multiple substances by an immunoassay, and in this case, a large number of mice are needed as a result of the sacrifice of animals at each time point of any responses to experimental treatments. In addition to this problem, it is impossible to investigate multiple time points, before and after responses to the treatment in the mouse of the same individual.

Recently, saliva is recognized as low-invasive sample for detection of physiologically active substances instead of plasma or serum. In this study for evaluation of stress response in the adult male mouse, we measured salivary corticosterone levels and salivary amylase activity, because glucocorticoids and salivary amylase have been known as biomarkers of stress response. However, in the small experimental animals such as mice and rats, few studies have been examined evaluation of salivary corticosterone and salivary amylase activity as a stress biomarker.

In order to clarify the whether salivary corticosterone is available to evaluate stress response in mice, 1. we confirmed that the corticosterone was detected in mouse saliva and that the glucocorticoid transferred from blood to saliva after administration of exogenous cortisol (chapter 2), 2. compared salivary secretion in mouse treated different anesthetic agents, and corticosterone levels between plasma and saliva in response to restraint stress, and evaluated saliva secretion in mouse treated with anticancer drug (chapter 3), and 3. examined adequate recovery periods from anesthesia for saliva collection between pre-stress and post-stress and also examine salivary corticosterone and amylase activity in mice (chapter 4).

1. Measurement of glucocorticoids levels using mouse saliva sample (chapter 2)

We confirmed that corticosterone in saliva was detected by enzyme immunoassay (EIA) and that glucocorticoids transferred from blood to saliva in mice treated with 2.0 mg/kg via intraperitoneal (ip) injection of cortisol. Cortisol is the glucocorticoid produced principally in humans but not in rodents. Saliva secretion was enhanced by pilocarpine hydrochloride (0.5 mg/kg ip). Obtained saliva and plasma samples used for measurement of cortisol levels by EIA.

Salivary corticosterone was detected by EIA and the cortisol was detected in plasma and saliva in mice as a result of cortisol injection. Conversely, in the mice treated with vehicle, the cortisol was not detected in saliva but it existed at very low levels in plasma. In this study, anti-cortisol showing 2% cross-reaction with corticosterone was used for EIA. The reason why cortisol detected in vehicle control group, might account for the cross-reactivity of anti-cortisol.

Results of this study revealed that the salivary corticosterone was detected by EIA, and the results indicate that the origin of cortisol detected in mouse saliva is considered as exogenous corticosteroid, and the

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cortisol could be transferred from blood to saliva via salivary glands. From these results, we concluded that the glucocorticoids in saliva might reflect to the levels of its blood circulation.

2. Investigation of anesthetic agent and stress conditions for evaluation of adrenal function using saliva sample of mouse (chapter 3)

We compared the effects of two different types of anesthetic agents (mixed anesthetic agents and pentobarbital) on salivary secretion. Mixed anesthetic agents consisting of medetomidine, midazolam and butorphanol are regarded as anesthetic agent instead of pentobarbital generally used in animal experiments. In this study, the mixed anesthetic agents were composed of medetomidine hydrochloride (0.3 mg/kg ip), midazolam (6.0 mg/kg ip) and butorphanol tartrate (7.5 mg/kg ip), and pentobarbital sodium was treated at a dosage of 40 mg/kg intraperitoneally injection. We also compared corticosterone levels between plasma and saliva in response to restraint stress with or without the mixed anesthesia. Mice were immobilized for 60 minutes by restrainer. Furthermore, we examined effect of anticancer drug (cyclophosphamide, 50 mg/kg ip) on salivary secretion and on salivary corticosterone levels.

Salivary corticosterone levels and volume of saliva were not significant differences between the mixed anesthesia group and pentobarbital anesthesia group. However, salivary protein levels in the mixed anesthesia group were significantly lower than the levels in the pentobarbital anesthesia group. The plasma corticosterone levels of restraint (60 min) group were significantly higher than the levels of the non-stress control group with or without anesthesia. The salivary corticosterone levels of restraint group were also significantly higher than the levels of the control group. Cyclophosphamide treated group did not show significant increase of salivary corticosterone levels compared to the vehicle control group. Moreover, volume of saliva secreted and salivary protein levels were not shown significant differences between control and cyclophosphamide groups.

Results of this study indicate that the mixed anesthetic agents are recommended agent for saliva sampling. The salivary corticosterone levels are significantly increased as a result of 60 minutes immobilization. The results suggest that salivary corticosterone levels reflect changes in plasma corticosterone levels caused by restraint stress in the mouse. Moreover, cyclophosphamide (50 mg/kg ip) does not significantly affect salivary secretion and on salivary corticosterone levels in mouse in the present study.

3. Investigation of the adequate recovery period from anesthesia for saliva collection (chapter 4)

We evaluated the adequate recovery period from anesthesia for saliva collection between pre-stress and post-stress in adult male mouse. In the investigations evaluating for recovery periods from anesthesia, four different time points (1, 3, 5 and 7 days) were set as a recovery periods. Salivary collection was divided into two fractions (0 ～ 20 and 20 ～ 40 min). Salivary corticosterone was determined by EIA and the amylase activity was measured using a dry chemistry system.

Restraint stress increased significantly corticosterone levels of saliva collected for 40 minutes in all four groups. On the other hand, the statistical evidence of corticosterone increase is more rigorous in a 7-day recovery group ($p < 0.001$) than the others ($p < 0.05$). Moreover, salivary amylase activities of prior fraction were shown significant increase in 3- and 7-day recovery groups by restraint stress, but not in 1- and 5-day recovery groups. Conversely, the activities of posterior fraction were shown significant decrease in 1- and 5-day recovery groups, and the activities in 3- and 7-day recovery group was unchanged by restraint stress.

Results of this study indicate that recovery period from anesthesia for saliva collection in stress experiment is preferable for 7 days in the same individual of mice. Furthermore, to evaluate salivary amylase activity in response to restraint stress using saliva sample, saliva sample in early period of saliva collection would be preferable.

In conclusion, we suggest that the collected saliva is available for EIA of corticosterone and that the salivary corticosterone levels reflect the plasma corticosterone levels, and it will be a useful less-invasive biomarker of physical stress in mice. Moreover, to evaluate restraint stress by salivary corticosterone level and amylase activity in mice under anesthesia, adequate recovery period from anesthesia is preferable for a week. Additionally, the present study may contribute to concepts of Reduction and Refinement of the 3Rs in small animal experiments.

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Characterization of cerebral ischemia-prone gerbils after unilateral carotid artery occlusion

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Cerebrovascular disease is the fourth leading cause of death in Japan, followed by malignant neoplasm, heart disease, and pneumonia. Approximately 60% patients died of cerebral infarction. In the past decade, the death rate has decreased following the development of new therapies, but the diseases remain intractable because of their serious after-effects. To overcome cerebral infarction, successive basic research using laboratory animals is imperative. In brain research, including that concerning cerebral blood flow and metabolism, the unilateral carotid artery occlusion (UCAO) method has been used for a long time in Mongolian gerbils (*Meriones unguiculatus*). The model in gerbils can easy produce cerebral ischemic insults in the ipsilateral hemisphere by UCAO, and the pathology after ischemic insults is closely similar to that in the human brain. However, the reproducibility of UCAO method is rather low (approximately 30%) because of variations in brain vascularity. We selected cerebral ischemia-prone gerbils in advance for which we had to resolve the following problem; the equalization of ischemic insult severity to obtain high reproducibility. In this study, we attempted to establish an original breeding colony with a high proportion of cerebral ischemia-prone gerbils and analyzed the characterization of cerebral ischemia-prone gerbils after UCAO.

First, we evaluated the utility of vaginal impedance to determine the estrus cycle in gerbils because obtaining an accurate result using the conventional vaginal smear methods, which is widely used in rats and mice, requires both time and skill. In gerbils, the impedance increased only during estrus and decreased during the later phases. This pattern in gerbils was the same as that in rats. There was no significant difference between the estrus cycle intervals of a gerbil as determined by the vaginal smear pattern and by impedance values. The results demonstrated that the vaginal impedance method is a useful tool for determining the estrus cycle in gerbils.

Second, to investigate the variation in the cerebral arterial circle (Willis' circle) in gerbils, we scored the animals by observing their behavior during the first 10-min period after UCAO. Ischemic-positive animals were selected on the basis of a stroke-index score of greater than 10 points. Three days after recirculation, the cell survival number in the hippocampal CA1 region was measured according to the standard procedure. The cerebral ischemia-positive rate for the experimental gerbils (MGS/SeaNvlu) was 36.0% (n = 113), which was significantly higher than that for the other two strains of commercially available gerbils (SLC:MON/JmsGbsSlc, 7.0%, n = 270; Kyudo: MGS/Sea, 27.7%, n = 358) ($P < 0.05$). The number of surviving neurons in the hippocampal CA1 region of the ischemic hemisphere tended to decrease as the neurological symptom score rose higher. The results demonstrated a significant correlation between the number of surviving neurons and neurological symptoms during occlusion ($P < 0.01$).

Finally, to elucidate the mechanism of variation development in the cerebrovascular structures in gerbils, we investigated the parent-child relationship in cerebral ischemia-positive animals based on the family tree of our gerbils (MGS/SeaNvlu). In addition, we examined genetic monitoring to clarify the relationship between variations in brain vascularity and genetic background using the skin grafting method within and between two strains (home bred and commercially available gerbil strains), which confirmed the difference in genetic background between cerebral ischemia-positive and -negative animals. Results showed that the

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ischemia-positive rate in F1 gerbils showing neurological symptoms induced by a transient UCAO was independent of the parents' phenotype. Furthermore, skin grafts between any combinations in the same strain, independent of the stroke-index score, survived more than 100 days. These results strongly suggested that the major histocompatibility complex (MHC) antigen of each animal within the strain is homogeneous. In contrast, skin grafts among the two strains were rejected within, on average, 8.8±2.4 days, indicating that the MHC between the two strains is different.

In conclusion, several factors, including genetic and other, might affect the mechanism of variations in the cerebrovascular structures in gerbils. However, uncertainty exists as to whether gerbils breeding specifically in our facilities show the cerebral ischemia-prone phenotype; environmental factors can affect gene expression at the transcriptional level in the process of gerbils' growth, producing variations in the cerebrovascular structure. Further research is required to investigate the embryonic stage of cerebrovascular development based on the breeding characteristics of gerbils. Finally, the cerebral ischemia-prone gerbils in this study could contribute to research in both cerebral ischemia and the development of cerebrovascular structures.

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Starch particles in the stomach contents as an indicator of feeding habits of wild boar (*Sus scrofa*)

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Introduction

To combat the increasing severity of crop damage caused by wild boars in recent years, a variety of defensive measures and population management methods are being investigated over multiple regions. An understanding of the relationship between wild boar populations and crops as a food source is critical in the regions where crop damage has become a problem. We have made an analysis of feeding habits, using stomach contents as a method for evaluating this relationship. However, in conventional feeding habit analyses, which are based on naked eye observations, only the kind of crop that has been damaged can be determined, and it is difficult to conclude whether the target crops were subject to multiple damage events or a single event. If such a grasp of the frequency of crop damage can be established, one can then determine whether or not crop damage is due to the group of boars in that region, allowing management of the causative population to be prioritized. In this study, we investigated whether it would be possible to gain an understanding of the duration over which materials were digested, based on changes in the characteristics of the gastric contents, in order to estimate the frequency of crop damage using feeding habit analysis.

Materials and Methods

In this study we examined sweet potato and Chinese yam, which are highly starchy crops of the kind that are preferable to wild boars.

The first step was to develop *experimental artificial digestive test I* to reproduce the digestion process of the wild boar from chewing to salivary digestion. Next, this method was used to produce artificial digestion products to mimic those of a wild boar. By comparing with digestion products collected from 44 wild boars, the ability of the artificial digestion products to reproduce the real samples was evaluated. Additionally, the possibility of using primarily naked-eye observations to gain understanding of elapsed digestion time was investigated.

In the second step, we focused on the properties of those starch particles that exhibited crop-specific morphologies, and investigated the temporal changes in their microscopic characteristics due to digestion. First, we investigated the relationship between elapsed digestion time and the number of starch particles shed from the periphery of the digestion products. Then, we focused on morphological changes in the starch particles due to digestion, and investigated the frequency of morphological changes in the digested starch by elapsed digestion time.

In the third step, we carried out *in vivo* trial experiments using indices and evaluation methods based on the previously developed *in vitro* experiments.

Results

Since it was found that many of the particles were similarly shaped when comparing the digestion products from *experimental artificial digestive test I* and the digestion products from wild samples, we concluded that it is possible to conduct experiments to reproduce oral digestion using the artificial digests prepared by this experimental system. In contrast, the morphology of digestion products obtained from stomach contents had decayed considerably, making interpretation of the temporal changes difficult.

The number of shed starch particles reduced significantly with elapsed digestion time for each of the

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test plants ($p < 0.01$). However, the reduction for the Chinese yam was not as pronounced as for the sweet potato. There were a number of morphological findings for the digested starch that suggested erosion of the surface from the early digestive stage, such as increased surface roughness and erosion of the extinction cross. Moreover, the number of particles exhibiting these changes increased with elapsed digestion time.

Additionally, the incidence of digested starch also significantly increased with elapsed digestion time for both sweet potato and Chinese yam ($p < 0.01$). After six hours of digestion, the morphology of digested starch particles in the *in vivo* experiments was often similar to the findings of the *in vitro* experiments; however, after 12 hours, the increase in surface roughness was markedly increased over the *in vitro* experiments. In addition, when comparing the incidence of digested starch *in vivo* and *in vitro*, large differences were found in values measured from the same time period, and in both cases were found to increase significantly with elapsed digestion time ($p < 0.01$).

Discussion

From comparisons of morphology between the digests from wild samples and those prepared using *experimental artificial digestive test I*, which mimics the oral cavity, we were able to manifest the exceptionally strong physical digestive action of chewing by wild boars. However, since the degree pulverization of the digestion products is severe for the wild samples, we determined that it would be difficult to grasp temporal changes through visualization alone.

In contrast, when focusing on the starch particles, it was revealed that the change in the number of shed starch particles was dependent on digestion time, and it seems likely that this could be used to ascertain temporal changes. However, changes in the number of particles was less for the yam when compared to sweet potato, which is likely to have been influenced by the viscous substances contained in the yam. Additionally, factors associated with performing the procedure also have a large influence, and so stability of the results remains a problem. Nonetheless, changes in digested particle morphology and incidence both depended on elapsed digestion time; thus, it seems that making consistent judgements regarding the temporal changes during the digestion process is possible mainly through understanding the changes in digested starch morphology.

Conclusions

This study suggests that by using starch particles in the stomach contents of wild boars as an indicator for grasping the changes in morphology that occur during digestion, it is possible to determine the elapsed digestion time of the originating crops. This means that, if the particles originate from just a single point in time, then the time when the animal came in contact with the crop can be estimated. Alternatively, if there exist particles at different stages of digestion, the frequency with which the animal comes into contact with the crops can be estimated. Thus, it is possible to estimate the frequency with which a wild boar damages a particular crop using the starch particles in the boar's stomach.

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Study in plantar pressure measurement and nursing practice during the postoperative recovery periods of hind limbs functions in small dogs

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In recent years, it is recommended to use the therapeutic exercise on animals with musculoskeletal diseases with the view of post-operative functional recovery and pain relief. Evaluations for standing posture and gait of cared animals performing therapeutic exercise are important, because they can not describe symptoms such as pains like human patients. Generally, we use force plates for evaluation of gait of animals with musculoskeletal diseases. However, the equipment can be used in limited places because it is too big to carry, and we need 1 hour for repeated measurements. These may be heavy burdens to the recovering animals.

In this study, we demonstrated plantar pressure measurement using a large flat area pressure distribution measurement system (the mat), focusing on the usefulness as an observation item for dogs in postoperative hind limb function recovery periods after surgical treatment. The mat consists of two-ply thin films and we can use it on a floor. The animals can walk on it freely without stress, so the mat is presumed to be a beneficial item for analysis of the dog's gait.

In chapter 1, we demonstrated plantar pressure measurement on 6 normal toy-breed dogs (Toy Poodle 4, Pomeranian 1, Yorkshire Terrier 1, age 2 to 7-years old, weight 2.8 ～ 4.6 kg) using the mat, to compare the percentage of 4-limbs loads pressures with them measured using a force plate. The results were as follows. The proportions of pressures were about 60 % in both forelimbs, about 40 % in both hind paws, about 30 % in a forelimb, and about 20 % in a hind leg. Collectively, it was approximately same with the results by measurement with a force plate. The mat is capable of pressure measurement under free walking and takes only 30 minutes to get the data. Thus, it was suggested that the mat is beneficial for post-operative gait analysis in dogs.

In chapter 2, we demonstrated gait analysis and plantar pressure measurement on 3 dogs (Toy Poodle 2, Maltese 1, age 2 to 13-years old, weight 2.3 ～ 3.3 kg) which had been diagnosed to be medial patellar luxation grade Ⅲ, surgically treated and underwent rehabilitation programs. The purpose was to examine if we can monitor their postoperative functional recovery with the mat. The gait analysis has revealed that grounding timing of the limbs had become more synchronized with time, and the pressure measurement with the mat has revealed that pressure of the affected limbs had recovered with time. However, the recovery processes were different among the animals. These results show that measurement with the mat was effective in monitoring postoperative functional recovery, in addition to traditional direct observation for limb grounding. Furthermore, it was suggested that it is possible to monitor postoperative recovery periods of animals with medial patellar luxation showing various kinds of symptoms. Moreover, it was indicated that we have to perform the measurement also in preoperative and after discharge periods, aside from the postoperative rehabilitation periods, because postoperative recovery processes are different among patients.

In chapter 3, we demonstrated gait analysis and nursing practice on a dog (Pomeranian, 1-year old, non-castrated male, 2.1 kg) which shows severe bilateral medial patellar luxation grade Ⅳ, for a long period from the preoperative to the re-examination days after discharge. We designed a nursing-care plan according

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to the postoperative processe, and performed nursing practices of the participant observation type. The gait analysis and measurement results indicated that the recuperative period was longer in the dog rather than those with unilateral medial patellar luxation, and the dog had recovered in 1 month. These results indicate that the recuperative periods are different depending on severity and/or numbers of affected limbs. Therefore, it was suggested that a long-term measurement with the mat is effective in postoperative monitoring to design suitable nursing practices.

In chapter 4, we demonstrated a long-term gait analysis and nursing practice on a dog (Toy Poodle, 1-year old, non-spayed female, 1.4 kg) which shows severe bilateral medial patellar luxation grade Ⅳ accompanying deformation of femurs and tibias, from the preoperative period to the re-examination days after discharge. We designed a nursing-care plan according to the operative procedure and postoperative processe, and performed a nursing practice accordingly. The gait analysis has revealed that the grounding timing had recovered 91 days later after discharge. However, the pressure did not recover within 4 months after surgery. These results suggest that longer periods are required for functional recovery in dogs which have received osteotomy of femurs or tibias with reduction of patellar luxation, in comparison with those receiving only patellar reduction.

Collectively, we conclude that gait analysis using the mat is beneficial for monitoring recovery periods of the dogs after surgical treatment of medial patellar luxation of various severity, and effective to design and perform suitable nursing practices of the patients which lead to appropriate assistance of individual animals.

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Effect of amount and component of dietary fat on incretin secretion in healthy cats

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Incretin is a gastrointestinal hormone which is secreted from the intestine. Incretin is secreted by dietary stimulation and which induce insulin secretion from pancreatic β cells. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are two primary incretin hormones. K cells (secreting GIP) and L cells (secreting GLP-1) has been reported to distribute on feline intestinal mucosa. Moreover, it has been confirmed that dietary fat is the biggest stimulating nutrients for secreting GIP in cats. However, the effects of dietary fat on incretin secretion in cats have not been evaluated. Therefore, the objective of this study is to investigate the effect of amount and component of dietary fat on incretin section in healthy cats.

1. Effect of weight control prescription diets on incretin secretion in healthy cats

Since excess intake of energy causes obesity, weight control prescription diet is generally low fat. Recently, moderate fat content prescription diet for weight reducing is developed in feline medicine. As such, we fed the control diet, weight control diet A (low fat) and weight control diet B (moderate fat) in 6 healthy cats. The fat content of each food is 4.0 g, 2.8 g, 3.6 g/100 kcal, respectively. 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 prior to 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 GIP and GLP-1 concentrations were measured.

No significant difference was observed in serum glucose, insulin and TG concentrations between the 3 diets. Serum NEFA concentration of postprandial 10 hours was significantly increased in the weight control diet B as compared with the control diet. The carbohydrate content of control diet was 10.5 g/100 kcal and that of weight control diet B was 8.7 g/100 kcal. Therefore, lower carbohydrate content (the weight control diet B) might promote fat mobilization. GIP $AUC_{0\text{-}10hrs}$ of the weight control diet A was lower than that of the control diet. On the other hand, GIP $AUC_{0\text{-}10hrs}$ of the weight control diet B was higher than that of the control diet. Although the weight control diet B had lower fat content than the control diet, GIP secretion level was significantly increased. When compared between the control diet and the weight control diet B, the control diet includes fish oil and vegetable oil and the weight control diet B includes coconut oil and animal oil. As such, the difference of fat ingredient might influence GIP secretion. No significant difference was observed in plasma GLP-1 concentrations between the 3 diets.

2. Effect of amount and component of dietary fat on incretin secretion in healthy cats

It is reported that high saturated fatty acid promotes GIP secretion in human. We fed the control diet (2.8 g of fat content/100 kcal) and the control diet with lard or bean oil (5.8 g of fat content/100 kcal) in 6 healthy cats. The lard consist high saturated fatty acid and the bean oil consist high unsaturated fatty acid. Each diet was fed by the latin square design for 7 days, and the blood sampling was conducted on the last day similar to Chapter 1.

No significant difference was observed in serum glucose and TG concentrations between the 3 diets. Serum insulin concentration of postprandial 1 hour in

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the control diet tended to be higher than the lard diet and the bean oil diet. Furthermore, NEFA $AUC_{0\text{-}10hrs}$ was significantly increased in the lard diet and the bean oil diet as compared with the control diet. The total calorie amount was similar between 3 diets. However, the ratio of carbohydrates was 36.2 % and 28.9 % in control diet and fat diets, respectively. Therefore, lower carbohydrate content might inhibit insulin secretion and promote fat mobilization in both fat diets. Plasma GIP concentration was significantly increased from postprandial 1 hour in the lard diet, and from postprandial 2 hours in the bean oil diet as compared with the control diet. In addition, GIP $AUC_{0\text{-}10hrs}$ in both fat diets were significantly higher than that in the control diet. However, there was no significant difference in GIP $AUC_{0\text{-}10hrs}$ between saturated fatty acid-rich lard diet and the unsaturated fatty acid-rich bean oil diet, which was not in agreement with human study. There was no significant difference in plasma GLP-1 concentrations between the 3 diets.

The result in Chapter 1 showed that the difference of fat ingredient might influence feline GIP secretion. Although we investigated the effect of dietary fat ingredient (lard and bean oil diet) in Chapter 2, there was no significant difference in GIP secretion level between diets. In Chapter 1, the control diet and the weight control diet B mainly include fish oil and coconut oil, respectively. Fish oil consist high long-chain fatty acid such as EPA and DHA and coconut oil consist high medium-chain fatty acid such as capric acid and lauric acid. As such, it was suggested that the carbon number of fatty acid might influence GIP secretion.

For elucidation of feline GIP secretion mechanism, it is necessary to investigate whether change in GIP secretion is occurred using different dietary fat such as short-chain, medium-chain or log-chain fatty acid.

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A study on oral care for dogs in veterinary nursing

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This study investigates the unclarified role of veterinary nurses in domestic canine oral care. In recent years oral care for dogs has been increasingly recognized in the field of veterinary medicine as a result of changes in both dog owner consciousness and canine longevity. Even so, it is the owner who is responsible for the oral care of a particular dog, not the animal itself. Accordingly, the primary role of a veterinary nurse in canine oral care has been assumed as providing assistance to the animal owner only. This study investigates the possibility of practical assistance by a veterinary nurse in addition to that by an owner of a dog through the initial use of an oral bacterial measurement counter to grasp the actual condition of a dog's mouth and then the subsequent application of a canine tooth brush as the means of oral hygiene maintenance by the owner. As outlined in Chapter 1, to understand the present role of veterinary nurse in relation to canine oral care, two questionnaire surveys as well as actual observations of canine mouths were carried out. Through these means, it became clear that there was a strong demand from the owners of middle or old age dogs for assistance from veterinary nurses in oral care and in ways of brushing teeth effectively. In addition, the survey revealed that without exception the veterinary nurses responding to the questionnaire expressed the belief that canine oral care was an absolute necessity. Moreover, the greater majority of the dogs under observation exhibited suspected or obvious oral afflictions. Not surprisingly, more than half of the veterinary nurses in the survey expressed their uneasiness over both general canine oral hygiene and the gingival and intra-oval contamination that was diagnosed specifically in the dogs under observation. A specific example of such oral affrications was exhibited by a beagle dog awaiting an operation for molar extraction. There was a deepening periodontal pocket in an intervention position and also adhesive plaque, and claque on the enamel of the first molar Mandible and the forth maxillary premolar. These findings suggested the need for research into the introduction and usage of a canine tooth brush for effectively cleaning the molars of middle to old age dogs whose owners had not previously employed such a device.

Chapter 2, outlines the subsequent research into the actual introduction of such a toothbrush for employment by such owners of middle and old age dogs. This research was carried out in four stages was follows. 1. The gingival effect of three commonly available types of canine tooth-brush; 2. The operational characteristics of six commercially marketed tooth brushes for dogs; 3. The potential application of a new prototype "jaw" canine toothbrush; 4. A phase of trials with the new prototype in brushing the teeth of six old-age beagle dogs unaccustomed to brushing. The result of the trial was that the new prototype toothbrush exhibited the strongest likelihood of beneficially removing plaque with the least movement interference by the dog. Accordingly, it is propose that a new canine tooth-brush based on the prototype would produce more beneficial results in oral care for old-age dogs and give greater ease of use for the owner of such animals. Chapter 3, focuses both on instruction given for hygiene assistance animal care in dental and nursing care facilities, and on the ascertainment of actual bacteria on dental plaque through the use of the oral bacteria measurement counter. To investigate the possibility of using this device as an observation tool two investigation were model. The first was into the suitability of using the counter as a valid measuring tool of E.coli. This investigation showed that the bacterium measurement counter evaluation exhibited a strong correlation across methods of culture and demonstrated a high level of reproducibility. The second investigation was into the

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use of the measurement counter as a suitable meay of evaluation the daily fluctuation of oral bacteria in the mouth of dogs. This investigation demonstrated also a strong correlation between the measured evaluation from device inspection of the method of canine bacterial culture. The measurement counter was therefore judge to be a useful device as a quantitative indicator of canine oral bacteria. Incidentally, the oral bacterial count of the dogs tended to highest in the morning, and lowest in the evening. From this it was hypothesized that performing canine oral care immediately after wake-up in the morning would reduce oral bacterial colonization during the day while brushing the tooth of dogs before sleep restrict bacterial growth in the mouth of a dog. From thus it was possible to gain an insight into more effective intervention method for canine oral care. According to the above research, the role of veterinary nurses in canine oral care, might include the necessity of ascertain the condition of the oral orifice through the use of a bacteria counter and the selection of such a device which would be most appropriate for a particular dog.

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Tissue specificity of biliverdin reductase gene expression in chickens

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Biliverdin reductase (BLVR) is the enzyme that converts biliverdin (BV) to bilirubin (BR). BV is a bile pigment in most of the birds and an eggshell pigment in several birds. Recently, it was predicted that BLVR does not fulfill its function as the enzyme in bird, described above. In the previous observation, *BLVR* mRNA was expressed in chicken liver, spleen, small intestine and kidney, respectively, especially was observed a strong signal in the chicken kidney. In addition, enzyme activity of BLVR was slightly detected in both chicken liver and kidney in this unpublished report. More recently, various pleiotropic functions of BLVR have been reported concerning cellular signaling, and so on. Therefore, the present study was examined about physiological significance of BLVR in chicken (cBLVR) without the metabolic conversion of BV to BR as the enzyme.

In the present study, White Leghorn laying hens were used as the experimental animals which does not contain BV as the eggshell pigment, and female Araucano fowl crossbred was also used, which contains BV as the same eggshell pigment. Following these sixteen organs, such as liver, gallbladder, spleen, pancreas, kidney (cranial, middle and caudal division), adrenal gland, duodenum, jejunum, ileum, colon, bone marrow, magnum, isthmus and shell gland of oviduct were collected from both White Leghorn laying hens and female Araucano fowl crossbred. These organs were dissected in both birds, immediately frozen in liquid nitrogen, and thereafter, stored at −80 C.

In experiment 1, in order to do the sequencing of White Leghorn's *cBLVR* mRNA, and to translate to amino acids, it was examine to compare to another amino acid sequences in various animals. As the results in experiment 1, amino acid sequence homology between chicken and mammals was 57.8～61.3 %. When nine animal species were compared, the highest homology in chicken was observed reptile and green sea turtle. Because birds and reptiles have common evolutionary feature, bile pigment is BV in both species, and birds and reptiles have hard-shell eggs and uric acid excretion. Therefore, birds and reptiles seem to have in common physiological function of BLVR.

In experiment 2, in order to examine the determination of *cBLVR* mRNA expression in 16 organs described above that collected from both White Leghorn laying hens and female Araucano fowl crossbred. As the results in experiment 2, the highest *cBLVR* mRNA expression was observed in kidney, and higher gene expression was also observed in small intestine in White Leghorn laying hens and female Araucano fowl crossbred. *cBLVR* mRNA expressions in another organs of both chicken were at the same level. From these results, the highest *cBLVR* mRNA expression in kidney of both chickens was common feature, regardless of the difference in phenotypes of eggshell color. The higher cBLVR mRNA expression was observed in kidney and intestine of chicken. Another research on *BLVR* mRNA expression in human and mouse were reported that not only kidney but also intestine play an important role of uric acid excretion. If the intestine in birds is also related to uric acid excretion, the higher *cBLVR* mRNA expression in kidney and intestine of White Leghorn laying hens and female Araucano fowl crossbred seemed to be caused by uric acid metabolism.

The higher *cBLVR* mRNA expression in kidney was observed, therefore, in experiment 3, in order to examine the determination of kidney *cBLVR* mRNA expression during embryonic stage (day 12 to day 21) and posthatched period (one-day to 21-day old), and

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also, adult at 226-day-old in White Leghorn chicken. No significant *cBLVR* mRNA expression was observed during embryonic stage and posthatched period until 21-day-old. So, it might be possible to increase kidney *cBLVR* mRNA expression due to sexual maturity, from 21-day-old to 226-day-old.

In experiment 4, in order to examine *cBLVR* mRNA expression site in kidney, *in situ* hybridization experiment was carried out. As the results in experiment 4, *cBLVR* mRNA expression site of White Leghorn kidney was obtained in renal tube. The renal tube of kidney in birds is the functional site concerning the secretion of uric acid. It is reported that uric acid caused the increment of reactive oxygen species in various cells. On the other hand, it is reported that the inhibition of cell inflammation and the production of antioxidant of BV and BR in mammalian BLVR. These results were suggested that BLVR in chicken kidney was considered to act as an important role of the cell protection.

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Research for factors which affect distribution of endoplasmic reticulum during *in vitro* maturation in pig

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Introduction

Most brand pigs have been improved giving priority to the meat quality than reproductive efficiency. Consequently, the litter size these pigs is relatively small. In order to dissolve this problem, a series of *in vitro* production procedures are useful, although there are higher polyspermy rate and lower blastocyst rate since *in vitro* maturation (IVM) might be incomplete (Yoshioka and Suzuki, 2006).

The serious problem is incomplete cytoplasmic maturation rather than nuclear maturation and it is not easily induced under the present conditions for IVM, (Gilchrist and Thompson, 2007). In the present study, we investigated dynamic relocalization of endoplasmic reticulum (ERs) which was one of intracellular organelle, to evaluate oocyte maturation with cytoplasmic maturation. ERs function as intracellular Ca^{2+} store during meiotic maturation and relate a rise of concentration of intracellular Ca^{2+} that is induced in oocyte after sperm penetration. In the previous study, dynamic relocalization of ERs during meiotic maturation has been reported. It was revealed that ERs were relocalized during maturation in annelida and echinoderm and distributed under plasma membrane in matured oocyte (Stricker, 2006). ER accumulation were observed close to cortical of *in vivo* matured porcine oocytes (Cran, 1985). In the present study, we investigated ER distribution in porcine oocyte before/after fertilization (from germinal vesicle stage to pronucleus stage) and examined condition of IVM by evaluating ER distribution as a index of cytoplasmic maturation to produce embryos efficiency.

Materials and Methods

Antral follicles of 4～6 mm in diameter were dissected from pig ovaries. Oocyte-cumulus-granulosa cell complexes (COCs) were isolated and cultured in various condition. To examine effect of dibutyryl cyclic adenosine monophosphate (dbcAMP), COCs were cultured in HP-POM containing 2.5 μg/ml follicle stimulating hormone (FSH), 2.5 μg/ml luteinizing hormone (LH) and 0 mM or 1 mM dbcAMP. To examine effect of different hormone, COCs were cultured in HP-POM supplemented with 10 IU/ml equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) or FSH and LH. Finally, to examine effect of different time to add hormone, COCs were cultured in HP-POM supplemented with FSH and LH for the first 20～22 h. For the subsequent 20～22 h after start of culture, COCs were moved to fresh culture medium, which did not contain hormone (FSH-LH group) or contained LH (FSH-LH/LH group), and cultured for 20～22 h(total 42～44 h). In each experiment, we investigated ER distribution in oocytes which were collected at 27 h (MI stage) and 42 h (MII stage) after start of culture.

For fertilization, cumulus cells of COCs cultured for 42 h were removed. After thawing frozen boar spermatozoa, motile spermatozoa were selected by percoll density-gradient method. Sperm motility and concentration were calculated and oocytes were inseminated for 6 h with 1×10^5/ml (spermatozoa/ml) final sperm concentration diluted with PFM. After insemination, oocytes were moved to PZM-5 drops and cultured for more 12 h. To investigate ER distribution, zona pellucida-free oocytes were fixed and immunostained using SelectFX Alexa Fluor 488 Endoplasmic Reticulum Labeling Kit and observed

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under the fluorescence microscope.

Result and Discussion

The distribution of ERs in cortical region of pig oocytes was classified five patterns; "entirety" and "partial" as forming clusters, "uniformity", "obscurity" and "cloud" as unforming clusters. In the present study, we evaluated that oocytes distributing ER clusters in cortical region (especially "entirety") at MI and MII stage were normal. Firstly, the ER distribution at MI and MII stage was not significantly different among groups when oocytes were treated 0 mM or 1 mM dbcAMP. This suggests that supplement of dbcAMP into IVM medium is not essential.

Secondly, we examined effect of different hormonal treatment in dbcAMP-free medium. The "entirety" rate of FSH-LH group was significantly higher than that of eCG-hCG group at MI and MII stage ($P<0.05$). This fact suggests that FSH-LH group observed normal ER distribution was suited. Finally, to generate similar hormonal kinetics *in vivo*, effect of different time to add hormone was examined. In FSH-LH/LH group, the "entirety" rate was significantly higher and "uniformity" rate was lower than those of control at MI stage ($P<0.05$). At MII stage, "entirety" rate was highest in each group.

The present study in pig demonstrated that ER distributed in cortical region as clusters during meiotic maturation, caused a peak of forming clusters at the MII stage and disappeared them at pronuclear stage. Moreover, concerning the condition of porcine oocyte maturation *in vitro*, our study suggested that the condition which COCs were cultured in HP-POM supplemented with FSH and LH for the first 20 ～ 22 h and moved to fresh hormone-free medium for the subsequent 20 ～ 22 h, was suitably.

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Studies on expression site of prolactin receptor gene and regulatory mechanism of its expression in the chicken anterior pituitary

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Prolactin (PRL) is a hormone secreted from the anterior pituitary. In birds, hormone-secreting cell types are distributed differently into two regions, the cephalic lobe and the caudal lobe. PRL-producing cells, lactotrophs, are present in the cephalic lobe. In birds, PRL exerts a variety of physiological functions such as crop milk production, osmo-regulation, induction and maintenance of incubation behavior, regulation of gonadal functions. These hormonal actions of PRL are mediated through PRL receptor (PRLR) in target cells. In chicken, PRLR gene expression is mainly regulated by transcriptional activation of two transcription start points (TSPs), 1A and 1G. Transcription from 1A occurs in wide-range of tissues including pituitary, whereas transcription from 1G occurs in limited tissues such as kidney and intestinal tissues. It has previously been shown that chicken PRLR gene abundantly expresses in both the cephalic and caudal lobes of the anterior pituitary, and the transcription is depend on the activation of TSP 1A. However, PRLR-expressing cell types and regulatory mechanism of the expression in the anterior pituitary are not known.

In this study, cell types expressing PRLR mRNA and the promoter region of TSP 1A in PRLR gene of the chicken anterior pituitary are investigated by *in situ* hybridization and luciferase assay, respectively. All procedures for animal experiments in this study were conducted in accordance with the provision for animal welfare of Nippon Veterinary and Life Science University.

Hens (White Leghorn, 10-months-old) were sacrificed by decapitation. The anterior pituitary was isolated, immediately frozen in liquid nitrogen, and stored at −80 ℃ until used. Total RNA was extracted from the tissue and was reverse-transcribed. The resulted cDNA was amplified by PCR with sense and antisense primers specific for PRL, GH, TSH-β, FSH-β, ACTH-, or PRLR. The amplified cDNAs were ligated to pGEM-T Easy plasmid vector and transfected into E. coli. The plasmid DNAs were extracted from the bacteria and purified, then subjected to synthesis of RNA probes for *in situ* hybridization using SP6 or T7 RNA polymerases. RNA probes were labeled with digoxygenin for PRLR mRNA and with fluorescein for PRL, GH, TSH-β, FSH-β, ACTH mRNAs, and were hybridized with each mRNA in pituitary sections. Red fluorescence of the digoxygenin-labeled RNA probe and green fluorescence of fluorescein-labeled RNA probes were detected by a fluorescence microscope.

PRL, TSH-β, and ACTH mRNAs were detected in the cephalic lobe, not in the caudal lobe. On the contrary, GH mRNA was detected only in the caudal lobe. FSH-β and PRLR mRNAs were found in both the cephalic and caudal lobes. PRLR mRNA distributed in the wide range of area of both the lobes and was co-localized with the most of individual cell type secreting PRL, GH, TSH, FSH, or ACTH. These results suggest that PRL has autocrine effect on the cells expressing PRL itself and paracrine action on the cells secreting other hormones through PRLR to regulate their hormone-secreting functions.

It has been known that transcription of PRL, GH, and TSH-β genes in the anterior pituitary are activated by a pituitary-specific transcription factor, Pit-1. A computer-based search for Pit-1 binding element in the 5'-upstream region of TSP 1A of chicken PRLR gene showed presence of four distinct elements (P1, P2,

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P3, and P4) having sequences resemble to consensus sequence of Pit-1 binding element. To examine promoter activity of the regions containing each element, luciferase assay was performed with GH3 cells derived from rat anterior pituitary. The cells are known to express GH, PRL, and Pit-1. Different lengths of the 5'-upstream regions of TSP 1A were ligated to pGL4 luciferase vector and the vector DNAs were transfected to GH3 cells by lipofection. Promoter activities were detected in all the 5'-upstream regions containing each of P1, P2, P3, and P4 element, and the activities of P1- and P4-containing areas were markedly higher than other areas. These results suggest that rat Pit-1 in GH3 cells acts on the P1 and P4 elements in the 5'-upstream regions of TSP 1A of chicken PRLR gene to activate transcription from TSP 1A. It has been shown that Pit-1 has two DNA binding domains, POU-homeo domain and POU-specific domain. Amino acid sequences of these two DNA binding domains of chicken Pit-1 show high sequence identity with those of rat Pit-1. In addition, sequences of chicken P1 and P4 elements are resemble to the consensus sequence of mammalian Pit-1 binding elements. These findings suggest that expression of PRLR gene in chicken anterior pituitary is regulated by Pit-1.

In conclusion, PRLR mRNA express in wide range of areas in both the cephalic and caudal lobes of chicken anterior pituitary with co-expressing with all cell types expressing individual pituitary hormones including PRL. The expression of PRLR mRNA in the anterior pituitary may be regulated by Pit-1 through its binding to P1 and P4 elements in the 5'-upstream region of TSP 1A of PRLR gene.

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Studies on the effect of various phosphates on myofibrils and actomyosin

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Water-holding capacities and binding properties contribute to deliciousness of meat products related to taste and texture. Water-holding capacities are the properties of meat which keep water in the muscular tissue or processed meat structure. Binding properties are the degree of binding between meat blocks by the paste made from partly solubilized meat by salt. It is basically important for the expression of that myosin (M) is extracted from myofibrils (mf) and formed gel network when it is heated.

Inorganic polyphosphates used as food additive in manufacture of meat products. Pyrophosphate (PYP) has been known to dissociate actomyosin (AM) into actin and myosin, improving the extraction of myosin from meat and enhancing binding properties and water-holding capacities of meat. Triphosphate (TRP) is presumed to be effective only after it is hydrolyzed into PYP by TRPase in meat[1].

On the other hand, organic monophosphates such as IMP were reported to dissociate AM into A and M[2]. An organic diphosphate such as ADP was reported to release thin-filament (AF) and thick filament (MF) from the restraints of mf and to liberate A and M from mf in the presence of IMP[3]. However, the details of the effect of inorganic polyphosphates except PYP and ADP on mf have been unknown. The dissociation of AM into A and M by IMP and TRP has been observed through changes of viscosity and solubility of proteins. However, there was no report to show changes of molecular weight directly.

The aim of this study is to elucidate these unsolved problems.

1. Effect of various inorganic polyphosphates on mf

Liberation of A and M from mf was investigated by the observation of A and M solubilized in the supernatant of 20 mM Tris-HCl (pH7.2)/0.2 M KCl/6 mM NaN_3 by SDS-PAGE. The solubilization of A and M are presumed to be induced by the dissociation of AM and release of those proteins from the restraints of mf.

TRP is known to contain about 10 % PYP. Thus, we compared the amount of liberated A and M in the various concentrations of TRP with the amount of those proteins in its one-tenth concentrations of PYP.

Although 0.8 mM PYP did not liberate A and M, 8 mM TRP liberated these proteins. Moreover, under these conditions, TRPase activity was not detected. Therefore, it is clarified clarified that TRP itself liberates A and M from mf instead of PYP contaminating to TRP or produced through hydrolysis of TRP.

SDS-PAGE pattern of supernatants obtained from mf incubated with various concentrations of TRP showed that A and M were liberated at high concentrations of TRP such as 8 ～ 32 mM. These results indicates that such a high concentration of TRP not only dissociates AM to A and M but also releases thin and thick filaments from the restraints of mf.

On the other hand, 4 mM TRP liberated A and M only in the presence of IMP, indicating that low concentration of TRP would have only the power same as ADP releasing thin and thick filaments from the restraints of mf.

Similar results were obtained for other polyphosphates.

When mf was incubated with 8 mM TRP, the small amount of A and M was liberated at 0 min-incubation and the amount of liberated proteins reached to

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maximum after 1 h-incubation, and then were kept constant. Thus, a period of 1 h was found to be necessary for the full liberation of both proteins.

Similar results were obtained for other polyphosphates.

2. Changes of molecular weight of AM in the presence PYP, TRP and IMP

AM was applied to Sepharose CL-2B (separation range of molecular weight (MW) ; 70 kDa- 40 MDa) gel permeation chromatography in the presence of these 8 mM phosphates. The obtained fractions were subjected to SDS-PAGE. The elution volume of AM was used as the void volume.

In the presence PYP, M heavy chain (200 kDa) and A monomer (45 kDa) were detected separately at the position of MWs 770 MDa and 2.4 MDa on the chromatogram. In the presence IMP, M heavy chain and A monomer were detected separately at the position of MWs 290 MDa and 2.4 MDa on the chromatogram. Thus, PYP and IMP were clearly shown to dissociate AM into A and M by the change of MWs. In addition, M (composed of two M heavy chains and M light chains, MW 500 kDa) and A were eluted extremely larger MWs than expected MWs of these molecules. These results suggest to be due to the polymerization of M heavy chains and A monomers or the fibrous structures of M and A.

On the other hand, in the presence of TRP, large amounts of M heavy chain and A monomers were detected at the position of void volume (MW larger than 770 MDa), which corresponds to AM. Thus, it was found that TRP had a power of solubilizing whole undissociated AM rather than dissociating AM into A and M.

3. Effect of ADP on mf

When mf was incubated with 1 mM ADP in the presence of 8 mM IMP, the liberation of A and M began after 15 min-incubation and the amount of proteins reached to maximum after 1 h-incubation and then kept constant. Thus, a period of 1 h was found to be necessary for the full liberation of both proteins by ADP.

Moreover, when mf was incubated with GDP that has a purine base same as ADP or UDP that has a pyrimidine base, the liberation of A and M was not observed. These results indicates that adenine base structure is required to release thin and thick filaments from the restraints of mf.

4. Structural changes of mf induced by various phosphates under phase-contrast microscope

Phase-contrast micrograph of mf incubated with various phosphates showed that ADP plus IMP induced narrowing of A-band and swelling of mf.

PYP and TRP also induced swelling of mf immediately after the addition. After 24 h A-band and I-band were indistinguishable and mf was almost completely solubilized.

It was considered that this difference between effects of ADP plus IMP and PYP/TRP was due to stronger solubilizing power of inorganic polyphosphates such as PYP and TRP than organic phosphates such as IMP and ADP.

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2) Okitani, et al., *Biosci. Biotechnol. Biochem.*, 72: 2005-2011 (2008)
3) Matsuishi, et al., *Anim. Sci. J.*, 87: 1407-1412 (2016)

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Effect of probaiotic bacteria on short-chain fatty acid production in the large intestinal fermentation

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The intestinal microbiota ferments undigested food materials to produce short-chain fatty acids, such as acetate, propionate and butyrate, and lactate. Short-chain fatty acids have some beneficial functions to host health. Butyrate, in particular, is most effective for promoting host health. The recent detection of receptors for short-chain fatty acids in the large intestine has enhanced the interest in their health beneficial functions. Lactate produced during the intestinal fermentation is rapidly metabolized to short-chain fatty acids by lactate-utilizing bacteria. Therefore, consumption of probiotic bacteria should stimulate short-chain fatty acids production due to metabolism of lactate produced by probiotic lactic acid bacteria to short-chain fatty acids by lactate-utilizing bacteria, and consequently consumption of probiotic bacteria bring about the health beneficial effects through stimulating short-chain fatty acids production. Thus, the stimulation of short-chain fatty acids production in the large intestine might be consider as one of the probiotic function. However, effect of probiotic bacteria on short-chain fatty acids production has not been well defined. In this study, effect of probiotic bacteria on short-chain fatty acids production in the large intestinal fermentation was investigated with *in vitro* fecal fermentation.

Experiment 1

Feces were collected from 5 healthy volunteers. They were anaerobically diluted to 25 times with phosphate buffer saline included L-cystein HCl monohydrate (0.5 %, w/v) as reducing agent (PBS + L) and squeezed through four layers of surgical gauze. A portion (6 ml) of the fecal diluents was mixed with equal volume of PBS + L contained fermentation substrates, and anaerobically cultured with 20 mM sodium lactate at 37 °C for 48 hours. After cultivation, the organic acids concentration and the copy number of bacterial 16S rRNA gene in the culture solution were analyzed. Organic acids were measured with ion-exclusion HPLC system. The copy number of bacterial 16S rRNA gene was measured with real-time PCR. The production of short-chain fatty acids and the copy number of 16S rRNA gene of fecal bacteria in the lactate culture was compared with those of the control (without sodium lactate) culture. The experiment was carried out in triplicate. Concentrations of propionate and/or butyrate in lactate culture were significantly higher than that of control culture. It was considered that the production of propionate and butyrate would be stimulated in the lactate culture. The 16S rRNA gene copy numbers of *Eubacterium hallii* and Bacterium SS2/1 which were lactate-utilizing butyrate-producing bacteria in the lactate culture were significantly higher than that in the control culture. The 16S rRNA gene copy numbers of the other bacterial group was not influenced by lactate. These results indicated that lactate was mainly metabolized to butyrate and/or propionate. *E.hallii* and Bacterium SS2/1 should contribute the butyrate production through metabolizing lactate in the colonic fermentation.

Experiment 2

Feces were collected from 3 healthy volunteers. They was anaerobically mixed and diluted to 25 times with PBS + L and squeezed through four layers of surgical gauze. A portion (6 ml) of the fecal diluents was mixed with equal volume of PBS + L contained fermentation substrates and, anaerobically cultured with probiotic bacterium at 37 °C for 48 hours. For this purpose, four probiotic bacteria were isolated from the commercially available fermented milks. They were

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identified with the sequencing of 16S rRNA gene. The suspensions of probiotic bacteria were prepared in 10^{10}, 10^{9} and 10^{8} cfu/ml, and 100 μl of those suspensions were inoculated into 12 ml of the fecal slurry. After cultivation, the organic acids concentration and the copy number of bacterial 16S rRNA gene in the culture solution were analyzed. Organic acids were measured with ion-exclusion HPLC system. The copy number of bacterial 16S rRNA gene was measured with real-time PCR. The production of short-chain fatty acids and the copy number of 16S rRNA gene of fecal bacteria in each probiotic culture were compared with those of negative-control (without probiotic bacteria) and positive-control (with sodium lactate, final concentration; 20 mM). The experiment was carried out in triplicate. The isolated four probiotic bacteria were identified as *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively. In positive control, the productions of butyrate and propionate were stimulated as observed in experiment 1. Except for *L.brevis*, the butyrate production was stimulated with 10^{10} cfu/ml probiotic inoculum. However, probiotic inocula lower than that bacterial number did not influence the short-chain fatty acids production. Regardless of bacterial number in the probiotic inocula, *L.brevis* did not influence the short-chain fatty acids production. These results suggested that enough number of probiotic bacteria stimulated butyrate production in the large intestine. However, this stimulation was difference among probiotic bacteria. The 16S rRNA gene copy numbers of bacteria were not influenced in all probiotic culture, so that the bacteria contributing butyrate production in probiotic culture were not determined.

In conclusion, lactate should metabolite to short-chain fatty acid by lactate-utilizing butyrate-producing bacteria, such as *E.hallii* and Bacterium SS2/1 in the large intestinal fermentation. Probiotic bacteria should stimulate short-chain fatty acids production, in particular butyrate, with participation of butyrate-producing bacteria other than *E.hallii* and Bacterium SS2/1.

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Research on improving Japanese dairy cattle for grazing: Possible utilization of imported NZ semen

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Introduction

Recently, there has been increased interest in Japan in expanding the use of pasture grazing for dairy cattle. Studies on establishing pasture and soil improvement are numerous, but there has been little research on improving the cattle for grazing. Holstein Friesian semen from the United States or Canada is widely utilized in Japan; however, some Hokkaido dairy farmers have begun importing semen from New Zealand (NZ). There are a number of studies indicating that Holstein Friesian cows derived from the NZ semen are much better adapted to produce milk in a grass-based system than those derived from the North American semen. The objective of this study was to determine the acceptability and usage of NZ semen among Japanese dairy farmers and to investigate any issues regarding the usage of NZ semen.

Materials and Methods

The research was performed in three parts. First, representatives from the NZ semen supplier as well as nine dairy farmers in Hokkaido, Japan who are customers of this firm were interviewed. Second, a questionnaire regarding the usage of NZ semen was administered to a total of 94 farmers in three areas in Hokkaido where pasture-based milking is common. Third, more extensive interviews were held with five farmers who indicated their interest (in the questionnaire) in using the NZ semen. The purpose of these interviews was to further clarify the reasons for their interest and the management profiles of these five farmers.

Results

The semen imported from NZ is produced by the Livestock Improvement Corporation (LIC). LIC began exporting semen to Japan after requests were received from dairy farmers who had visited NZ. Nine dairy farmers using the NZ semen were interviewed. The answers given revealed that the average herd size on these farms was 59.2 dairy cattle and that the average amount of land under management was 45.1 ha, including 13.1 ha of grazing land. This is more grazing land than the average used by other farmers in Hokkaido. Farmers in all nine dairy farms graze their cows in pastureland which is unique and uncommon in Japan.

The nine farmers interviewed gave three main reasons for introducing the NZ semen into their management: (1) improvement in cattle body conformation and leg health so that the animals would be better suited to grazing and more efficient in producing milk from grass, (2) improvement in fertility, and (3) decreasing the inbreeding coefficients in their herds.

The questionnaire was administered to the 94 farmers in the three typical grassland dairy farming areas in Hokkaido. Of these, there were 30 farmers who showed interest in using the NZ semen, but only 17 expressed a definite intention to use it. Most farmers are not planning to use NZ semen because they are not sure if it will help them. The management profiles of these 17 farmers were not significantly different from those of the remaining farmers surveyed.

Five dairy farmers were chosen for more extensive interviews from among the 17 farmers who expressed definite interest in using the NZ semen. Four of the five farmers said that their goal was to produce low-cost

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fresh milk from pasture while feeding the cattle with lower amounts of concentrate feeds. Two of the five farmers have already used the NZ semen in their herds with the goal of improving the conformation and leg/foot health of the cattle so that they are better suited for grazing. They also wish to improve the animals' efficiency in converting grass to milk. However, while the other three farmers also feel the need to improve their cattle for pasture-based milking, they have not yet tried using the NZ semen because of the cost and lack of information. These three farmers said that they need more information about improvements seen in cows derived from the NZ semen before they would decide to use it.

Discussion

This study has shown that there is interest among Japanese dairy farmers in using the NZ semen to improve their herds. These farmers want to improve the conformation and health of their herds, as well as the efficiency of producing milk in a grass-based system. Two issues must be addressed to encourage the usage of NZ semen in Japanese dairy farming. First, more data must be collected regarding improvements in dairy cattle sired using the NZ semen. Second, the NZ semen is not widely or easily available. Today, farmers who want to use the NZ semen have to purchase it without help from agricultural cooperatives. Wider distribution through agricultural cooperatives, veterinarians, and artificial inseminators may increase the availability and usage of NZ semen.

Conclusion

There is an increasing interest among Japanese dairy farmers in using the NZ semen and adopting a grass-based system to produce low-cost fresh milk, which is uncommon in Japan. This study suggests that there are two issues hindering the usage of NZ semen. More data on herd improvement and better distribution channels for the NZ semen are needed to increase its usage among Japanese dairy farmers.

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Preparation of a *Lactococcus lactis* expression vector, pNZ8148, and its stability in host cells

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Introduction

Lactic acid bacteria have environmental advantages to other bacteria by producing organic acids and bacteriocin. Those antimicrobial actions are traditionally utilized for food preservation. Thus the lactic acid bacteria are indispensable in broad areas of fermented food production industry.

Lactic acid bacteria are generally known for their roles as "good bacteria" in small intestine. They are believed that they have an ability of improving intestinal health by suppressing growth of other harmful bacteria in intestine and colon with their antimicrobial action during their stay in intestine for a certain period of time.

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

Lactococcus lactis is a homo-fermentative bacterium that is used for the production of 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 Altzheimer's disease e.t.c 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 considered to be great advantages over genomic integration systems.

In this report, establishment of a transformation method using gene expression vector, pNZ8148, for *L. lactis*, establishment of plasmid preparation method, stability of pNZ8148 in host cells, and analyses of the copy-numbers of the plasmid by comparing with *E.coli* MC1061, which is also a host strain for pNZ8148.

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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) was 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)-containing medium. The plasmid was prepared from the transformed NZ9000 and MC1061 with the alkaline lysis silica membrane method.

＜Stability of pNZ8148 in the strains＞

To estimate the host stability of pNZ8148, the transformed cells at the indicated time points were inoculated on cm-free ager medium. 100 of the colonies that appeared on the ager medium were then re-inoculated into both cm-free ager medium plates and cm containing ager medium plates. Stability of the plasmid was estimated by comparison of the appearance number of colonies in both media.

＜Copy-number measurement of pNZ8148 in both strains＞

The total DNAs were prepared from the transformed cells. 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 the each strain was calculated from the area sizes of the bacterial genome and plasmid DNA in the graph, and from their DNA molecular weight.

Results and Discussion

＜Plasmid preparation from both strains＞

From the results in this study, it was confined that the plasmid can be prepared using an alkaline lysis silica membrane method from the transformants.

By RNase processing, it was also confirmed that the plasmid with higher purity could be obtained from NZ9000.

In the transformation of NZ9000, it was also found that the repair of the cell wall after electroporation requires magnesium chloride and calcium chloride, if they are not included in the medium the transformation could not be successful.

＜Stability of pNZ8148 in the host strains＞

From the results, pNZ8148 was found to be highly stabile in NZ9000. There observed that almost no loss of the plasmid even if they were cultured for 72 hour in cm-free medium. On the other hand, MC1061 when cultured for 24 hours in cm-free medium percentage of pNZ8148 holding cells dropped about 80%. After 72 hours, it dropped 70%.

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

The plasmid copy number of MC1061 was calculated to be approximately 13 times higher than that in NZ9000. It seems necessary to consider the difference of the preparation stage of total DNA.

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Papain-catalyzed synthesis of oligo-tyrosine peptides

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Introduction

Some tyrosine (Tyr)-containing di- or tri- peptides derived from food protein sources are known to exhibit specific bioactivities such as antihypertensive, anxiolytic, and analgesic effects. Among them, Tyr-Tyr was isolated from royal jelly hydrolysates as a potent angiotensin I-converting enzyme (ACE) inhibitor, which probably functions like as antihypertensive agents. Since the solubility of Tyr-Tyr in aqueous media is far higher than free Tyr, it is expected not only to inhibit ACE but also to supply Tyr as a synthetic starting material of catecholamine.

Commercially available dipeptides are generally synthesized by chemical methods. In the case of chemical synthesis of Tyr-containing peptides, the hydroxy group of Tyr side chain as well as the amino- and carboxy groups of amino acids are commonly protected. This blocking process sequentially requires acid- or base-catalyzed removal of the protecting groups. Side reactions and formation of byproducts cannot be excluded through these chemical reactions. Thus, the synthesized peptides should be purified and are often too expensive to be readily used. To overcome the problems of chemical methods for peptide synthesis, protease-catalyzed reactions have been attracting a lot of attention.

It has been already reported that papain polymerized L-Tyr ethyl ester (Tyr-OEt). In our previous study, the papain-catalyzed polymerization of Tyr-OEt in aqueous media was efficient for synthesis of oligo-Tyr peptides with degrees of polymerization (DP) from 2 to 4. Tyr-Tyr (DP2) and Tyr-Tyr-Tyr (DP3) showed high inhibitory activities for ACE from rabbit lung with IC_{50} values of 34 μM and 51 μM, respectively. However, water-insoluble Tyr polymers with DP from 5 to 10 rapidly accumulated during the papain-catalyzed reaction, lowering yield of oligo-Tyr peptides.

The aim of the present study is to increase yield of oligo-Tyr peptides in the papain-catalyzed polymerization of Tyr-OEt. We investigated the effects of reaction temperature, pH and initial Tyr-OEt concentration on the DP and yield of peptide products, and also kinetically analyzed the mechanism through which papain elongated oligo-Tyr peptides to Tyr-polymers. Furthermore, for the purpose of effective utilization of the enzyme, we tried to construct a reaction system using immobilized papain.

Mechanism of papain-catalyzed peptide synthesis

Papain is an endoprotease with a cysteine residue in the active center. The active site of papain comprises seven subsites, each of which specifically accommodates a single amino acid residue of peptide substrate. The subsites locate on the both sides of the active center, S1-S4 on the one side and S'1-S'3 on the other. Starting polymerization of Tyr-OEt, the S1 subsite of papain accommodates one molecule of the substrate to form an acyl-enzyme intermediate (EA). If the amino group of another molecule of Tyr-OEt bound to the S'1 subsite causes nucleophilic addition to the carbonyl carbon of EA, Tyr-Tyr with ethyl ester will be synthesized by deacylation called aminolysis. In such a reaction process, two different deacylation pathways, hydrolysis and aminolysis, occur competitively. Therefore, optimization of the reaction condition is important to increase yield of the peptides.

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1. Optimization of papain-catalyzed synthesis of oligo-Tyr peptides

Tyr-OEt and 30 μM papain were mixed in 200 mM Na-phosphate buffer containing 1 mM dithiothreitol. The reactions were performed at different reaction temperatures (25 ～ 50 ℃) and pHs (6.0 ～ 7.5), with initial Tyr-OEt concentrations (25 ～ 100 mM). After a 72-hr reaction, the DP and yield of peptide products were analyzed by RP-HPLC. As a result, the most highest yield of oligo-Tyr peptides was achieved in the reaction conducted at 30 ℃ and pH 6.5, with 75 mM Tyr-OEt.

2. Kinetic analysis of mechanism of papain-catalyzed Tyr-OEt polymerization

As described above, the papain-catalyzed synthesis of oligo-Tyr peptides from Tyr-OEt is based on acyl-transfer reactions. To understand its mechanism, in particular through which the oligopeptides were elongated, we analyzed the efficiencies of Tyr-OEt for both EA formation and aminolysis. Analysis of the correlation between initial Tyr-OEt concentration and rate of Tyr-OEt reduction (in the early reaction time) indicated that the EA formation was a rate-limiting step. On the other hand, DP3 and DP4 were more quickly consumed by papain than Tyr-OEt, providing tyrosine and oligo-Tyr peptides, but not Tyr polymers. This result suggested that oligo-Tyr peptides preferentially reacted with papain as acyl donors. Comparing aminolytic activities of Tyr-OEt, DP2 and DP3 using *Nα*-benzoyl-L-arginine ethyl ester as an acyl donor, we found that Tyr-OEt caused aminolysis more effectively than the others.

Taken together, it was suggested that Tyr-OEt at higher concentrations contributed to the rapid elongation of early-synthesized oligomers, acting as a nucleophile for the aminolysis. Therefore, lower concentrations of Tyr-OEt were favorable to obtain oligo-Tyr peptides in good yields.

However, the lower initial concentration of Tyr-OEt for the reaction was, the lower papain efficiency was. In order to improve papain availability for the synthesis of oligo-Tyr peptides, application of the catalyst immobilized on an insoluble carrier to the reaction may be a promising strategy.

3. Preparation of immobilized papain and its application to synthesis of oligo-Tyr peptides

The enzymes bound to or trapped in insoluble carriers, so called immobilized enzymes, are generally more robust and easier to handle than free ones. Therefore, we tried to prepare the immobilized papains. Immobilization was performed using two kinds of supports, chitosan and cyanogen bromide-activated Sepharose (CNBr-Sepharose). The amount of absorbed papain and its residual activity were measured.

As a result, about 95 % of treated papain was bound to each of two carriers with residual activity of 40 ～ 45 %. In the initial use of papain immobilized on chitosan for synthesis of oligo-Tyr peptides, 23 % of Tyr-OEt remained. Meanwhile, the substrate was completely consumed by papain on CNBr-Sepharose, giving 65 ～ 80 % yield of oligo-Tyr peptides through 10-time reactions. The immobilized papain on CNBr-Sepharose was also resistant to repetitive use, compared with that on chitosan. However, the induction period was observed in the reaction, suggesting that the diffusion of substrate and products was restricted. In order to increase papain availability, other supports which not only keep the enzyme activity but also allow the efficient diffusion of substrate and products are desirable.

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Establishment of experimental conditions for leptin secretion induced by feeding in adult male mouse

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Introduction

Leptin is 16kDa peptide hormone encoded by obese (*ob*) gene and secreted predominantly in white adipose tissue. Leptin regulates body weight by suppression of food consumption, and feeding is a one of major stimulation of leptin secretion. It is necessary for fasting time periods to examine the postprandial leptin secretion, but many previous reports have set 24h as fasting time, and there is no report examined the adequate fasting time for the postprandial leptin secretion in the experimental animals.

Ethical guidelines for animal experiments suggests that fasting time periods should be shortened, and we investigated adequate fasting time periods (24h, 18h, 12h, 6h) before the refeeding schedule (3h) for leptin secretion. In the present study, diet-induced leptin secretion was examined in adult male mouse using plastic cage and wire meshed cage. In the plastic cage, the increased level of leptin in response to refed stimulus was suppressed in 24h fasting group, and this phenomenon was not observed in wire meshed cage. In 24h fasted groups of the plastic cage, wood beddings were confirmed in the stomach, suggesting that this bedding intakes might interrupt the leptin secretion in response to feeding stimulus. We assumed bedding intakes might delay the time of peak value of leptin induced by feeding in 24h fasted groups of the plastic cage, and refeeding time periods were set at 4.5h and 6h in addition to 3h. We also focused the two different factors; the adequate fasting time periods or the adequate amounts of food intakes, and examined which factor is more important for leptin secretion.

Materials and Methods

Male ddY mice (12-13w) were used in this study. In the first experiment, animals were divided into three groups for examining the effect of prolonged fasting time periods on leptin secretion: satiated, fasted and refed groups. Fasted group were starved for 6h, 12h, 18h (this group were carried out in plastic cage) and 24h. Refed group were allowed access to food *ad libitum* for 3h after each starvation period. In the second experiment, animals were divided into four groups in both cages. The animals were fasted for 24h, and were refed for 3h, 4.5h and 6h after fasting (24h).

In the pair-feeding experiment, mice were divided into two different groups. One group was fasted for 12h, and the other was allowed access to restricted amounts of food which the 6h fasted group took for 3h during refed periods (pair-fed group). Mice were killed by decapitation for determination of leptin concentrations in plasma by enzyme immuno-assay.

Results and Discussion

Plasma leptin concentration decreased significantly in fasted groups compared to nonfasted group, except for the 6h fasted group in wire-meshed cage. Plasma leptin concentrations significantly increased in the refed groups treated with fasting over 12h, but those did not increased the refed group treated with 6h fasting group. These results demonstrate that the shortest period of fasting time to observe leptin secretion induced by refeeding might be 12h in adult male mice.

In the fasting and refeeding experiment, the plasma leptin level showed the highest value in refeeding (3h) after 24h fasting in wire-meshed cage, but in plastic cage, the higher value of plasma leptin was observed

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in refeeding (3h) after 12h fasting than in refeeding after 24h fasting. As the results of investigating the plasma leptin concentration at 4.5h and 6h in addition to 3h refeeding time after 24h fasting in both plastic cage and wire-meshed cage, leptin secretion was elevated significantly in all refed groups (3, 4.5 and 6h refed groups) in both cages. However, the observed peak of leptin secretion was different between plastic cage and wire-meshed cage. In plastic cage, plasma leptin concentration was elevated significantly in 4.5h and 6h refed groups compared to 3h refed group. The peak levels of plasma leptin was observed at 4.5h refed group, whereas the peak of leptin was observed at 3h refed group in wire-meshed cage. In previously reports have shown that postprandial leptin secretion was mediated by insulin, and the insulin secretion was stimulated by a rise in blood glucose. Therefore, this data indicates that wood bedding in the stomach suppresses the absorption of nutrition such as glucose and results in interfered with leptin secretion.

In the last experiment, we focused the two different factors; the adequate fasting time periods or the adequate amounts of food intakes, and examined which factor is more important for leptin secretion. In response to restricted (amounts of food which the 6h fasted group took for 3h during refed periods) feeding after 12h fasting, plasma leptin concentration increased in pair-fed group compared with 12h fasted group. This result show that the more important factor for leptin secretion might be the adequate fasting time periods than the adequate amounts of food intakes.

In conclusion, we suggest that experimental conditions for postprandial leptin secretion should be set at 12h fasting time periods and also be housed in plastic cage with wood bedding in adult male mice.

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Identification of prolactin-responsive gene in mouse brain

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Prolactin (PRL) is a peptide hormone mainly synthesized in the anterior pituitary. In mammals, PRL plays important roles in physiological functions such as mammary gland development, osmo-regulation, immune regulation, gonadal function. In addition, PRL is involved in brain functions including maternal behavior, stress tolerance, gonadptropin secretion. These functions are mediated by PRL receptor (PRLR) of the target cells. The medial preoptic area (MPOA) in hypothalamus is known to be an critical region for maternal behavior, but the mechanism of PRL action on MPOA is not known.

In this study, PRL-responsive genes in the MPOA region are investigated. First, cDNA microarray analysis of the MPOA region of PRL-deficient (PRL-KO) and high PRL-secreting day-5 lactation mice was performed. Second, expression levels of microarray-positive mRNAs in diestous and day-5 lactation mice were determined by real-time PCR. Third, the responsivity of expression of these mRNA to PRL administration into PRL-KO mice was examined by real-time PCR. Finally, expression sites of PRL-responsive mRNAs in the MPOA region are determined by *in situ* hybridization. All procedures for animal experiments in this study were conducted in accordance with the provision for animal welfare of Nippon Veterinary and Life Science University.

The MPOA region was isolated from the brain section of mice and cDNA microarray analysis was performed by Agelent Array: Whole Mouse Genome DNA microarray analysis. Expression levels of 590 mRNAs were higher (more than double) in day-5 lactation mice than in PRL-KO mice, and 497 mRNAs showed higher expression level in PRL-KO mice than in day-5 lactation mice. Among the microarray-positive mRNAs, 21 mRNAs were confirmed to be PRL-responsive mRNA by RT-PCR analysis, and two mRNAs encoding follistatin and kisspeptin were subjected to further analyses. Follistatin is a neural peptide that inhibits actibin function to stimulate secretion of follicle-stimulating hormone from the anterior pituitary. Kisspeptin is a peptide that known to be synthesized at the arcuate nucleus and the anteroventral periventricular nucleus. Kisspeptin participates in the stimulation of secretion of gonadotropin-rereasing hormone and lutenizing hormone. Expression levels of follistatin and kisspeptin mRNAs in the MPOA region of diestros (physiological hypoprolactinemia status) and day-5 lactation (physiological hyperprolactinemia status) mice were determined by real-time PCR. The expression levels of follistatin were significantly higher at day-5 lactation, whereas kisspeptin mRNA levels were significantly higher at diestrous. To confirm the PRL-responsiveness of these two mRNAs, PRL was administrated to PRL-KO mice and expression levels of the mRNAs were examined by real-time PCR. Bovine PRL having hormonal activity equivalent to mouse PRL was subcutaneously administrated to PRL-KO mice daily for 4 days with low (50 μg) or high (100 μg) dose. PRL administration showed no effect on the follistatin mRNA expression. This result suggests that other factors are involved in the stimulation of follistatin mRNA expression in the MPOA area at hyperprolactinemia status during lactation. Expression levels of kisspeptin mRNA was significantly reduced by the PRL administration of both the low and hjgh doses, indicating that expression of kisspeptin gene was suppressed by PRL.

To clarify the expression sites of kisspeptin and PRLR mRNAs in the MPOA region, *in situ* hybridization was performed with the brain section of the MPOA area containing the preoptic periventricular nucleus (PVpo). The brain sections were prepared from diestrous, PRL-KO, and PRL (100 μg)-administrated PRL-KO mice. RNA probes for PRLR and kisspeptin mRNAs were labeled with fluorescein and digoxigenin,

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respectively. After the hybridization, green fluorescence of fluorescein-labeled antisense RNA probe and red fluorescence of digoxygenin-labeled antisense RNA probe were detected by a fluorescence microscope. PRLR mRNA was detected in the MPOA with almost equal intensities among the diestrous, PRL-KO, and PRL -administrated PRL-KO mice. On the other hand, kisspeptin mRNA was mainly detected in the PVpo with higher intensity in PRL-KO mice than diestrous mice. The intensity in PRL-KO mice was reduced by the PRL administration. Little of co-expression of PRLR and kisspeptin mRNAs was observed. These results suggest that PRL suppresses kisspeptin gene expression in the PVpo through unknown factors derived from PRLR-expressing neurons in the MPOA.

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Effects of dietary amino acids status on nutritional physiology of growing field voles

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Filed voles (*Microtus arvalis*) are expected to use for various nutritional study, because they are herbivore utilize gastric bacteria and have specific characteristics in carbohydrate metabolism. Therefore, they have been estimated nutritional requirement, especially energy and protein to prepare the feeding standard.

Amino acid (AA) requirements of voles have been estimated too using semi-purified diet replaced half dietary casein, protein source, to the crystalline AA mixture consisted of the same pattern AA as casein. However, in methionine (Met) dose response experiments, growth retardation was observed in only voles fed diet containing Met at the same level to casein. Above fact might be due to interaction between dietary AA pattern and gastric bacteria of vole because milk had been recognized as the determination factor of gastrointestinal bacteria.

Thus, in this study, in order to test that hypothesis, effects of methionine levels and status on growth of voles were examined by three experiments.

In Experiment 1, effects of methionine levels on growth of voles were examined when dietary Met was protected to bacteria using protected Met (Pro-Met). 30 growing field voles at 6 weeks of age were divided into 5 groups with 6 voles, and fed experiment diets during 7 days. At the end of feeding trial, voles were bled from the hart to determine plasma AA concentrations. Experiment diet was consisted of casein, crystalline AA, starch, and sucrose. Dietary non-protein methionine was supplied as pro-Met, and 5 level of Met from 1.5% to 3.5% per protein were formulated compensated glutamic acid.

In Experiment 2, to clarified effects of dietary Met status on time course of AA contents in the stomach and plasma AA concentrations when Met was maintained as the same as casein, 12 voles at 6 weeks of age were divided into 3 groups with 4 voles, and were fed experiment diets for 3 days. After then, they were bled from the hart to determine plasma AA concentration.

In Experiment 3, in order to clarify the specificity of voles to dietary AA composition, plasma AA pattern were compared between voles and mice after administration of casein pattern AA. 30 voles and ICR mice at 20～30 weeks of age were divided 6 groups with 5 animals each, and were administered casein formed AA solution or distilled water orally after 4 hours fasting, and then they were bled from the heart after 0, 1, or 4 hours after AA administration to determine plasma AA concentration.

Body weight gain (BWG) of voles increase with increasing dietary Met to 2.5% of protein, and then decreased in Experiment 1. Growth retardation was not observed in voles fed the diet containing Met as the same as casein. It may be suggested microbial protection of Met prevented growth reduction of voles, and gastro bacteria might be due to growth retardation of voles fed casein pattern AA diet.

In Experiment 2, there were no differences in BWG and feed intakes among all treatments. However, total AA contents was lower in voles fed Pro-Met diet than others, while plasma AA were higher in in voles fed Pro-Met diet. Hence Met status might affect movement of AA.

In Experiment 3, almost of AA were increased until 1 hour after AA administration in both mice and voles, and then decreased at 4 hours in mice but remained constant in voles.

These results suggested that there was interaction between dietary AA pattern as the casein and gastric bacteria, and it affected growth of field voles.

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