

Studies on bovine viral diarrhoea virus quasispecies detected in RK-13 cell line

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The rabbit kidney RK-13 cell line, originating from ATCC, has been confirmed to be a bovine viral diarrhoea virus (BVDV) contaminated culture. The contaminated virus was found to be noncytopathogenic, showing characterization of exaltation of Newcastle disease virus (END phenomenon positive) in cell cultures. As a result, the existence of BVDV in the RK-13 cell line was stable, according to either estimates of virus titre ($10^{4.6 \pm 0.5}$ TCID₅₀/ml) or ratio of BVDV positive infected cells ($71.9 \pm 3.12\%$), over 6 subsequent passages. Moreover, this stability in persistent infection was also observed in extra and intra-cellular virus, as well as in the RK-13 cell line.

The parent RK13 strain detected in the RK-13 cell line was found to be composed of two different biological types, namely, RK13/E⁺ strain, which shared the same END⁺ characterization with parent RK13 strain, as a major population strain and RK13/E⁻ strain, as a minor population isolated by reverse plaque formation method. RK13/E⁻ strain showed intrinsic interference against VSV, but no CPE, nor any END phenomenon. After four times biological cloning for RK13/E⁺ and RK13/E⁻ strains, by END and interference methods respectively, isolated strains showed different amplification titers in different host origin cultures (bovine, swine and rabbit), forming different growth curves depending on cell type. The highest reproduced virus titre was in bovine origin (MDB-SY and BT cells) for both strains, with titres of RK13/E⁺ and RK13/E⁻ strains reaching up to 10^8 and 10^6 TCID₅₀/ml in bovine cells, respectively. Overall, RK13/E⁺ strain consistently maintained higher virus titre than RK13/E⁻ strain throughout one step and multistep growth curve courses, in bovine origin cell cultures. In spite of the difference in END characterization between

RK13/E⁺ and RK13/E⁻ strains, similar antigenicity against BVDV antisera and identical nucleotide sequence of the 5' UTR between both isolates were observed.

In comparison to other BVDVs, RK13/E⁺ and RK13/E⁻ strains can grow in rabbit cells *in vitro* by transient and/or persistent infection, and in rabbit *in vivo*. This indicates the presence of adaptation and/or attenuation to rabbit origin, which is considered to be a main prerequisite for selection to be an attenuated vaccine strain. Similar to CSFV/GPE⁻ vaccine strain, which adapted to primary guinea pig kidney cells, RK13/E⁻ strain may also be considered as a possible candidate as a seed virus for a BVDV vaccine.

Based on phylogenetic analysis of viral RNA, it became clear that RK13/E⁺ and RK13/E⁻ strains belong to BVDV-1b genotype, and are close to Argentinean and American BVDV strains. Sequence comparison results, between RK13/E⁺ and RK13/E⁻ strains, demonstrated only four different amino acid substitutions within the open reading frame. These substitutions may determine the potential key for differentiating between END⁺ and END⁻ strains.

The CVA method was developed as a novel practical method, offering sensitivity, accuracy and increased safety, for the titration of BVDV END⁺ and END⁻ viruses, using RK13/E⁻ and RK13/E⁺ strain as competitor virus, respectively, as compared with conventional methods.

Overall, the present study introduces new BVDV strains, which maintain adaption to rabbit origin cells, different END characterization, and only four amino acid substitutions difference in the ORF genome sequence between them. Furthermore, it introduces a novel and practical method for the titration of BVDV using these aforementioned strains.

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Studies on the proliferation of canine pituitary corticotroph adenomas.

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Cushing's syndrome is a common endocrine disease in dogs that results from excessive cortisol secretion by the adrenal cortex. Approximately 80–85% of Cushing's syndrome in dogs are Cushing's disease due to adrenocorticotropin (ACTH)-secreting corticotrophic adenoma. Polydipsia, polyuria, abdominal distention and skin lesions such as alopecia are common symptoms of Cushing's disease, which results from a chronic overproduction of cortisol. Hence, most dogs with Cushing's disease are treated clinically to inhibit cortisol excess. However, in Cushing's disease, expansion of the pituitary tumor may lead to neurological signs because of the intracranial mass effect. In veterinary clinical medicine, the prevalence of advanced imaging modalities, such as computer tomography (CT) and magnetic resonance imaging (MRI), has enabled a detailed visualization of the pituitary. However, most dogs with Cushing's disease are treated clinically to inhibit a chronic overproduction of cortisol without visualization of the pituitary in Japan.

In humans, resection of the pituitary corticotroph adenoma with the transsphenoidal surgery is first choice for treating Cushing's disease. A number of reports demonstrated the occurrence of Nelson's syndrome, in which pituitary adenoma growth was accelerated, resulting from the disappearance of cortisol secretion that occurred after bilateral adrenalectomy in Cushing's disease patients. Several reports have shown that the inhibition of cortisol secretion in dogs with Cushing's disease may accelerate the growth of pituitary adenoma. However, whether changes similar to those observed in Nelson's syndrome appear in dogs with Cushing's disease is controversial.

In the present study, we evaluated the relation between tumor size and endocrinological tests result to determine the capability of pituitary tumor size prediction

without CT or MRI. Additionally, we investigated the expression of the proliferation markers, and evaluate their relationship with tumor size in canine pituitary corticotroph adenomas. Moreover, the effect of inhibition of cortisol secretion from the adrenal cortex by trilostane on hypothalamic corticotrophin releasing hormone (CRH) secretion, and the effects of CRH on the proliferation of pituitary corticotroph was evaluated to clarify the mechanism of corticotroph hypertrophy and hyperplasia after trilostane treatment in healthy dogs. In addition, the effect of CRH on the proliferation of canine pituitary corticotroph adenoma was evaluated to assessing the potential risk of Nelson's syndrome in dogs with Cushing's disease.

1. Relationship between pituitary tumor size and endocrinological test results in dogs with Cushing's disease.

It is difficult to predict the size of pituitary corticotroph tumors size in dogs with Cushing's disease without advanced imaging modalities, such as CT or MRI. The purpose of this study was to examine the relationship between endocrinological test results and pituitary size in dogs with Cushing's disease. Endocrinological test results and tumor size (measured as the pituitary height/brain area (P/B) ratio) were examined retrospectively in 67 dogs with pituitary corticotroph adenomas of various sizes.

There was a correlation between P/B ratio and basal-ACTH concentration ($r = 0.717$; $P < 0.001$). Dogs with P/B ratio $> 0.31 \times 10^{-2} \text{ mm}^{-1}$ had higher concentrations of basal-ACTH than dogs with P/B ratio $\leq 0.31 \times 10^{-2} \text{ mm}^{-1}$ (mean \pm S.D. concentration $133.8 \pm 92.2 \text{ pg/ml}$, $n=20$ versus $34.6 \pm 27.9 \text{ pg/ml}$, $n=7$; $P < 0.01$). With a threshold of 47.7 pg/ml of basal-ACTH concentra-

tion, the estimated sensitivity and specificity to predict the enlargement of pituitaries (P/B ratio $> 0.31 \times 10^{-2} \text{ mm}^{-1}$) were 85% (95% confidence interval [CI], 74-89%) and 86% (95% CI, 55-97%), respectively. We interpret these data as indicating that measurement of basal-ACTH might be of value in the characterization of tumor size in dogs with Cushing's disease.

2. Ki-67 and minichromosome maintenance-7 (MCM7) expression in canine pituitary corticotroph adenomas.

The ratio between pituitary height and the area of the brain (P/B) has been used to evaluate the pituitary size in dogs with Cushing's disease. A P/B ratio > 0.31 indicates an enlarged pituitary, while a P/B ratio ≤ 0.31 indicates a non-enlarged pituitary. The aim of this study was to investigate the expression of proliferation markers Ki-67 and minichromosome maintenance-7 (MCM7) in canine corticotroph adenomas in enlarged and in non-enlarged pituitaries, and to evaluate their relationship with the size of canine pituitary corticotroph adenomas.

Ki-67 and MCM7 expression in ACTH-positive tumor cells was determined by dual-labeling immunohistochemistry in resected corticotroph adenomas from 15 dogs with Cushing's disease. The mean \pm SD Ki-67 labeling index (LI) was $0.55 \pm 0.59\%$ in corticotroph adenomas with non-enlarged pituitaries and $1.6 \pm 0.6\%$ in adenomas with enlarged pituitaries. The MCM7 LI in corticotroph adenomas with non-enlarged pituitaries and in adenomas with enlarged pituitaries was 2.9 ± 2.2 and $10.9 \pm 3.7\%$, respectively. The Ki-67 LI and MCM7 LI were significantly greater in the adenomas with enlarged pituitaries than in the adenomas with non-enlarged pituitaries ($P < 0.01$ and $P < 0.01$, respectively). The MCM7 LI was significantly greater than the Ki-67 LI in adenomas ($P < 0.01$). The Ki-67 LI was positively correlated with the MCM7 LI ($r = 0.820$, $P < 0.01$), and the P/B ratio was positively correlated with the Ki-67 LI ($r = 0.560$, $P = 0.03$) and the MCM7 LI ($r = 0.854$, $P < 0.01$). In conclusion, canine corticotroph adenomas in enlarged pituitaries show greater proliferation potential than do adenomas in non-enlarged pituitaries.

3. The effect of trilostane on cerebrospinal fluid CRH concentration and pituitary corticotroph proliferation.

Most dogs with Cushing's disease caused by pituitary corticotroph adenoma are treated clinically to inhibit a chronic overproduction of cortisol, and the efficacy of this treatment has been reported. However, the suppression

of cortisol secretion by trilostane has reported to cause an enlarged pituitary due to corticotroph hyperplasia in healthy dogs. The aim of this study was to investigate the effect of inhibition of cortisol secretion by daily trilostane administration on the hypothalamic CRH secretion, and the effects of CRH on the proliferation of pituitary corticotroph in clinically normal dogs.

Dogs were administered 5 mg/kg trilostane twice a day every day for 4 weeks ($n = 6$). Before the administration of trilostane the mean \pm S.E.M. cerebrospinal fluid (CSF) CRH concentrations of the dogs was 260 ± 11.6 pg/ml. During the administration of trilostane, the CSF CRH concentrations showed significantly increased after trilostane administration. The CSF CRH concentrations at every 2 weeks were 337.8 ± 20.7 (2 weeks) and 366.2 ± 19.5 pg/ml (4 weeks), respectively. Anterior pituitary cells obtained from trilostane-treated dogs were cultured in a primary serum-free condition. Proliferation of corticotroph was detected by monitoring the cellular uptake of 5-ethynyl-2'-deoxyuridine and immunocytochemical detection of the adrenocorticotrophic hormone. Treatment with CRH (5nM) significantly stimulated the corticotroph proliferation ($P < 0.01$). Moreover, Antalarmin, a CRH receptor 1 inhibitor, significantly inhibited the cell proliferation induced by CRH ($P < 0.05$). These results indicate that pituitary enlargement due to corticotroph hyperplasia after trilostane administrations in healthy dogs was induced by the increased CRH stimulation.

4. The effect of CRH on the proliferation of canine pituitary corticotroph adenoma in vitro.

CRH is believed to play an important role to accelerate the growth of pituitary adenoma in the Nelson's syndrome. Moreover, drugs which inhibit the cortisol secretion is known to cause this phenomenon in humans. The aim of this study was to investigate the effect of CRH on the proliferation of canine pituitary corticotroph adenoma, and assessing the potential risk of Nelson's syndrome in dogs with Cushing's disease.

Pituitary corticotroph adenoma cells obtained from dogs with Cushing's disease treated by transsphenoidal hypophysectomy were cultured in a primary serum-free condition. Proliferation of corticotrophic tumor cells was detected by monitoring the cellular uptake of EdU and immunocytochemical detection of the adrenocorticotrophic hormone. Treatment with CRH (5nM) significantly stimulated the corticotroph adenoma proliferation ($P < 0.05$). Moreover, Antalarmin, a CRH receptor 1 inhibitor, significantly inhibited the cell proliferation in-

duced by CRH ($P < 0.05$). These results suggest that proliferative effect of CRH on canine pituitary corticotroph adenoma was CRHR1 dependent. Treatment with cortisol (100nM) did not alter the proliferation of corticotroph adenoma proliferation. This indicates that corticotroph adenoma cells have resistance to glucocorticoids, which is a characteristic feature of Cushing's disease. These results may help to understand whether changes similar to Nelson's syndrome appear in dogs

with Cushing's disease.

From these studies, it has been suggested that the inhibition of cortisol secretion by trilostane may increase the risk for accelerating the growth of corticotroph adenomas in dogs with Cushing's disease. Moreover, these results may also help to understand the pathophysiology of Nelson's syndrome, and development of tumor-targeted therapeutic agents.

Studies on isolation of canine mesenchymal stem cells and their differentiation to insulin producing cells

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The aim of this study was to create insulin producing cells from canine mesenchymal stem cells (MSCs). To understand insulin endocrine mechanism in canine MSCs, we isolated the canine bone marrow and adipose tissue derived mesenchymal stem cells and compared phenotype of both cells and cloned transcription factors related to pancreatic islet beta-cell differentiation and insulin production to analyze their functions.

1. We investigated the protein and mRNA expression profiles of bone marrow derived mesenchymal stem cells (BMSCs) and adipose tissue derived mesenchymal stem cells (ASCs). Expressions of CD29, CD44, and CD90 as mesenchymal tissue markers were positive on the both cells surfaces, while expressions of CD34 and CD45 as hematopoietic tissue markers and SSEA and TRA as embryonic stem cell markers were negative in both cells surface. Both MSCs showed similar pattern of their cell surface markers. mRNA expression of the stem cell markers, Oct3/4, Sox2, and Nanog, in canine BMSCs and ASCs were investigated using the qRT-PCR. Oct3/4 mRNA showed similar expression levels between BMSCs and ASCs. However, mRNA expression of Sox2 in BMSCs tended to be higher than in ASCs. In addition, mRNA expression of Nanog in ASCs was 2.5-fold higher than in BMSCs. Nanog is required to maintain the undifferentiated state and for the self-renewal of stem cells. In fact, several study reported that self-renewal activity of ASC is higher than that of BMSC. However, pluripotency of BMSC and ASC is not difference in differentiation into osteoblast, chondrocyte and adipocyte. We performed immunostaining assay with OCT3/4 and SOX2 in MSCs. In both BMSC and ASCs, OCT3/4 was detected in the nuclear fraction, whereas SOX2 was detected in the cytoplasmic fraction. In general, transcription factor is localized in nuclear fraction. In canine ESC experiment SOX2,

was detected in nuclear fraction. However, several transcription factors were known to be localized in the cytoplasmic fraction such as the Tead and FOXO families. These transcription factors were inactive state. Hence it is possible that they inactivate SOX2. Localization of SOX2 protein may lead to lack of proliferation of MSCs *in vivo* as well as maintenance of pluripotency of MSCs *in vitro*. Further studies are required to characterize canine MSCs with respect to the expression of other proteins and mRNAs.

2. Pancreatic and duodenum homeobox 1 (PDX1), beta cell transactivator 2 (BETA2) and V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) have been reported to show important roles in activation of the insulin gene promotor establishing beta cell specific insulin expression, and in the regulation of beta cell differentiation in many animal species. However, the precise molecular mechanism underlying the activity of PDX1, BETA2 and MAFA of canine is unknown. We performed cDNA cloning for full length of canine Pdx1 to give a molecular characterization in canine insulin gene expression. The canine Pdx1 cDNA consisted of 99 bp of 5'-untranslated region (UTR), 849 bp of coding region, and 550 bp of 3'-UTR. A deduced 283 amino acid sequence of canine Pdx1 displayed high overall sequence identity with Pdx1 of human (92.9%), bovine (90.0%) and mouse (87.2%). Canine Pdx-1 mRNA expression levels in various tissues (namely, tissue of cerebral cortex, duodenum, heart, kidney, liver, pancreas, skeletal muscle, spleen, and stomach) derived from a 2 year old male beagle were profiled by qRT-PCR. The highest level of expression was observed in the duodenum. In addition, high gene expression was observed in pancreas, stomach and liver. We observed high level of canine Pdx1 mRNA expression in gastrointestinal tissue, and it suggested

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that Pdx1 may be a marker of endodermal. And we performed a promoter assay to investigate a function of obtained Pdx1, Beta2 and Mafa cDNA. Significant promoter activity was observed within the -583 bp 5'-upstream region of canine insulin gene with Chinese hamster ovary cells. In addition, Pdx1 appeared to have a strong synergistic effect with Beta2, but only an additive effect with Mafa. These results suggest that canine Pdx1 play an important role in expression of insulin gene. Pdx1 and Beta2 mutations have been reported to cause maturity onset diabetes of the young. Mafa decreased expression and/or DNA binding activities gradually deteriorates pancreatic beta cell function. In future studies, we hope to clone and examine PDX1, BETA2 and MAFA from Type 1 DM suffering dogs more in depth in an attempt to determine if mutations exist which prevent or inhibit successful binding to insulin promoter region.

3. We established insulin producing cells from canine BMSC transiently expressed canine PDX1, BETA2 and MAFA by gene transfer using lipofection. Real-time PCR analysis revealed that insulin mRNA expression was observed and increased in the transfected cells. ELISA and immunostaining revealed that insulin protein was ex-

pressed detected in cytoplasmic fraction. These results showed that co-transfection of Pdx1, Beta2 and Mafa induced insulin producing activity in canine BMSCs. This finding provides clue to basic research for insulin produce mechanism. However, in our result insulin protein were not detected in the medium and this indicated the insulin producing cells have no activity to secret insulin. In general, insulin secretion was originated from beta-cell glucose metabolism increase, resulting insulin granule exocytosis via several pathways activated. Therefore we need to confirm that these pathways work functionally and to search for appropriate condition including transfection, cell culture or maintenance period for suitable to insulin secretion.

At present, induced pluripotent stem (iPS) cell play an important role in human regenerative medicine. However, application of iPS cells to regenerative veterinary medicine is difficult because the generations of iPS cells are very difficult and expensive. On the other hand, accessibility and safe of MSCs were very attractive strategy for application to veterinary clinical field. Our results provide fundamental information to regenerative medicine in veterinary clinical field.

Studies on the regeneration therapy for canine spinal cord injury using autologous bone marrow-derived mononuclear cells.

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Causes of spinal cord injury are classified into two categories : external causes, such as traffic accident and fall, and internal causes, including disc herniation and spinal cord tumor. No medical treatments are currently available for regeneration of damaged spinal cord and restoration of lost motor function. Though various efforts have been made in this field, high-dose methylprednisolone is the only option in the current clinical practice. However, recent studies have raised questions against the use of high-dose methylprednisolone because it is not very effective considering the severity of adverse effects.

With remarkable progress of regenerative therapy today, many studies have evaluated cell transplantation as a new therapy for spinal cord injury, using various cells including Schwann cells, embryonic stem cells, macrophages, neural stem cells, olfactory ensheathing cells, induced pluripotent stem cells, bone marrow stromal cells, etc. However, clinical application of cultured cell transplantation is still hindered by many major issues, such as quality control of culture cells, potential risk of bacterial infection, concern for tumorigenesis, high cost, equipment and special technique required. In addition, cell culture is time-consuming, making it difficult to obtain cells at the right timing for transplantation in the treatment of spinal cord injury. To circumvent this problem, we conducted a clinical study with a focus on the transplantation of bone marrow-derived mononuclear cells, which can be obtained simply by centrifugation without performing cell culture.

The present report describes the clinical study of bone marrow-derived mononuclear cell transplantation in acute and chronic spinal cord injury patients, conducted to develop a practical spinal cord regenerative therapy and to elucidate the mechanism by which the mononuclear cells regenerate the spinal cord.

1. Epidemiological study on spinal cord injury due to thoracolumbar intervertebral disc herniation in miniature dachshunds (Chapter 2)

Extensive epidemiological studies have been performed on canine intervertebral disc herniation (IVDH) mainly in western countries, but little data is available on the epidemiology of canine IVDH in Japan. In particular, no studies are available on the epidemiology of IVDH in miniature dachshunds, which have a high incidence of IVDH, in and outside Japan. Therefore, we considered it necessary to figure out whether epidemiological trend in IVDH in miniature dachshunds in Japan is similar to that reported in the past, and to review the recovery rate in the most severe cases and assess whether spinal cord regeneration therapy is applicable to those cases.

We examined the epidemiological trend in Japanese miniature dachshunds and found out that the trend was similar to what has been reported in foreign countries, however, males had a higher incidence than females. The recovery rate in the most severe cases (neurological grade 5) was 56.5%, which was significantly lower than the recovery rate of 100% in grade 3 and 4 cases, indicating that spinal cord regeneration therapy is applicable.

2. Study on effects of disc material extrusion on the neurological severity and outcome/recovery (Chapter 3)

Precise assessment of the degree of spinal cord injury is important in predicting outcome in animals with paraplegia due to IVDH. An assessment system to predict outcome of paraplegia is required, because animals not recovering after surgery will suffer permanent hind-limb paraplegia and urination disorder. Such assessment system is yet to be established.

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This chapter describes a retrospective study on CT and 3DCT imaging data to identify parameters that influence the recovery rate after surgical treatment for IVDH.

The study found out that the outcome was worse in animals that had extruded disc material diffused extensively in the spinal canal in the cranial to caudal direction. Because more mechanical energy is required to spread extruded disc material more extensively, the degree of extruded disc material spreading was likely to reflect the degree of primary injury in the spinal cord.

Based on the above results, it was considered that the extent of disc material spreading in the cranial to caudal direction was associated with a lower recovery rate, identifying it as a potential predictor of outcome.

3. Therapeutic efficacy of autologous bone marrow-derived mononuclear cell transplantation in miniature dachshunds with spinal cord injury due to IVDH (Study on acute phase spinal cord injury) (Chapter 4)

Animals with paresis or paraplegia with pain sensation show a high recovery rate after surgical decompression ; improvement of symptoms has been seen in approximately 95% of such cases. However, animals with paraplegia and loss of pain sensation exhibit a low recovery rate, ranging from 33% to 76% in various reports.

This chapter describes a controlled clinical study in dogs with severe thoracolumbar IVDH (paraplegia with analgesia), in which bone marrow-derived mononuclear cells (BM-MNCs) were transplanted immediately following surgical decompression, using control dogs that underwent surgical decompression alone.

The study demonstrated a significantly higher ambulatory recovery in the BM-MNC transplantation group compared to the control group (88.9% vs. 56.5%, $p < 0.05$).

Based on these results, it was considered that BM-MNC transplantation improved the recovery rate in severe IVDH cases probably through neuroprotective effect in the acute phase of spinal cord injury.

4. Therapeutic efficacy of autologous bone marrow-derived mononuclear cell transplantation in miniature dachshunds with spinal cord injury due to IVDH (Study on chronic phase spinal cord injury) (Chapter 5)

In cases functional recovery was not achieved after surgical therapy for IVDH, such as hemilaminectomy, permanent hind-limb paralysis remains (chronic spinal cord injury) for which no curative therapy is available.

This chapter evaluates clinical evaluation of BM-MNC transplantation using Texas Spinal Cord Injury Scale (TSCIS) in 3 cases of chronic spinal cord injury (miniature dachshunds), which had had paraplegia and analgesia due to thoracolumbar IVDH and did not show improvement in motor function after 6 months of surgical therapy.

TSCIS score was 0 at the first visit for all cases. After BM-MNC transplantation, the score improved to 11 (at Day 240), 5 (Day 240), and 4, respectively.

These results indicated that spinal cord regeneration therapy by BM-MNC transplantation is effective for chronic spinal cord injury, for which little has been reported on effective therapy.

5. Analysis of morphology and surface markers of transplanted bone marrow-derived mononuclear cells (Chapter 6)

The clinical study results described in previous chapters strongly indicated that BM-MNC transplantation is effective for spinal cord injury in dogs. To make this therapy more effective, it is required to understand the mechanism by which BM-MNC transplantation stimulates the regeneration of spinal cord. First, we characterized canine BM-MNCs using smear samples and flow cytometry to examine the types and ratios of cells contained in BM-MNCs.

The analysis revealed that canine BM-MNC fraction was a heterogeneous population, containing many CD4⁺, CD8⁺, CD14⁺, CD29⁺, CD34⁺, and CD90⁺ cells.

These results indicated that BM-MNCs are a mixture of CD4⁺, CD14⁺, CD29⁺, CD34⁺, and CD90⁺ cells, which help regenerate the spinal cord and are likely to be involved in the functional recovery.

6. Examination of mRNA expression levels for various cytokines in BM-MNCs (Chapter 7)

The previous chapter examined the types and ratios of cells contained in BM-MNCs as a first step to elucidating the mechanism by which BM-MNCs stimulate spinal cord regeneration. BM-MNC fraction was a mixture of various cell types, such as CD4⁺, CD14⁺, CD29⁺, CD34⁺, and CD90⁺ cells, and no single cell type dominated. Because it was considered difficult to elucidate the mechanism based on cell types, next we focused on cytokines and analyzed the expression of mRNAs for IL-4, IL-6, IL-10, and HGF in transplanted BM-MNCs.

The analysis demonstrated higher levels of mRNAs for HGF, IL-10, and IL-4, which are known to regenerate and protect the nerves, compared to that for IL-6, which pro-

motes secondary injury in the spinal cord.

These results confirmed that BM-MNCs used for transplantation had higher levels of mRNAs for HGF, IL-10, and IL-4 than that of inflammatory cytokine IL-6. In particular, HGF mRNA was highly expressed, indicating strong involvement of HGF in the regeneration and protection of spinal cord by BM-MNCs.

7. Analysis of HGF concentrations in the cerebrospinal fluid after bone marrow-derived mononuclear cell transplantation (Chapter 8)

HGF has been reported to stimulate therapy for injured spinal cord, but no study has been reported on HGF distribution in the spinal cord and cerebrospinal fluid (CSF) in dogs. This chapter describes a study that monitored HGF concentrations in CSF samples obtained periodically after BM-MNC transplantation to evaluate the effect of transplantation. CSF samples were periodically collected (immediately prior to and at Days 1, 2, 3, 7, 14, 21, and 28 after transplantation) from 3 of the study dogs in chapter 4, after obtaining the owner's consent, as well as from 2 healthy laboratory Beagle dogs, and the CSF samples were analyzed for HGF concentrations using ELISA.

The analysis demonstrated increased HGF levels for a

certain period (peaking at Day 3) after BM-MNC was transplanted into the subarachnoid space of the spinal dura mater.

Only a limited number of samples have been analyzed to date, and additional cases should be studied for definitive assessment. However, in acute and chronic spinal cord injury in dogs, BM-MNC transplantation is likely to induce spinal cord regeneration by increasing HGF level in CSF.

Many studies have been conducted on spinal cord regeneration, but therapeutic procedure that leads to radical treatment is yet to be developed. In this circumstance, development of novel therapeutic method, such as regenerative therapy, is awaited. Unlike other types of cells that require culturing, BM-MNCs are free of disadvantages, such as bacterial infection, tumorigenesis, and high cost, and can be transplanted at the right timing (acute or subacute phase) in the treatment of spinal cord injury because cell culture is not involved.

The present study demonstrated the efficacy of BM-MNC transplantation in canine spinal cord injury (both acute and subacute), and this technique is expected to help improve patients' QOL as a new therapeutic method.

Studies on the effect of canine aquaporin 5 on the tear secretion

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Keratoconjunctivitis sicca (KCS) in dogs is an abnormality of visual function caused by lack of water in the tear layer that results in chronic inflammation of the cornea and conjunctiva. Autoimmune disease of topical gland nictitating membrane and lacrimal gland has been recognized as a cause of KCS, and the local administration of immunosuppressive drugs has been used to treat pathological changes in tear production glands and clinical symptoms.

The pathology of canine KCS is similar to that of Sjogren's syndrome in humans. Sjogren's syndrome is an autoimmune disease associated with dry eye and dry mouth due to chronic inflammation of the lacrimal and salivary glands. Histopathologically, changes in the distribution of aquaporin 5 have been reported in the lacrimal glands of patients with Sjogren's syndrome.

Aquaporins are water channel proteins that participate in the rapid water movement that occurs in the membrane of various epithelial and endothelial cells. Aquaporin 5 is expressed at the apical site of acinar epithelial and ductal epithelial cells in the lacrimal glands of the mice and humans.

The purpose of this study was to examine the involvement of aquaporin 5 in the tear secretion of dogs and to begin to elucidate the pathogenesis of KCS in dogs. Initially, we showed the genetic sequence of canine aquaporin 5 and inferred the amino acid sequence. Based on these data, we attempted to obtain structural information regarding canine aquaporin 5 since data regarding the structure and function of aquaporin 5 in dogs is not available. Thereafter, we performed absolute quantification by using real-time PCR in order to compare aquaporin 5 mRNA expression in the lacrimal and nictitating membrane glands of the dogs to infer the association between mRNA expression and tear production. We used western blotting and immunohistochemical staining to confirm the presence and to investigate the distribution, respec-

tively, of aquaporin 5 in the lacrimal and nictitating membrane glands of healthy dogs. Finally, the distribution of aquaporin 5 in the nictitating membrane glands of immune-mediated KCS was compared to that of healthy beagle dogs. Image analysis software was used to compare the positive region of aquaporin 5 lacrimal and nictitating membrane glands of healthy dogs to this region in the nictitating membrane glands of immune-mediated KCS dogs.

1. Aquaporin 5 cloning in dogs

We deciphered the genetic sequence of canine aquaporin 5, inferred the amino acid sequence, and attempted to analyze the structural information of canine aquaporin 5 since structural and functional data for aquaporin 5 in dogs is not available. Based on sequence analysis of the unknown region of aquaporin 5 cDNA from the lacrimal and nictitating membrane glands taken from a healthy dog, we analyzed the structural information of canine aquaporin 5 from the estimated amino acid sequence. We determined that the open reading frame of canine aquaporin 5 consists of 795 bases and the amino acid sequence consists of 265 amino acids. Structural analysis of canine aquaporin 5 revealed characteristic aquaporin 5 sequences such as a NPA motif and SRRTS arrays that encode cAMP-protein kinase A phosphorylation site; these characteristics are consistent with those of aquaporin 5 in other mammals. In addition, the amino acid sequence of canine aquaporin 5 showed 94.7% sequence homology with the human aquaporin 5, 90.2% sequence homology with mouse aquaporin 5, and 90.9% sequence homology with rat aquaporin 5. The homologies between the nucleotide sequence and the predicted amino acid sequence of the dog and human aquaporin 5 was higher than the homologies of dog and mouse or dog and rat aquaporin 5. The high homology between canine and human aquaporin 5 suggests that the dog can serve as an animal model of human aquaporin 5 related diseases.

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Aquaporin 5 appears to have functional importance in dogs because the sequence has a very high homology with other mammals, especially in domains with important functions.

2. Aquaporin 5 mRNA expression in the lacrimal and nictitating membrane glands of the dog

In order to compare the expression level of aquaporin 5 mRNA in the lacrimal and nictitating membrane glands that produce tears in dogs, we performed absolute quantitative analysis by using real-time RT-PCR on isolated from the lacrimal and nictitating membrane glands of healthy dogs. We synthesized cDNA from the mRNA (0.5 ng) extracted from the lacrimal and nictitating membrane glands of 8 healthy dogs. The expression levels of aquaporin 5 mRNA were significantly greater in the nictitating membrane glands than in the lacrimal glands. Thus, if equal amounts of nictitating membrane gland tissue and lacrimal gland tissue were compared the nictitating membrane glands would have a greater ability to produce tears.

3. The expression and distribution of aquaporin 5 protein in the lacrimal and nictitating membrane glands of dogs.

Because the expression and distribution patterns of aquaporin 5 in the lacrimal and nictitating membrane glands of dogs were unknown, we confirmed the expression of aquaporin 5 using western blotting and the distribution of aquaporin 5 by immunohistochemical staining in these glands in 5 healthy dogs. Western blotting revealed clear, single bands at approximately 29 kD in the lacrimal and nictitating membrane glands of a dog, thus confirming the expression of the aquaporin 5 protein in these glands in dogs. In addition, since the size of the aquaporin 5 protein in the lacrimal and nictitating membrane glands of a dog were observed to be the same size as the aquaporin 5 protein in rats, the aquaporin 5 antibody used in this study showed cross-reactivity with dogs.

Immunohistochemical staining in the lacrimal and nictitating membrane glands of healthy dogs revealed that aquaporin 5 was localized at the apical site of acinar epithelial and ductal epithelial cells. In particular, aquaporin 5 was strongly expressed at the apical site of acinar epithelial cells in the ductal epithelium cells. The expression pattern of aquaporin 5 in the lacrimal and nictitating membrane glands in the healthy dogs was different from the expression pattern of aquaporin 5 in the lacrimal gland in mice. Aquaporin 5 was found to localize more strongly at the ductal epithelial cells than at the apical

site of the membranes of acinar cells in mice lacrimal glands. However, dogs showed an opposite pattern, with aquaporin 5 localizing more strongly at the apical site of membranes of acinar cells than at the ductal epithelial cells in the lacrimal and nictitating membrane glands. The stronger expression of aquaporin 5 at the apical site of acinar epithelial cells, compared to the ductal epithelial cells, is considered advantageous for the liquid production. Thus, the strong expression of aquaporin 5 in the apical site of acinar epithelium cells might be important for a high amount of tear production in dogs.

4. The distribution of aquaporin 5 in the nictitating membrane glands of KCS dogs.

We used aquaporin 5 immunohistochemical staining to investigate whether the distribution of aquaporin 5 was altered in the nictitating membrane glands of KCS dogs. Immunohistochemical staining revealed markedly decreased expression of aquaporin 5 in the nictitating membranes of glands collected from immune-mediated KCS dogs. We obtained consent from dog owners prior to obtaining tissues. In contrast to our observation of strong aquaporin 5 expression at the apical site of acinar epithelial cells and ductal epithelial cells in the nictitating membrane glands of healthy dogs, the expression of aquaporin 5 was significantly reduced at these sites in the nictitating membrane glands of immune-mediated KCS dogs. A comparison of aquaporin 5 positive regions between lacrimal glands and nictitating membrane glands in healthy dogs did not reveal significant differences. In contrast, statistically significant difference were observed in aquaporin 5-positive regions between the lacrimal and nictitating membrane glands in healthy dogs and the nictitating membrane glands in immune-mediated KCS dogs. The expression of aquaporin 5, which is involved in the regulation of exocrine secretion, was significantly decreased in the nictitating membrane glands of immune-mediated KCS dogs.

The above results indicate that aquaporin 5 is present in the lacrimal and nictitating membrane glands of the dog, which is consistent with observations in other animal species. The expression pattern of aquaporin 5 in the lacrimal and nictitating membrane glands indicates that aquaporin 5 may be involved in tear secretion in dogs. Furthermore, aquaporin 5 was reduced significantly in the nictitating membrane glands of dogs with immune-mediated KCS. Taken together, these observations suggest that aquaporin 5 is involved in tear secretion in dogs and that reduction of aquaporin 5 expression causes a deficiency of aqueous tear in immune-mediated KCS dogs.

Studies on effect of rCaIFN- γ on the propofol-isoflurane anesthesia-induced suppression of anti-tumor immunity in dogs

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Recently, aging of companion animals such as dogs and cats has progressed as well as human, and the clinical cases of tumors have dramatically increased. It is well known that general anesthesia is essential in order to perform the bioimaging diagnosis using MRI, CT etc., the surgical operation or radiation therapy to the case suffered from the tumor. In human medicine, immunity ability decreases by adding the surgical stress which includes anesthesia and an operation to the case suffered from the tumor, transition and the rate of recurrence of a tumor after anesthesia or postoperative will increase (Vallejo et al.). Moreover, it has also been reported that the lymphocyte apoptosis of a dog is promoted by general anesthesia and the decrease of lymphocyte count reduction and the immunity ability accompanying it takes place (Yamada et al.). Therefore, the influence of general anesthesia to antitumor immunity in dogs with the tumor has not been clarified yet, although the decrease in immune ability by general anesthesia is common in human and dogs. In this research, the influence of the propofol-isoflurane anesthesia, which is popular as general anesthesia in dogs, on antitumor immunity was examined in order to establish the recipe which prevents the fall of antitumor immunity ability.

1. Influence of the combination of propofol-isoflurane anesthesia on natural killer cytotoxic activities of peripheral blood lymphocytes in dogs (Chapter 2)

The natural killer (NK) cytotoxic activity of NK cell, NKT cell, killer T cell, CD8 positive T-lymph cell etc, which is a kind of natural immunity and which targets tumor cells, was used as an index of antitumor immunity. The peripheral blood lymphocyte (PBLs) was extracted in dogs, and the NK cytotoxic activity was measured us-

ing the rose bengal method, of which target is the cell line of the canine thyroid cancer, before and after the propofol-isoflurane anesthesia. After the introductory anesthesia by intravenous propofol, the maintenance anesthesia was performed by isoflurane inhaled for 3 hrs at clinical concentration of 2% with pure oxygen. As a result, the significant fall of the NK cytotoxic activity and the number of peripheral blood lymphocytes was observed 24 hrs after anesthesia ($p < 0.05$), indicating that the propofol-isoflurane anesthesia affects antitumor immunity in dogs.

2. Influence of the combination of propofol and 1 hr-isoflurane anesthesia on NK cytotoxic activities of PBLs in dogs (Chapter 3)

To prevent the fall of the antitumor immunity by the propofol-isoflurane anesthesia, the influence of the shortening of the maintenance anesthesia by isoflurane on the antitumor immunity ability was considered from 3 hrs in Chapter 2 to 1 hr. After the introductory anesthesia by intravenous propofol, isoflurane was inhaled at clinical concentration of 2% with pure oxygen for 1 hr as the maintenance anesthesia. As a result, the significant fall of the NK cytotoxic activity was not observed 24 and 72 hrs after anesthesia. Moreover, the number of peripheral blood lymphocytes did not decrease by the maintenance anesthesia for 1 hr, although it significantly decreased by the maintenance anesthesia for 3 hrs ($p < 0.05$). Therefore, it was suggested that the short duration of isoflurane inhalation did not prevent the antitumor immunity in dogs.

3. Effect of rCaIFN- γ on NK cytotoxic activities of PBLs in healthy dogs (Chapter 4)

Since NK cells of human and mice are activated by IFN, antitumor immunity is expected to be reinforced by IFN. As a method of controlling the decrease in antitumor immunity ability, its attention was paid to the canine recombination type interferon gamma (rCaIFN- γ) formulation. First, the effect of the incubation time and the concentration of IFN- γ on the NK cytotoxic activity was examined using PBLs obtained from dogs in vitro. As a result, the time- and concentration-dependent enhancing effect of IFN- γ on the NK cytotoxic activity of PBLs was observed. And the next, the effect of rCaIFN- γ on the NK cytotoxic activity of PBLs was examined using the healthy beagle. The NK cytotoxic activity of PBLs was enhanced intentionally ($p < 0.05$) by rCaIFN- γ 24 hrs after 10,000-units/kg medication. Moreover, the number of PBLs was increased intentionally ($p < 0.05$), indicating the reinforcement of antitumor immunity by rCaIFN- γ . When IFN- γ was added again to PBLs 24 hrs after rCaIFN- γ medication, the significant reinforcement of the NK cytotoxic activity was not observed. Therefore, the further studies will be needed on this phenomenon.

4. Effect of IFN- γ pretreatment on the suppression of the NK cytotoxic activity induced by the propofol-isoflurane anesthesia in canine PBLs (Chapter 5)

In small animal clinic, rCaIFN- γ formulation is using for the treatment of atopic dermatitis in dogs as well as that of several viral infection diseases and tumors. In addition to the effect of rCaIFN- γ on the NK cytotoxic activity of PBLs under conscious condition (Chapter 4), the effect of fCaIFN- γ under the combination of propofol and isoflurane anesthesia was examined in dogs. The influence of the combination of propofol and isoflurane anesthesia for 3 hrs on the NK cytotoxic activity of PBLs was examined 24 hrs after 10,000-units/kg of rCaIFN- γ . Moreover, the susceptibility of PBLs to rCaIFN- γ before and after anesthesia was also examined. As a result, the significant decrease in the NK cytotoxic activity and the number of PBLs 24 hrs after anesthesia was observed in the control group same as the result of Chapter 2. rCaIFN- γ administered 24 hrs before anesthesia significantly inhibited the decrease in the NK cytotoxic activity 24 and 120 hrs after anesthesia. Moreover, the number of PBLs did not change at all after anesthesia in the CaIFN- γ treated group, indicating that rCaIFN- γ admin-

istered 24 hrs before anesthesia inhibited the decrease in the number of PBLs induced by the anesthesia.

5. NK cytotoxic activity, influence of anesthesia/surgical stress and susceptibility to rCaIFN- γ in tumor disease dogs (Chapter 6)

In this report, we examined the influence of propofol-isoflurane anesthesia on the NK cytotoxic activity of PBLs in the healthy dogs in vitro, and the effect of rCaIFN- γ as a regulator until the previous chapters. In this chapter, the NK cytotoxic activity of PBLs 24 hrs before anesthesia and after anesthesia/operation was measured in dogs with tumor disease, and compared with that in healthy dogs. In addition, the medication effect of IFN- γ on PBLs of a tumor disease dogs was also examined. The target animal was standard home-breed dogs who had been seen in the Nippon veterinarian and life science university animal medical center to cure the tumor. Dogs were divided into the malignant tumor disease dog and the benign tumor disease dog according to the histopathological diagnosis of the excised tumor. Although the specification of anesthetization was not carried out in particular for the clinical case, isoflurane was used as the maintenance anesthesia in all the cases. The normal value of the NK cytotoxic activity of a healthy dog was calculated and averaged using the baseline values of the beagle in the previous examination ($n=48$), and compared it with those of a tumor disease dog. As a result, change of the NK cytotoxic activity was not observed before and after anesthesia in both the malignant tumor disease dog and the benign tumor disease dog, although the NK cytotoxic activity in both tumor disease dogs 24 hrs before anesthesia and after anesthesia/operation were lower than that in the healthy dog. As for the medication effect of rCaIFN- γ to PBLs of malignant and benign tumor disease dogs, the enhancing effect on the NK cytotoxic activity was observed before anesthesia, but not 24 hrs after anesthesia/operation.

It was shown that NK cytotoxic activity which is the antitumor immunity ability of a dog decrease by general anesthesia from the above result. Moreover, reducing the decrease by medication shortening of anesthesia time or 24 hours before anesthesia of rCaIFN- γ was shown. It is necessary to inquire, such as a given dose, frequency of administration, etc. of the rCaIFN- γ , it was suggested strongly that rCaIFN- γ can reduce the danger of postoperative rate of a recurrence and metastasis of tumor to the tumor disease dogs.

Studies on canine atopic dermatitis

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Atopic dermatitis in dogs (Canine atopic dermatitis : CAD) is a multifaceted disease associated with exposure to environmental allergens such as pollen, and mold. It is difficult to identify and diagnose the cause of this condition and to cure since it is complex interactions between the host genetics and their environment in many cases. Characteristics of CAD are that the risks of developing clinical symptoms differ between species and yet the onset of this condition is observed at a young age. Relevance for the breed, sex, environment factors and clinical symptoms have not been studied in Japan. The purposes of the current study are to investigate possible pathogenic mechanisms of CAD and its current status in Japan. The results of this study provide clinically relevant basic information on CAD, which may help overcome those animals with CAD and humans suffering from the Japanese cedar pollinosis.

1. Survey results about age of onset and clinical symptoms of atopic dermatitis in dogs. (Chapter 2)

The objectives of the present study were to characterize the age of onset and clinical signs of CAD using descriptive epidemiology. Medical records of 2,338 dogs diagnosed of CAD by veterinarians from 996 hospitals were categorized to gender, geographic distributions, breeds, diet, and smoking status of the owner. Backward elimination in multiple regression analysis was performed, and non-significant variables ($p > 0.05$) were eliminated from the models. Mean age of onset in CAD was 2.56 years old (0.05 SEM). Earlier age of onset was associated with breeds, spayed female dogs, dogs living with a cat, and dogs owned by a smoker ($p < 0.05$). Various clinical signs were associated with breeds, intact female dogs, dogs living with a cat and dogs owned by a smoker ($p < 0.05$). In conclusion, this study explored an association between age of onset and clinical signs in

CAD and breeds, sex or environmental factors.

2. Nationwide survey results of allergen sensitization in canine atopic dermatitis (Chapter 3)

This study analyzed serum allergen-specific IgE (specific IgE antibody : sIgEab) levels in dogs suffering from atopic dermatitis (AD) in Japan. From the results the situation of positive allergen sensitization was examined and the relevance of the mutual positive allergen was investigated. Comparison of positive rate in the environmental allergen, house dust mite showed the highest values 92.6%. This result was consistent with other studies reported in humans and dogs in Japan and Australia. Comparison of positive rate in food allergen, rice (brown) and poultry (chicken & turkey) accounted for the top two categories. This result was consistent with the main raw material in the dog food market. It was revealed that the most frequently used source of protein as dog food materials tended to indicate a high positive rate (Provide range). Among the aggravation agent of AD, each sIgEab value of house dust mite and staphylococci showed stronger positive correlations with the number of positive allergens. Those agents were suggested the possibility of increasing the number of allergen in sensitized individuals by some sort of function.

3. Study of serum allergen-specific IgE testing in normal and atopic young dogs (Chapter 4)

The purpose of this study was to compare the serum allergen-specific IgE levels in 10 atopic and 15 normal young dogs. The strength and the number of positive allergen in both groups were investigated. After comparing the number of positive allergen and average sIgEab level of the normal dog group with the atopic dog group of the young age, almost all of the allergens in atopic dog group showed statistically significantly high level (give range). These results suggest the relevance of these pos-

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itive allergen and the onset of AD. Rice (brown), poultry (chicken, turkey, duck/wild duck) and sheep showed a high rate of positive reaction in atopic dogs. These results suggest that it will reflect the large number of opportunities for exposure as sensitization to food antigens in juvenile stage. Among the aggravation agent of AD each sIgEab level of house dust mite and staphylococcus showed a positive correlation between the numbers of positive allergen in Chapter 3. Also in the study of this chapter, each sIgEab level of house dust mite, Staphylococcus, Malassezia and Tobacco showed that there were positive correlations with the number of positive allergen. Those agents were suggested the possibility of increasing the number of allergen in sensitized individuals by some sort of function. Only in cedar pollen from all the environmental allergen, the average sIgEab level of normal dogs was significantly higher than the level of atopic dogs. It is possible that the timing of serum collection and the vegetation differences in various regions may have influenced this fact. Because the serum from normal dogs were collected from only one area from spring to summer and the serum from atopic dogs were collected from nationwide from Hokkaido to Kyushu district in autumn and winter.

4. Basic study for the creation of dogs experimentally sensitized to Japanese cedar pollen (Chapter 5)

The establishment of next generation therapy which lower than the side reaction of hyposensitization therapy and which enhanced the effect of hyposensitization therapy is desired for overcoming Japanese cedar pollinosis in humans. This basic study was carried out to establish a model of cedar pollen sensitization in dog based on the results for analysis and the current state. There are some reports about dogs showing a positive reaction to cedar pollen antigen but few dogs show symptoms of Japanese cedar pollinosis in clinical case. It was necessary to make the independent sensitization model using beagle sas experiment system, because the autogenesis example of a dog sensitized in cedar pollen alone was extremely rare. Elevated levels of sIgEab has been confirmed by continuous allergen exposure through the lymph nodes after nasal exposure but nasal exposure to cedar pollen alone did not show any clinical symptoms. From these results, it was suggested that the state of the mucosal barrier and the genetic predisposition were more important factors for the establishment of clinical nasal sensitization models rather than antigen exposure conditions. Mast cells including granules of positive with

toluidine blue stain were seen in tissue of the lesion showed a positive for patch test reaction. These results suggest possibility of the immediate hypersensitivity reaction. It was necessary to find out why level of antigen-specific IgE against cedar pollen showed a negative reaction after all. There was a possibility of either unsustainable sensitization due to lack of continuous exposure in addition to resection of sensitized lymph nodes or transition to the delayed-type hypersensitivity reaction due to sensitization to continue more than six months. The skin reaction tests were conducted to confirm that. But the results were negative for both immediate and delayed-type reaction. Therefore, it was suggested that to maintain the sensitization state in the model dog was not possible.

5. Dermal exposure test for the dogs experimentally sensitized to Japanese cedar pollen - Measurement of Transepidermal Water Loss (TEWL) (Chapter 6)

After passing through the process in Chapter 5, the amount of water evaporation (Transepidermal Water Loss : TEWL) through the skin site of allergen exposure were measured and evaluated for the newly dogs experimentally sensitized to Japanese cedar pollen alone.

Before and after the exposure, serum cedar pollen antigen-specific IgE levels were measured by the two different methods (Company A : immunofluorescence assay ; absolute concentration, Company B : enzyme immunoassay ; Relative concentration) and the results were compared. After observing the skin symptoms at each site of exposure, TEWL was measured five times at each site using the portable moisture transpiration meter (VapoMeter[®]). Measurements were calculated by the mean \pm standard deviation and were analysed by Tukey-Kramer multiple comparison procedure. Sensitized dog showed the symptoms of dermatitis at the site of exposure but non-sensitized dogs were asymptomatic. TEWL of exposure site of sensitized dog showed a significantly higher value on the comparison of exposure site of non-sensitized dogs and control site of sensitized dog ($p < 0.05$). Before and after the test, each serum IgE levels of sensitized dogs were higher in both the two companies. High serum cedar pollen IgE dog showed inflammatory response induced by exposure to cedar pollen alone and showed significantly higher TEWL. These results suggest that the onset of dermatitis induced allergen exposure is likely also involved in the state of the skin barrier as well as sensitization. Tape-stripping was carried out in order to damage the skin barrier function

assuming normal skin to interfere with the invasion of allergens. Several reports indicate that dermal sensitization occurs easily in the condition of atopic dermatitis and of low skin barrier function. These results suggest that dermal exposure after the injury the barrier function of skin by tape-stripping is effective in the maintenance of closer to nature sensitization for sensitized dog. It was reconfirmed that measurement of TEWL is useful for objective assessment of skin condition. TEWL is considered to be meaningful as clinical procedure because of non-invasive and less burden to the animal.

6. Evaluation study of hyposensitization therapy for canine atopic dermatitis using the drug score (Chapter 7)

Steroid therapy is a common treatment of canine atopic dermatitis (CAD). Although its suppressive effect on pruritus has been recognized, many side effects that have occurred by relying on the long-term steroid administration. In contrast, hyposensitization therapy (allergen-specific immunotherapy) is positioned as a sole treatment option that encourages the natural healing of allergy in the WHO Position Paper, and is recognized as the best long-term therapy of CAD in veterinary medicine in Europe and the United States. Therefore, we investigated the effects of hyposensitization therapy on CAD and recorded clinical symptom scores and drug treatment scores. We have also examined the effect of steroid dose reduction in clinical symptoms and 13 months after treatment and one month prior to treatment are compared. Hyposensitization therapy was induced into 11 cases of CAD maintained in steroid therapy. Hyposensitization treatment resulted in significant

reduction of the steroid dosage was achieved. 9 out of 11 cases (81.8%) have been evaluated in more than effective for hyposensitization therapy. In order to compare the before and after treatment in steroid dose and the drug score, Wilcoxon's signed rank test was performed, a significant difference was seen in both ($p < 0.05$).

Skin barrier disruption and immune imbalance are two of the major factors to explain the pathogenic mechanisms of CAD. By the observations in these studies, two things have been revealed. Immune imbalance can be induced by increasing allergen-specific IgE levels with repeated exposure of antigen, and hyposensitization therapy is useful for the improvement of clinical symptoms and reduction of steroid dose. It has been shown that skin barrier disruption can be induced by treatment to increase the TEWL as repeated tape-stripping. For the facts of creating experimental sensitized models were possible but the duration of sensitization was difficult, it was considered that some important factors such as genetic predisposition is present in addition to skin barrier disruption and the presence of allergen-specific IgE. Furthermore, it is considered that sustained stimulation of the third deterioration factors other than the skin barrier and immune balance should be necessary for the duration of the dermatitis model.

Further research is expected towards the establishment of treatment method in which enables to avoid or eliminate the effect of the third deterioration factors such as oxidative stress of environmental chemicals.

We hope that current findings will not only add to the general knowledge base of atopic dermatitis (AD), but also to help further develop for overcoming AD in humans and dogs.

Epidemiological studies on metallo- β -lactamases and extended-spectrum β -lactamases producing Gram-negative organisms isolated from bovine mastitis and feces among dairy farms in Japan

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The major aims of the present study were to evaluate the prevalence of plasmid- and chromosome-encoded MBL producers (*S. maltophilia*), MDRP strains, and CTX-M and CMY producers among dairy cattle in Japan. CTX-M producers in particular represent an emerging problem in human and veterinary medicine, as well as public health on a global scale.

Chapter 1 described the mastitis *P. aeruginosa* isolates that exhibited high susceptibility to antipseudomonal drugs. The mastitis *S. marcescens* isolates exhibited high susceptibilities to antimicrobial agents other than ampicillin, first to second generation cephalosporins, tetracycline class. Neither MBL producers nor MDRP strains were detected among these mastitis isolates. They differ from human clinical isolates from both antibiogram and phenotypic perspectives.

In Chapter 2, 11 *S. maltophilia* isolates from an outbreak of bovine clinical mastitis on one farm and two *S. maltophilia* isolates from two other farms had acute mild local mastitis and showed favorable outcomes. Most of these isolates exhibited susceptibility to SXT, chloramphenicol, minocycline, and levofloxacin. The 11 isolates derived from 9 cows in one herd over a 7-month period exhibited a closely related ERIC2 type, and were involved in a herd outbreak of mild mastitis. The incidence of *S. maltophilia* in bovine mastitis was extremely low. Bovine mastitis *S. maltophilia* isolates were similar to human isolates in an antibiogram.

In Chapter 3, the 28 isolates of four genera and four *Enterobacteriaceae* species (i.e., *E. coli*, *K. pneumoniae*, *C. freundii*, and *E. cloacae*) harbored genes of CTX-M-2 (42.9%), CTX-M-15 (25.0%), CTX-M-14, CMY-2, and

CTX-M-2/15/14 with CMY-2. The incidence of *Enterobacteriaceae* producing CTX-M or CMY-2 in bovine feces was 28 (3.1%) of 897 fecal samples. The herd prevalence of these *Enterobacteriaceae* was 20 (5.2%) of 381 dairy farms. The 23 *E. coli* isolates belonged to 18 diverse STs, and exhibited 21 PFGE patterns. Many CTX-M-15-producing *E. coli* isolates were resistant to ceftazidime and fluoroquinolones.

In Chapter 4, genes of three CTX-M clusters (i.e., CTX-M-15/-2/-14) had spread among 68 isolates of four genera and five *Enterobacteriaceae* species (i.e., *E. coli*, *K. pneumoniae*, *K. oxytoca*, *C. koseri*, and *E. aerogenes*) from 59 cows with mastitis on 26 dairy farms. The predominant ESBL producers causing mastitis were CTX-M-2-producing *K. pneumoniae* and CTX-M-15-producing *E. coli*. The incidence of *Enterobacteriaceae* producing CTX-M in bovine mastitis was 68 (0.035%) of 197,002 cows, or 68 (0.024%) of 289,000 mastitis milk samples. The herd prevalence of these *Enterobacteriaceae* was 26 (2.6%) of 1,000 dairy farms. The *E. coli* isolates and *K. pneumoniae* isolates showed diversities of PFGE patterns and MLST types, and RAPD patterns, respectively. The clinical courses of the 59 cows with mastitis caused by CTX-M producers were consistent with cases of conventional coliform mastitis. Taken together, the herd prevalence of four CTX-M or CMY-2-producing genera and seven *Enterobacteriaceae* species in bovine feces and mastitis between 2006 and 2011 was 45 (4.5%) of the 1,000 dairy farms.

Producers of plasmid-encoded MBLs and MDRP strains were not isolated in the present study because carbapenems are not approved for cattle in Japan. In ad-

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dition, these multidrug-resistant organisms would have spread little beyond nosocomial environments. On the other hand, the results of the present study indicate that dairy cattle are a reservoir of CTX-M and CMY-2 genes. In addition, these genes have been spreading to coliform bacteria involving in clinical and subclinical mastitis. The three CTX-M clusters (i.e., CTX-M-1, CTX-M-2, and CTX-M-9) had been emerging among dairy cattle in Japan. The common CTX-M sequence types of bovine *E. coli* and *K. pneumoniae* isolates in the present study are similar to other human and animal isolates in Japan and the rest of the world, with the exception of the predominance of CTX-M-2. The clonal spread of CTX-M may be due to oxyimino-cephalosporins, fluoroquinolones, and first- to second-generation cephalosporins that were approved for cattle. In addition, CTX-M genes are derived from *Kluyvera* spp., which live in dairy environments including soil and animal intestinal tracts. Moreover, CTX-M-producing *E. coli* and *K. pneumoniae* strains have spread beyond nosocomial environments and the three CTX-M clusters have spread worldwide. The CTX-M genes are easily transferred by genetic mobilization units (e.g., plasmid, complex class 1 integrons and transposons). However, the infection sources and the routes of transmission of CTX-M and CMY-2 producers have not yet been identified, and the relationship between the clonal spread of CTX-M or CMY-2 and ceftiofur and other antimicrobial agents used in our clinics or epidemiological factors was not determined in the pres-

ent study. Further studies using PCR-based replicon typing of plasmid DNA are needed to elucidate the relationship between dairy cattle-derived CTX-M and human-derived CTX-M.

CTX-M-producing *E. coli* strains rarely contaminate retail beef, and no study has reported the contamination of retail dairy products with CTX-M producers. These findings suggest that bovine *bla*_{CTX-M}, *bla*_{CMY-2}-positive Enterobacteriaceae may pose a low risk to public health. However, CTX-M and CMY-2 producers are emerging as multidrug-resistant organisms in livestock medicine and a serious public health problem. First, CTX-M-producing *E. coli* and *K. pneumoniae* strains live in animal feces, sawdust bedding, and other environments on dairy farms. Thus, improvements in bedding hygiene and milking practices and disinfection of dairy environments and boots of veterinarians and other workers on dairy farms may prevent their clonal spread and reduce the incidence of mastitis caused by CTX-M-producing coliform bacteria. Second, the prevention of bovine infectious diseases by vaccination and improvements in animal hygiene and animal feeding are needed to reduce the occurrence of bovine infectious diseases and use of antimicrobials. Third, the routine monitoring for CTX-M-producing coliform bacteria derived from feces and mastitis by screening test and the prudent use of oxyimino-cephalosporins and fluoroquinolones are necessary to prevent the clonal spread of CTX-M.

Clinical approach to ovary and oviduct disorders in the green iguana (*Iguana iguana*)

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Mature female green iguanas (*Iguana iguana*) become estrus and lay eggs once a year. The clutch size will be up to 70 eggs. Signs of estrus are loitering, digging behavior and can also be diagnosed by abdominal distention brought by numerous follicles on their ovaries or by eggs in their oviducts which will induce anorexia and respiratory distress. These symptoms are commonly seen in normal female green iguanas. Generally, these symptoms will fade out after oviposition. But some will collapse or die before oviposition if their general condition is poor. Therefore, we need to establish the methods of evaluating their general condition and master the techniques of diagnosing their pathologic conditions.

As ovary and oviduct disorders are commonly seen in green iguanas, ovariosalpingectomy is recommended in particular cases. However, surgical operation on iguanas has high risk without accurate examination.

In this study, we reviewed the diagnosing methods to know the condition from their clinical symptoms, and retrieved availability of complete blood count and biochemical examination and image diagnosis on histopathologically searched ovaries and oviducts extracted from female green iguanas. Histopathologic indexes are conducted to classify the pathological condition of ovary and oviduct on their estrous stage. Stasis of follicles and egg binds are classified as "functional disability" and lecithal ascites, granulomatous ovaritis, salpingitis, and neoplasm of ovary are classified as "morphological disability".

Since surgical operation will be the option of ovary and oviduct disorders caused by morphological disability, it is

important to diagnose the ovary and oviduct condition before the operation by non-invasive method.

If abdominal pains are seen during the clinical symptom observation other than signs of estrus, there is high possibility of carrying ovary and oviduct disorders caused by morphological disability.

Complete blood count and biochemical examination does not have an ability of diagnosing ovary and oviduct disorders specifically, but able to evaluate the general condition. To avoid the risk of surgical operation, complete blood count and biochemical examination is suggested to be done before ovariosalpingectomy on every individual.

By using both X-ray and ultrasound examination, it is able to evaluate the condition of ovary and oviduct objectively. Especially, these image diagnosis methods were highly efficient to diagnose ovary and oviduct disorders caused by morphological disability.

Early evaluation of general condition and confirmation of clinical condition are the most important work in clinical approach to evaluate and treat mature female green iguanas. During the observation period which will usually last for several months, periodical complete blood count and biochemical examination, X-ray and ultrasound examination are recommended to confirm the condition of ovary and oviduct. When a morphological disability is found, ovariosalpingectomy will be the option.

This study provided the classification method of ovary and oviduct condition of female green iguanas, and contributed the diagnosing methods in clinical approach.

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Studies on the development of the new extra-luminal prostheses (Parallel loop line prostheses : PLLP) for the surgical treatment of canine tracheal collapse, and its usefulness

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Tracheal collapse is a tracheal and bronchial disease which occurs in dogs, cats, and humans. Although, traumatic injuries, endotracheal tumors, extratracheal tumors which compress the trachea, and tracheomalacia has been suggested to influence the occurrence of tracheal collapse in dogs, little is known about the pathogenesis of canine tracheal collapse. In dogs, tracheal collapse was first reported by Dr. Baumann R., and since then, the disorder has been reported throughout the world. The condition is commonly encountered in small-breed dogs, such as Yorkshire terrier and Pomeranian with increased frequency at 2-3 years old younger dogs or 8 years and older senile dogs. Labored respiration during inspiration, productive or non-productive cough and goose sounding type cough called goose-honking cough are common symptoms of the disorder. Severe tracheal collapse cause respiratory distress on inspiratory and expiratory phase, and progression of the disorder lead to lethal outcome because of respiratory distress and cardiac failure. Therefore, surgical treatment has been needed to restore the collapsed trachea to normal tracheal diameter.

Various surgical procedures for tracheal collapse have been described, including placement of extraluminal prostheses using modified external syringes, and endotracheal stenting introduced from human medicine. However, complications associated with these procedures are frequent. Thus, long-term follow-up results after these surgeries were unsatisfactory. Because of these factors, the author developed an extraluminal prostheses using acrylic linear which has advantages in tissue affinity, less risk of postoperative complications, facilitate rapid adaptation to individual patients. In the present study, I evaluated tissue affinity of the material used for the prostheses. Moreover, dogs with tracheal collapse were treated

by surgical correction using the prostheses to evaluate the efficacy. Additionally, pathological study was conducted in trachea at necropsy of the dogs with tracheal collapse. In addition, the occurrence of the disorder in Japan was examined retrospectively.

1. Retrospective research on the epidemiology of canine tracheal collapse in Japan : 300 dogs without respiratory disorders.

In this chapter, dogs kept in standard home were surveyed on the presence or absence of tracheal collapse to clarify the occurrence frequency and age at onset of the disorder in Japan. Three-hundred dogs presented to a veterinary hospital without complaints of respiratory disorders were included in the study. Tracheal collapse was diagnosed by cervical and thoracic radiography at inspiratory and expiratory phase, and tracheal endoscopy under general anesthesia. Moreover, severity of the tracheal collapse was graded according to the classification described by Dr. Tangner and Hobson. Narrowing of the trachea was also evaluated by sky view of the radiograph, and diagnostic value of the view was examined.

Incidence rate of the tracheal collapse in 300 dogs without respiratory symptoms such as cough and abnormal respiratory sound was 30.3 %. Severity of the tracheal collapse found in these dogs were graded 1 to 3. It was interestingly that most of these dogs with tracheal deformation were young small-breed dog. Moreover, Sky view of the cervical and/or thoracic radiography was useful in the early diagnosis of tracheal collapse.

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2. Retrospective research on the epidemiology of canine tracheal collapse in Japan : 250 dogs with tracheal collapse.

In this chapter, 250 dogs with tracheal collapse treated by surgical correction were surveyed to clarify the generation status of the disorder in Japan. Severity of the tracheal collapse was graded as described in the last chapter. As the results, 3.6% of the dogs were considered as grade 1, 13.6% were graded as 2, 41.2% were graded as 3, and 41.6% were graded as 4. Small-breed dogs, such as Yorkshire terrier, Pomeranian, and Chihuahua were accounted for 88% of the all dogs. The majority being males, and the median age and body weight were 7 years and 3.4 kg, respectively. Before surgery, cough, honking cough, and respiratory distress were found in 96.8%, 55.2%, and 52.4% of the dogs, respectively. Although, the severity of tracheal collapses were associated with clinical symptoms, there was no relations in some dogs. Therefore, it had been suggested that tracheal collapse was potential in these dogs. The degree of collapse determined by sky view of the radiograph and endoscopy with the compression of neck were similar. Although, both methods increase the degree of collapse, the sky view of radiograph do not requires general anesthesia. Thus, sky view of the radiograph was considered as a superior method to determine the strength of trachea and early diagnosis of the collapse.

3. Immunohistochemical assessment of the tracheal cartilages in dogs with tracheal collapse.

In dogs, tracheal collapse is a common disorder. However, little is known about the pathogenesis. Although, decrease of the cartilage cells, alternation of the cartilage matrix such as replacement of hyaline cartilage by fibrous tissues and fibrous cartilage has been reported, no studies have investigated the alternation of collagen. Therefore, the aim of the present study was to investigate the proteoglycan, cartilage calcification, collagen type 1 and 2 in the healthy and collapsed tracheal cartilage with histochemically and immunohistochemically procedures, and evaluate their relationship with the pathogenesis of tracheal collapse. In the collapsed tracheal cartilages, cartilage cells and matrix determined by Alcian Blue staining, collagen type 1 in the cartilage cells, collagen type 2 in the cartilage matrix of tracheal cartilages were decreased, and collagen type 1 in the marginal region of tracheal cartilages were increased. Since, these changes were suspected as a primary alteration of collapsed cartilages, it had been suggest-

ed that inherited factors may be related to pathogenesis of the tracheal collapse.

4. Inspection and tissue affinity of the material in Parallel Loop Line Prostheses (PLLP).

The material used in PLLP for surgical treatment was plastic optical fiber (POF), which was not developed for medical purposes. Thus, analysis of the component of the material was carried out, and safety and tissue affinity were evaluated in the healthy dogs and the dogs with the tracheal collapse. Dogs were implanted the POF in subcutaneous tissue and PLLP was placed to peritracheal region. Tissue affinity of the POF was evaluated histopathologically.

As the results, acrylic linear of POF had double-layered structure, the core was polymethylmethacrylate (PMMA), and the clad layer was copolymer of polyvinylidenedifluoride (PVDF) and polytetrafluoroethylene (PTFE). These materials were used as intraocular lens and vascular suture, and they were widely known as a safety for the biochemical materials. Histopathological examination revealed the capsulation which suggest the minimize reaction to the implants. Other factors suggesting the rejection responses were not observed. These results indicates that the PLLP has superior tissue affinity.

5. Progress in PLLP as a surgical treatment for canine tracheal collapse.

Newly-developed extraluminal prosthese PLLP using acrylic linear was indicated to dogs with tracheal collapse, and effectiveness on biological function derived from the unique figure was compared with traditional surgical treatment. Moreover, postoperative survival was analyzed after PLLP in the treatment of 250 dogs with tracheal collapse.

PLLP was developed by using the thermal reversibility of plastic optical fiber, and casted in a staggered cylindrical shape. The 250 dogs included in the study were treated by restoring cervical trachea with PLLP. In 25 of the 109 dogs with caudal collapse of thoracic trachea were also treated by restoring thoracic trachea with thoracotomy procedure. Longest survival time after surgery was 4,178 days (11years and 5 month). The median survival was 956 days. The estimated survival rate was 96% at 14 days and 86% at 6 month. These survival rate were higher than the previous reports using extraluminal prostheses or endotracheal stenting. The median survival of the dogs with tracheal collapse graded as 1, 2, 3, and 4 were 857 days, 1698 days, 964 days, and 877 days, respectively. In all grades, fatal cases were older than the

survival cases at the time of surgery. Therefore, postoperative survival has less relation with the severity of tracheal collapse. Moreover, it has been suggested that the advanced age were associated with a reduced survival time after surgery. Without combination of bronchial collapse or other respiratory disorders, cough and respiratory distress were clearly improved after surgery, and PLLP placement provided a better prognosis.

From these studies, pathophysiology of tracheal collapse has been partly understood. Moreover, superior tissue affinity and durability of the PLLP has been shown. These results suggest that the treatment with PLLP placement can be a radical cure for canine tracheal collapse which has been considered as an intractable disorder, and can provide a favorable prognosis in dogs with tracheal collapse.

Comparative studies on behavioral characteristics and expression levels of prolactin receptor gene in the choroid plexus between Wistar and Wistar-Imamichi rats

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Wistar-Imamichi is a strain separated from Wistar rats based on the constant estrus cycle and passive nature, which are excellent characteristics as a laboratory animal. However, genetic feature correlating with the behavioral characteristics is not known. Behavior is regulated by endocrine system as well as central nervous system. Prolactin is a peptide hormone mainly synthesized in the anterior pituitary, and exerts many physiological functions such as lactogenesis, ovary function, immune function, and osmoregulation. Prolactin is also known to act on the central nervous system to enhance maternal behavior and stress tolerance. In lactating Wistar-Imamichi, ultrasonic sound of pups enhances prolactin secretion and maternal behavior of the mother. In the present study, the correlation between the prolactin action on the brain and passive nature of Wistar-Imamichi was investigated by behavioral and molecular analyses.

First of all, Wistar and Wistar-Imamichi were subjected to an open field test. The activities in the novel open field of Wistar-Imamichi was significantly lower than that of Wistar at both juvenile and adult stages. These results may reflect the passive nature of Wistar-Imamichi. Next, maternal behavior was compared between Wistar and Wistar-Imamichi. Pups were separated from their mother rats for fifteen minutes at day 3 of lactation and maternal behaviors of the mother rats toward returned pups were examined. No difference was observed for licking, retrieving, and crouching behaviors between both rat strains, whereas nest-building after the returning of pups was significantly delayed in Wistar-Imamichi compared to Wistar. Since a major purpose of nest-building is to hide the pups, the delay of nest-building may also reflect the passive nature of Wistar-Imamichi.

In the open field test, activities of female rats were

higher than those of male rats in both strains. As a sex steroid hormone, estrogen, is known to influence on some kinds of behaviors, effect of 17β -estradiol (E_2) on the activity at the open field test was examined in female Wistar and Wistar-Imamichi. Ovariectomy significantly reduced the activity at the open field test in both rat strains. Administration of E_2 significantly increased the activity in Wistar-Imamichi but not in Wistar. These results suggest that estrogen sensitivity of Wistar-Imamichi is higher than that of Wistar.

Estrogen stimulates the expression of prolactin gene in the anterior pituitary gland. Therefore, effect of estrogen on the activity of Wistar-Imamichi in the open field test may be mediated by prolactin. The effect of prolactin on the central nervous system is depend on the prolactin receptor (PRLR) in the choroid plexus, which is a site of receptor-mediated prolactin transport from the blood to cerebrospinal fluid. There are five alternative first exons, i.e., E1₁, E1₂, E1₃, E1₄, and E1₅, and expression of PRLR in the choroid plexus is regulated by transcriptional activation of E1₃, E1₄, and E1₅. Real-time PCR analysis revealed that E1₃, E1₄, and E1₅-PRLR mRNA levels increased in the choroid plexus in male and female rats during postnatal development and lactation, with markedly higher level of E1₄-PRLR mRNA. In the developmental and lactating stages, expression levels of these three mRNA species in Wistar-Imamichi were greatly higher than those of Wistar. Plasma prolactin levels of male and female Wistar-Imamichi increased during the postnatal development. These results suggest that prolactin action on the brain of Wistar-Imamichi is stronger than that of Wistar due to higher efficiency of incorporation of blood prolactin into the cerebrospinal fluid through larger amount of PRLR in the choroid plexus.

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In conclusion, Wistar-Imamichi shows lower activity in the novel open field and delay of nest-building during lactation compared with Wistar, whereas expression levels of PRLR mRNA in the choroid plexus during postnatal development and lactation are higher in Wistar-Ima-

michi than in Wistar. These findings suggest that enhanced prolactin action on the brain through PRLR in the choroid plexus may account in part for the passive nature of Wistar-Imamichi.

Effects of the Intestinal Microflora on Antioxidant Enzyme Activity in Mouse Intestine

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The westernization of dietary habits is associated with an increase in the number of patients with inflammatory bowel disease (IBD) and colon cancer in Japan. Oxidative stress is believed to play a key role in the pathogenesis of IBD and colon cancer-related intestinal damage. While physiological concentrations of reactive oxidative species (ROS) *in vivo* are known to be involved in cell signaling pathways and survival from invading pathogens, unbalanced and elevated levels of ROS may contribute to the development of various diseases, such as IBD, cancer, hypertension, diabetes, atherosclerosis, inflammation, and premature aging. Antioxidant enzymes play an important role in preventing oxidative stress by acting on the ROS generated *in vivo*. Superoxide dismutases (SODs) are the most important antioxidant enzymes in the antioxidant defense system against the superoxide ($O_2^{\cdot-}$). At present, three distinct SOD isoforms have been identified in mammals. One SOD isoform has Cu and Zn in its catalytic center (CuZnSOD) and exists in the intracellular cytoplasmic compartment, the second has manganese (Mn) in its catalytic center and exists in the mitochondria of aerobic cells, and the third is an extracellular SOD. SODs are the first line of defense in dismutation of excess $O_2^{\cdot-}$, which causes tissue disorders because SODs convert $O_2^{\cdot-}$ to molecular oxygen and H_2O_2 . H_2O_2 is subsequently catabolized to water by catalase (CAT) and glutathione peroxidase (GPx). Of the different SOD enzymes, CuZnSOD is the most abundant and widely distributed enzyme in several tissues.

One-hundred trillion bacteria, representing hundreds of species, live in homeostasis within the intestinal immune system. Maintenance of homeostasis in the intestinal microbial milieu is critical for preserving health. IBD is a group of idiopathic syndromes in humans marked by unrestrained gastrointestinal inflammation, and the gut

microbial community is significantly altered in IBD patients. Therefore, interactions between oxidative stress and the intestinal microflora may be central to the development and severity of IBD. However, little is known regarding the interactions between intestinal microflora and antioxidant enzyme activity. In the gastrointestinal tract, SOD activity has been reported to determine the therapeutic SOD value in an experimental model, but the effect of intestinal microflora on antioxidant enzyme activity remains unclear.

In the present study, the association between intestinal microflora and antioxidant enzyme activity, particularly SOD activity, was investigated in the intestinal tissue of germ-free (GF) mice. GF mice appear to be a useful model for investigating the possible contribution of bacterial flora to SOD activity in the intestine.

1. Antioxidant enzyme activities and gene expression in the intestinal tract of GF mice and conventional (CV) mice

This study used some GF mice to examine the effects of intestinal microflora on SOD activity in the cecum. In immature or adult GF IQI strain and FVB/N strain mice, the SOD activity, protein expression, and mRNA expression of some tissue were used and compared with those in CV mice. SOD activity as well as protein and mRNA expression in the ceca of GF IQI and FVB/N mice were significantly higher than those in the ceca of CV mice. Consistent with these results, total SOD activity in the ceca of conventionalized (CVz) mice decreased to the total SOD activity levels observed in the ceca of CV mice. Furthermore, no differences in SOD activities in the liver, thymus and spleen were observed between GF and CV mice. These results suggest that the antioxidant defense system in the mouse cecum is influenced by the in-

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testinal microflora that downregulate SOD activity.

Subsequent examinations confirmed that the upregulation of SOD activity in GF mice occurred in the cecal mucosa using an SOD activity assay and immunohistochemistry. These results clearly demonstrate that the total SOD and CuZnSOD activities in the cecal mucosa of GF mice were significantly higher than those in the cecal mucosa of CV mice, but not in cecal tissue other than the mucosa. Furthermore, immunohistochemistry results revealed that CuZnSOD expression in the cecal mucosa was high in all GF mice. These results support the finding that upregulation of SOD activity occurs in the mucosa of GF mice. In addition, total SOD activity in the cecal mucosa of pseudo-GF mice treated with antibiotics (1,500 U/ml penicillin-G and 2 mg/ml streptomycin sulfate for 4 days) was significantly higher than that in cecal mucosa of CV mice. Thus, these data confirmed that SOD activity is influenced by the mouse intestinal microflora that downregulate SOD activity in the cecal mucosa.

Next studies examined the effects of the intestinal microflora on CAT and GPx activities in the cecal mucosa. The CAT activities in the cecal mucosa of GF mice were significantly higher than those of CV mice. CAT mRNA expression showed similar tendencies. In contrast, The GPx activities in the cecal mucosa of GF mice were significantly lower than those of CV mice. GPx mRNA expression showed similar tendencies. The data suggest that these antioxidant enzymes are differentially influenced by the intestinal microflora in the cecal mucosa. In addition, SOD activities at five sites in the intestinal mucosa (duodenal, jejunal, ileal, cecal, and colonic) were higher in GF mice than those in CV mice. Total SOD and CuZnSOD activities at the five intestinal mucosa sites in GF mice were significantly higher than those in CV mice. CuZnSOD mRNA expression showed similar tendencies with respect to SOD activity in the intestinal mucosa. Thus, these data suggest that SOD activity was influenced by the intestinal microflora in the intestinal mucosa of all mice.

2. Influence of intestinal microflora on SOD activity in mouse cecal mucosa

In this study, the gnotobiotic mice were used to examine specific intestinal microflora that downregulate SOD activity in the cecal mucosa. Gnotobiotic mice were pro-

duced by inoculating *Escherichia coli* E-17, *Lactobacillus*, *Bacteroides*, or chloroform-treated feces (CHF) into GF IQI mice. Murine CHF was prepared from a 1 : 100 fecal suspension taken from CV IQI mice in 3% chloroform, as previously described. The *Lactobacillus*, *Bacteroides*, or CHF cultures were orally inoculated into GF mice that were previously administered *E. coli* E-17. Activity in the cecal mucosa of GF and *E. coli*, *Lactobacillus* or *Bacteroides*-treated mice was significantly higher than that in CHF, CVz, and CV mice. In addition, CuZnSOD mRNA expression in the cecal mucosa of GF and *E. coli* mice was significantly higher than that in CHF and CV mice. These results suggest that SOD activity in the cecal mucosa was downregulated by CHF inoculation.

Next studies examined the transcription factor that upregulates CuZnSOD mRNA in the cecal mucosa of GF mice. Transcriptional regulation of CuZnSOD mRNA involves several transcription factors. These transcriptional factors, such as thyroid hormone receptor β , nuclear factor-kappa beta (NF - κ b), selective promoter factor 1 (SP1), and CCAAT-enhancer-binding protein alpha (C/EBP α), play important roles in regulating the constitutive or inducible CuZnSOD expression levels. TR β 1, NF - κ b, SP1, and C/EBP α mRNA expression levels were significantly higher in the cecal mucosa of GF IQI mice than those in the cecal mucosa of CV mice, suggesting that transcriptional regulation of CuZnSOD mRNA is influenced by the intestinal microflora in mouse ceca. However, identification of the specific transcription factor that upregulates CuZnSOD mRNA in GF cecal mucosa remains unclear.

3. Conclusion

These results suggest that the intestinal microflora downregulate SOD activity in the cecal mucosa. Furthermore, the results suggest that SOD activity in the cecal mucosa was downregulated by CHF inoculation. In addition, CAT and GPx activities in the cecal mucosa were differentially regulated by the intestinal microflora. Finally, the results indicated that the transcriptional regulation of CuZnSOD mRNA was influenced by the intestinal microflora in the mouse cecum, but the mechanism by which intestinal microflora downregulated CuZnSOD activity in the mouse cecal mucosa remains unclear. This is the first report on the relationship between antioxidant enzyme activity and intestinal bacteria in mice.

The effect of carbohydrate quantity on postprandial plasma glucose concentrations and insulin requirements in dogs.

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Foods that contain carbohydrate have the greatest effect on blood glucose compared to foods that contain protein or fat. Carbohydrate Counting (Carbo count) is a meal planning technique for managing blood glucose levels and has been applied human patients with diabetes mellitus. Carbo count can help to keep diabetic patient's blood glucose levels in the target range.

In veterinary medicine, diabetic dogs were treated with insulin and dietary therapy. Dietary strategies for diabetic dogs were almost based on their ideal daily energy requirement (amount of calories/day). However, relationship between carbo count and insulin requirement has not fully understood. Therefore, the purpose of this study is to investigate whether contents of carbohydrate affect their post prandial glucose concentration and insulin requirement in healthy and diabetic dogs.

The purpose of the second chapter is to investigate whether different amount of carbohydrate affects their postprandial plasma glucose and insulin concentration in healthy dogs. In the present study, four dogs maintained in our laboratory for research, were used. All dogs were fed on diet with three regimen, 1 ; commercial diet (c/d wet diet, Hills-Colgate Japan Ltd., Tokyo, Japan), 2 ; c/d wet diet with tapioca flour and 3 ; c/d wet diet with cornstarch flour to change the total amount of carbohydrate.

Significant difference was not observed in mean temporal glucose concentration and glucose area under the curve (AUC) between three diet regimen. Insulin AUC with tapioca flour and cornstarch flour diet was tends to higher than that with c/d wet diets. Healthy dogs did not increase in postprandial glucose concentration by feeding carbohydrate added diet. It might be considered that high insulin secretion allow maintain normoglycemic

status in healthy dogs.

The purpose of the third chapter is to investigate whether amount of carbohydrate affects their insulin requirement in three diabetic dogs. All dogs were fed on two different amount of carbohydrate food (c/d wet diet and c/d wet diet with cornstarch flour), and then maintained normoglycemic status by connecting to an artificial pancreas apparatus. Insulin requirement in c/d wet diet with cornstarch flour in three diabetic dogs was 31, 20, 59% higher than that in c/d wet diet, respectively. The amount of carbohydrate divided by total unit of rapid action insulin (1 carbo ; amount of carbohydrate/unit) which calculated by artificial pancreas was estimated as 6.6, 11.3 and 12.6 g (average of 10.2g) for three diabetic dogs, respectively. Therefore, it is suggested that carbo count in diabetic dogs has individual difference by their insulin sensitivity.

The purpose of the fourth chapter is to investigate whether average carbo count (1 carb = about 10 g) in the third chapter is useful for estimating insulin requirement in two diabetic dogs treated with intermediate acting insulin preparation. Both dogs were given three regimen, 1 ; c/d wet diet (control diet) with intermediate acting insulin (NPH insulin) (control), 2 ; c/d wet diet containing cornstarch with intermediate acting insulin (cornstarch) and 3 ; c/d wet diet containing cornstarch with intermediate acting insulin and rapid acting (cornstarch+R). Estimated dose of rapid acting insulin (R) was calculated by carbo count. Mean glucose concentrations in dogs with cornstarch were higher than those in control dogs. However, the glucose concentration of cornstarch+R was lower than that in control. This results showed that supposed value for carbo count (1 carb, [1 unit] = about 10 g) is greater effect for decreasing blood

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glucose concentrations as we expected. Therefore, we should further studies for confirming appropriate carbo count using more appropriate numbers of diabetic dogs.

In conclusion, these results indicate that the total amount of carbohydrate in diet was considered as important factor for determining insulin requirement in dia-

betic dogs. However, it is suggested that carbo count with each diabetic dog have individual difference, therefore, future studies include increasing sampling size, evaluating another breed and testing with clinical setting rather than laboratory setting in diabetic dogs.

Evaluation of salivary Chromogranin A concentration as an indicator of stress in dogs

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When animals (including human) were stressed, two reactions is occasioned in vivo. One is Hypothalamic-Pituitary-Adrenal system, called HPA system. When the system is activated, cortisol is secreted from the adrenal cortex. Another one is Sympathetic nervous-Adrenal Medullary system, called SAM system. When the system is activated, release of catecholamine into the blood is promoted.

Currently, in research and clinical practice, cortisol has been often used as an indicator of stress reflects the activation of HPA system. Cortisol reacts to both physical and mental stress. But it reacts to only severe mental stress. In order to cover this shortcoming, salivary Chromogranin A is used as an indicator of stress in human. Chromogranin A is acidic glycoprotein that is stored in the sympathetic, and adrenal medullary chromaffin cells. Further, in humans and rats, there has been also reported that this concentration is increased by the response of SAM system. However, in dogs, it has not been clearly demonstrated that salivary Chromogranin A can be used as an indicator of stress. And it has not been established the reaction mechanism and characteristics of Chromogranin A.

In chapter 1 of this study, we examined salivary Chromogranin A is available as an indicator of stress in dogs. In chapter 2, it was examined whether salivary Chromogranin A concentration in dogs increases due to SAM system response as well as humans and rats. In chapter 3, we examined the reaction of Chromogranin A when the dogs are given an exercise stress and mental stress.

In chapter 1, we performed fake ultrasonography examination as mild stress in dogs, and measured concentrations of salivary Chromogranin A and cortisol which has already been used as an indicator of stress. The Chromogranin A concentrations showed a significant

positive correlation in the cortisol concentrations. It was thought that Chromogranin A were elevated in response to stress. It has been suggested that Chromogranin A has a high credibility as stress indicators. And salivary Chromogranin A concentrations could be measured by less amount of saliva than salivary cortisol concentrations. It has been suggested that it's easy to measure a small amount of saliva (e.g. small breed), and sample of continual collection. However, Chromogranin A responded almost the same as cortisol in this study. It was thought that Chromogranin A might have the reaction of HPA system as well as cortisol.

In chapter 2, it was examined whether salivary Chromogranin A of dogs showed the reaction of SAM system as well as humans and rats, or showed HPA system reaction. Each dog was administrated noradrenaline preparations acting on the adrenal medulla, and synthetic ACTH preparations acting on the adrenal cortex, and then we measured the value of salivary Chromogranin A and cortisol. The cortisol concentrations showed increase as administrated synthetic ACTH preparations. The Chromogranin A concentrations showed increases as administrated noradrenaline preparations. It was thought that salivary Chromogranin A of dogs was subject to effect of SAM system and cortisol was subject to HPA system. And it has also shown that salivary Chromogranin A concentrations increased within 5-10 minutes since got stimulated of SAM system. These results provided that stress of fake ultrasonography examination in chapter 1 activated both HPA and SAM system to an equal degree.

In chapter 3, in order to investigate the characteristics of stress as an indicator of Chromogranin A, we examined the reaction of Chromogranin A when given an exercise stress and mental stress. And in order to investi-

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gate the difference between Chromogranin A and cortisol, we measured value of Chromogranin A and cortisol when given the stress consisting mainly of exercise such as running, and the stress such as holding as much as possible not to give exercise. As a result, cortisol has been shown to increase than Chromogranin A when gave exercise stress, and Chromogranin A has been shown to increase than cortisol when gave holding stress as mental stress. These results provided that HPA system reacted dominantly due to exercise stress, and SAM system reacted dominantly due to mental stress. It was thought that we can guess how the stress works individually by measuring Chromogranin A and cortisol when

given the stress.

As a result, it was found that salivary Chromogranin A could be used as stress indicators in dogs, and showed SAM system reaction as well as humans and rats. Compared to cortisol, Chromogranin A indicated a lower concentration when exercise stress given, and indicated a higher concentration when mental stress given. These results provided that by comparing measured Chromogranin A and cortisol, we can determine not only the various life stimuli whether has become stressful, but also the stress whether has worked as mental stress. In addition, it was thought that the concentration of Chromogranin A leads to an objective evaluation of mental stress.

Study of structure and expression of *Uncoupling Protein 4 and 5* genes in Dog

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Uncoupling Proteins (UCPs) present in the inner mitochondrial membrane are a proton transporter family and implicated in non-shivering thermogenesis, regulation of energy and reactive oxygen species production by driving the ATP synthase pathway via regulation of the proton electrochemical gradient. Of five structural homologues (UCP1 to UCP5), UCP4 and UCP5 are principally expressed in the central nervous system (CNS). Their physiological function has not yet been established, but they regulation of oxidative stress and Ca^{2+} homeostasis. Therefore they are performed a case-control study in neurodegenerative disease as Alzheimer disease and schizophrenia. In dog, despite of dog UCP1-3 have already been identified, however, dog UCP4 and UCP5 have few research. Here we report that analysis of structure and expression of dog UCP4 and UCP5. The dog *UCP4* and *UCP5* cDNAs have 972 bp and 978 bp ORF,

which encoded a protein of 323 and 325 amino acids. GenBank accession number for these sequences were registered as AB780976 (*UCP4*) and AB612119 (*UCP5*). It showed that dog *UCP4* and *UCP5* gene has 9 and 10 exons by comparison with the gene structure in this study and dog genome sequences. Expression of *UCP4* and *UCP5* genes analyzed by RT-PCR, real-time RT-PCR (only *UCP5*) and fluorescent immunohistochemistry. By RT-PCR analysis, it was found that both *UCP4* and *UCP5* were seemed to express predominantly in CNS. In addition, fluorescent immunohistochemistry confirmed that *UCP4* and *UCP5* were almost all investigated dog tissue (up to 23). The results showed that the *UCP4* and *UCP5* expressed ubiquitously in dog.

Our findings may provide useful information to reveal physiological function for *UCP4* and *UCP5* genes.

Molecular pathological analysis about the association of Bovine papilloma virus with oncogenesis in Equine sarcoid

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Equine sarcoids are the most common skin tumors in horses and donkeys, and this disease is characterized by the proliferation of dermal fibroblasts. Infection with bovine papillomavirus types 1 and 2 (BPV-1 and -2) has been implicated as the major etiological factor in equine sarcoids. Although BPV DNA was previously detected in 86%-100% of equine sarcoids using PCR, the association between BPV infection and oncogenesis remains unclear. In this study, to clarify the involvement of BPV in development of equine sarcoids, we performed histopathological analysis of five cutaneous tumors suspected of being equine sarcoids and molecular pathological analysis for detection of BPV DNA and localization of BPV DNA and its protein. Furthermore, for the analysis of the association between BPV infection and oncogenesis of equine sarcoids, we immunohistochemically investigated the expression of the platelet-derived growth factor β receptor (PDGF- β R), which is reported to bind to BPV E5 in tumors. The five cutaneous tumors suspected of being equine sarcoids were histopathologically analyzed using H&E staining. As a result of H&E staining, hyperkeratosis, acanthosis, rete pegs formation, and picket fence pattern were observed in the epidermis and storiform pattern of growth in the dermis of all tumor samples. On immunohistochemistry, the tumor cells were positive for vimentin and negative for S-100, GFAP, α -SMA, and factor VIII for all five tumor samples. On the basis of these results, it was concluded that these five cutaneous tu-

mors were equine sarcoids. BPV DNA was detected in all five cases of equine sarcoids using PCR. Sequencing of the BPV E5 region of BPV DNA revealed the presence of BPV-1 in four tumor samples and BPV-2 in one tumor sample. Localization analysis of BPV E5 DNA performed using in situ PCR revealed that tumor cells in the dermis of four tumor samples were positive, and two cases among them were positive in epidermis. In immunohistochemical staining using anti-BPV L1 antibody, positive expression was observed in tumor cells in the dermis of two tumor samples; however, no positive expression was observed in the epidermis of all samples. Although BPV L1 protein was not detected in the epidermis, BPV E5 DNA was detected. It has been suggested that the virus was unproductive and in the latent infection state. On the other hand, having detected BPV L1 protein in tumor cells, we considered that the virus was productive. In addition, the oncoprotein of BPV is completely involved in oncogenesis of equine sarcoids. PDGF- β R expression in the tumor cells was immunohistochemically analyzed to determine the association between oncogenesis of equine sarcoids and BPV infection. PDGF- β R expression was observed in four samples of equine sarcoids. On the basis of this result, we concluded that this receptor was involved in equine sarcoid formation. The present study revealed the association between BPV infection and oncogenesis of equine sarcoids.

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A study on the regions with high body pressure and decompression using an urethane foam mat with low rebound properties in beagles under general anesthesia in supine and lateral positions

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In modern veterinary medicine, animals are sometimes given general anaesthetic agents even when the treatment, examination, and local surgery do not require anaesthesia. General anaesthesia is beneficial in that the animals can receive veterinary care safely and painlessly. However, the safety of the use of anaesthetics cannot always be ensured because of its sedating effect on the central nervous system. Therefore, to ensure safety and the smooth progression of surgery under anaesthesia, veterinary nurses assist in anaesthesia induction, scrub/circulating nursing and placing the animal in surgical position in order to obtain optimal surgical view, respectively. However, the surgical position, a non-physiological position, increases the risk of developing pressure ulcers when retained for a long time. Therefore, the prevention of pressure ulcers during surgery under general anaesthesia is considered one of the most important issues in operative care of patients. In humans, it is a known fact that high body pressure occur in the supine, lateral, prone, and lithotomy positions.

However, no report has been published on body pressures during each surgical position in dogs under general anaesthesia. In this study, the body pressures in beagles under general anaesthesia were compared between different surgical positions. Chapter 2 compares the body pressures in the supine position ; and chapter 3, the body pressures in the lateral position. Chapters 4 and 5 describe decompression studies in the supine and lateral positions, respectively, using urethane foam mats with low rebound properties.

Chapter 2 describes how high body pressures and body weight ratios were obtained by category and com-

pares the body pressures in the beagles under general anaesthesia in the supine position. High body pressures were observed in the head, scapular, and iliac regions. I speculated that the beagle dogs under general anaesthesia in the supine position had a risk of developing pressure ulcers during surgery. Chapter 3 describes how high body pressures and body weight ratios were obtained by category and compares the body pressures in the beagles under general anaesthesia in the lateral position. High body pressures were observed in the head, brachial, cubital, lateral, and greater trochanter regions. Beagle dogs under general anaesthesia in the lateral position were speculated to have a risk of developing pressure ulcers during surgery. Chapter 4 describes decompression using a urethane foam mat with low rebound properties. Using a urethane foam mat, I was able to reduce the high body pressures in certain regions in the beagle dogs under general anaesthesia in the supine position. In the future, however, it is necessary to consider changing the thickness of the urethane foam mat. In addition, the animals fixed in the supine position manifested a region of high pressure compared with those placed in the other positions. Chapter 5 describes decompression using a urethane foam mat with low rebound properties. By using a urethane foam mat, I was able to reduce the high pressure in specific regions in the beagle dogs under general anaesthesia in the lateral position. In a future study, pressure reduction must be considered to compensate the partial dispersion of tool of body pressure. In veterinary nursing care, it may be necessary to further evaluate animal patients and determine a safe fixed position, without being bound by the conventional fixing

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method.

Taking both strategies in consideration, I used a urethane foam mat to reduce the high pressures in specific regions during the supine and lateral positions. Therefore, developing interventions aimed at reducing pres-

sure in a fixed position are warranted. Based on the present results, it can be speculated that the beagle dogs under general anaesthesia in the supine and lateral positions had a risk of developing pressure ulcers during surgery.

Non Tuberculous Mycobacteriosis in farmed Thread-sail filefish, *Stephanolepis cirrhifer*

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Recently, mycobacteria distinct from *M.tuberculosis* complex and *M. leprae* have been referred to as nontuberculous mycobacteria (NTM). NTM are universally present in the soil and water, and have been isolated from a wide variety of organisms including vertebrates (mammals, birds, reptiles, amphibians and fish), invertebrates, and protozoa as well. Some species of NTM have been recognized as a cause of human disease such as skin and respiratory disorders.

Infectious diseases of fish caused by NTM have been reported in more than 165 species of saltwater, brackish water and fresh water fishes. These species were also classified into ornamental, cultured and wild fishes.

Thread-sail filefish (*Stephanolepis cirrhifer*), classified into Monacanthidae Tetraodontiformes, is a marine food fish cultured mainly in western part of Japan. Because of its delicious filet and liver served as fresh "SASHIMI" or stew, the market price of this species is remarkably higher than those of the other culturing fish species. Therefore, it has been anticipated as a new high-valued culturing species in Japan.

In 2009, a disease outbreak occurred in a captive population of thread-sail filefish reared in a net cage at Ehime Prefectural Fisheries Research Center. From the abdominal cavity of diseased fish, acid-fast bacteria were continuously isolated. The diseased fish characteristically showed the abdominal distension and the reddening of cloaca. These gross features were similar to those of NTM infection in cultured yellowtail, however; histopathological features of the present case quite differed from those in the other NTM infections of fish. The most distinctive histopathological feature was a formation of numerous pyogenic granulomas, consisted of liquefied central cores surrounded by several layers of spindle shaped epithelioid cells, on the serosal regions of

abdominal organs (mainly intestine). The numerous acid-fast bacilli were observed in the central cores of these lesions. From the results, it was suspected that the present case might be a novel fish NTM infection accompanied with the characteristic pathological features comparing with the previous NTM infections in fish.

Bacterial isolates of the present case were strongly positive with Ziehl-Neelsen stain, and formed colonies of type S / R within 5 days on Ogawa medium and/or Middlebrook7H10 medium. However, all isolates did not showed photochromogenicity. These findings indicated that the isolates should be classified into Runyon Group IV, rapid growing mycobacteria in the light of Runyon classification. The optimal growth temperature of the isolates was within the range of 25 to 30°C. This might reflect the epidemiological characteristics of the present case occurred in summer season.

Artificial infection test was conducted with the isolate against healthy thread-sail filefish by intraperitoneal administration. As a result, almost all fish died within three weeks after inoculation. This acute infection might correlate with the inoculated isolate which was classified into rapid growing Runyon Group IV. Dead and diseased fish showed apparent abdominal distention associated with accumulation of ascites in the abdominal cavities, and numerous white nodules on the serosal surface of abdominal organs, mesentery and peritoneum. Histopathologically, these nodules were pyogenic granulomas corresponding to those found in the spontaneous case, although the granulomas were not observed in the parenchyma of abdominal organs. From the results, it was suggested that the isolate used in this study could exhibit strong pathogenicity against thread-sail filefish.

The results of DNA-DNA hybridization (DDH) test of the selected isolates using DDH mycobacteria "KYOKU-

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TO” kit suggested these isolates could be classified into *Mycobacterium chelonae-abscessus* group, however ; it was also apparent that further classification could not be conducted with the kit. On the other hand, DNA sequence analysis of the isolates demonstrated that they were closely related to *M.chelonae*. Because *M. chelonae* has been recognized as a human pathogenic NTM, it was suggested that the isolates should be examined in the light of public health.

Taking into account the public health aspects and properties of those isolates, we conducted susceptibility testing of the isolates. In addition, two strains of human-derived *M.chelonae* and the reference strain of *M. chelonae* were also donated for the tests, by courtesy of Hansen's Disease Research Center, National Institute of Infectious Diseases. According to the procedure that is described in the document issued by the Clinical and Laboratory Standards Institute (CLSI), the test was carried out by broth microdilution method using 96-well flat-bottom plates. In total of 21 agents including anti-*M. tuberculosis* drugs were prepared for the tests. The reference strain of *M. chelonae* was already identified to be

sensitive to all of these agents. The fish-derived isolates were sensitive to 17 out of the 21 agents examined, and resistant to four agents. The thread-sail filefish examined in this study were not clinically treated with the four agents. Therefore, it was considered that the resistant characters of the fish-derived isolates were not acquired. Two strains of human-derived *M. chelonae* showed similar sensitivities with the fish-derived isolates, with the exception of some agents clinically administered to the human patients from whom the strains were isolated.

In this study, I showed that this case was the first report of NTM infection in thread-sail filefish, and the pathological features were quite different from those in the previous cases. In addition, biological and genetic analyses revealed that the isolated acid-fast bacilli were closely related to *M. chelonae* recognized as human pathogenic NTM. From these results, it was strongly suggested that the present case and isolated strains should be further studied in the light of public health, by mutual communication of aquatic medicine and human medicine.

Basic research in the establishment of an *in vivo* bio-imaging system using fluorescent marker proteins in the transgenic animals

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Transgenic (Tg) animals that express fluorescent proteins, such as green fluorescent protein (GFP) or red fluorescent protein (DsRed), are widely used in organ regeneration and transplantation research. We aimed to evaluate the usefulness of fluorescent proteins in a noninvasive *in vivo* bio-imaging system (IVIS). We developed a novel GFP Tg mouse strain and investigated the whole body GFP expression patterns. We also studied the applicability of DsRed2 to the bio-imaging system.

A novel GFP Tg mouse was developed to elucidate the properties of GFP in mammalian cells. A fusion DNA construct expressing GFP under the control of a CAG promoter was injected into the pronuclear cell of a BDF1 mouse to generate a GFP Tg mouse line. The GFP expression profile at each developmental stage from early embryo to adulthood was observed using fluorescence microscopy, IVIS, and flow cytometry. GFP expression was ubiquitous and abundant in each tissue and at every developmental stage. The peripheral blood cells of the GFP mouse consisted of more than 90% GFP-positive cells. We report the successful generation of a GFP Tg mouse line, which is useful for studying cell migration and differentiation in biomedical research.

We also investigated the relationship between DsRed2 expression and an endogenous hepatic gene expression in the DsRed2 Tg rat to elucidate the usefulness of DsRed2 in a bio-imaging system. DsRed2 expression is controlled by an albumin enhancer/promoter, and DsRed2 expression was detected exclusively in the adult male liver, resulting in sexual dimorphism. We examined the relevance of regulation between the sexually dimorphic DsRed2 expression and the hepatic drug-metabolizing enzyme, cytochrome P450 (CYP) expression. Sexually dimorphic CYPs are regulated by pituitary growth

hormone (GH) secretory patterns. It is well known that the sex hormone level at perinatal period determines the pattern of GH hormone in adult rats same as that of sex hormone. To reverse the GH secretory pattern in both sexes, male and female Tg rats were gonadectomized under isoflurane on postnatal day 1, and testosterone propionate (TP, 0.25mg/animal s.c.) was administered to ovariectomized (OVX) females immediately following surgery. DsRed2 expression levels were quantified using fluorescence microscopy and IVIS, and the mRNA expression levels of male-dominant CYP2C11 and female-dominant CYP2C12 were detected by reverse transcriptase polymerase chain reaction (RT-PCR) at 9 weeks after treatment. The volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) was measured to confirm the effects of the gonadectomy and TP treatment during the perinatal period. The OVX and TP injected (OVX/TP) 9-week-old female Tg rats exhibited elevated DsRed2 expression, which was undetected in normal Tg female rats of the same age. However, 9-week-old castrated male Tg rats did not express hepatic DsRed2, whereas normal adult Tg males expressed high levels of hepatic DsRed2. With regard to sexually dimorphic CYPs, the OVX/TP females expressed high male-dominant CYP2C11 mRNA, and the castrated males expressed high female-dominant CYP2C12 mRNA. The SDN-POA volume among the OVX/TP female rats was the same as that of normal male rats, whereas castrated males rats exhibited SDN-POA volumes that were equivalent to normal female rats. The expression between DsRed2 and sexually dimorphic CYPs were significantly correlated in each animal. This result suggests that sexually dimorphic DsRed2 expression is regulated by the GH secretory pattern similar to endogenous sexually di-

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morphic CYP expression. We propose that changes in CYP mRNA expression can be easily detected using this Tg rat line.

In conclusion, this research demonstrates that fluorescent proteins could be a useful tool to detect gene expression using IVIS.

Influence of the single nucleotide polymorphism (SNPs) of beta-3 adrenergic receptors on canine obese condition

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Obesity is a condition that excessive fat is accumulated in white adipose tissues, caused by the higher energy intake overcoming its consumption. In human beings, diabetes, hypertension and hyperlipidemia related to obesity are getting increased and these conditions are named metabolic syndrome. The study in obesity focusing on the causes and mechanism is important to prevent and treat these conditions. Concerning the causes of obesity, now in the spotlight are genetic factors in addition to environmental factors. Uncoupling protein 1 (UCP1), beta-2 adrenergic receptor (β 2AR), G-Protein-coupled receptor 120 (GPR120) and beta-3 adrenergic receptor (β 3AR) are known as human obese related genes. In particular, it is proved that Trp64Arg variation with β 3AR gene makes daily energy requirement 200 kcal lower in comparison with wild-type people.

In the veterinary medicine, obesity is a most common metabolic disorder too. There are some breeds more likely to get weights epidemiologically, but obese-related genes have not ever been proved in cats and dogs. In this study, we investigated gene variations of β 3AR in dogs focusing on relationship with obesity. Regarding the single nucleotide polymorphisms (SNPs) found in canine β 3AR genes, we analyzed the genotype frequency in comparison with their breed variations, body condition scores (BCS) and the body weight change.

Chapter 1 : Search for the SNPs of β 3AR genes in various dog breeds.

We analyzed the sequences of β 3AR genes in 45 dogs consisting of 22 breeds. As a result, we found three synonymous substitutions and six non-synonymous substitutions, but no breed-specific SNP was detected. The SNP of C1121G (Pro374Arg) was more common in the dog breeds reported to be more likely to get weight. The

SNP of A1184C showed higher genotype frequency rather than that of wild-type sequence reported in the database, then it was estimated that the sequence with this SNP is a major one in dog species. The amino acids located in human Trp64Arg SNP were arginine in all the dogs tested in this study, which were obese-type in human beings.

Chapter 2 : Genotype frequency of the β 3AR gene SNPs in the dogs with various body condition scores.

We analyzed the frequency of the β 3AR gene C1121G SNPs in 81 dogs containing 3 breeds. As a result, the gene frequency of the C1121G was higher in the lower BCS groups, suggesting that this SNP should increase the metabolism in dogs. In addition to it, we found a new SNP, C749T (Ser250Phe) which was non-synonymous substitution too. The gene frequency of the C749T was higher in the higher BCS groups, suggesting that this SNP should decrease the metabolism in dogs contrary to that in C1121G. Based on the results, these two SNPs are likely to be candidate obese-related genes in dogs.

Chapter 3 : Phenotypic effect of the C749T SNP on the weight gain in the dogs.

The SNP of C749T is possible to be an obese related gene in dogs, but we had not checked the effects of environmental factors in the chapter 2. To clarify this possibility, we analyzed the phenotypic effect of the SNP using the experimental beagle dogs kept in a same environment. Three dogs with the SNP and other 3 dogs without the SNP were fed on a similar fed schedule, i.e. the energy intakes were set to $90 \pm 5\%$ of their daily energy requirements for 12 weeks. When we compared

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the change of their body weights, only the wild-type dogs (without the SNP) showed a significant decrease in body weights since week 9. We concluded that the C749T SNP might function to decrease the metabolism, then the body weight did not decrease.

Conclusion

In the present study, we found the seven non-synonymous substitutions in the canine β 3AR genes. We could not prove any breed-specific SNPs in this study, but the SNP of C749T was likely to decrease the metabolism and

the C1121G was likely to increase it based on the results comparing with the BCSs. When we fed the dogs with or without the C749T SNP in a same condition, the body weights of the dogs with C749T SNP did not change, but those without the SNP decreased significantly. We concluded that these SNPs are candidate obese related genes in dogs. It is necessary to increase the sample size to confirm the present results. But on the horizon, a genotype-based feeding program for dogs should be established like that in human medicine.

Identification of taste receptor T1Rs families in cattle

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The T1R family of taste receptors comprises G protein-coupled receptors that traverse the plasma membrane seven times and is reported to be receptors of sweet and umami substances and amino acids. This family consists of T1R1, T1R2, and T1R3. The T1R1/T1R3 heterodimer is specific for umami taste, and the T1R2/T1R3 heterodimer is specific for sweet taste. Thus the response characteristic changes with the combination of T1Rs receptors. In the cattle T1R family, few genome analysis and polymorphism studies have been conducted, but molecular genetic elucidation has not been extensively carried out. Therefore the present study identified the nucleotide sequences of mRNA to make the taste receptor T1Rs family of cattle clear at a gene level and investigated expressions of the mRNA in taste buds.

Holstein cattle were used as the study model. The nucleotide sequences of T1R1, T1R2, and T1R3 were deduced from total RNA isolated from the tongue containing circumvallate papillae. T1R nucleotide sequences were translated into amino acid sequences and homology searches were conducted against human and mouse T1Rs. Structural analyses were also performed. Cross-sections were prepared using the tongue contain-

ing circumvallate papillae, and *in situ* hybridization was performed using cattle T1R1, T1R2, and T1R3 cRNA probes.

Cattle T1R1, T1R2, and T1R3 mRNAs were 2526 bp, 2514 bp, and 2571 bp, respectively. Homology searches yielded nucleotide sequence similarities of 81.4% and 76.9% for T1R1, 82.4% and 78.0% for T1R2, and 78.6% and 72.5% for T1R3 in humans and mice, respectively, while amino acid sequence similarities were 76.0% and 70.5% for T1R1, 75.3% and 71.1% for T1R2, and 71.4% and 69.3% for T1R3 in humans and mice, respectively. Structural analyses revealed that these three amino acid sequences had a seven-transmembrane domain characteristic of G protein-coupled receptors. Moreover, cattle T1R1, T1R2, and T1R3 had cysteine-rich and long ligand-binding domains characteristic of the T1R family. *In situ* hybridization demonstrated T1R1, T1R2, and T1R3 expression in taste buds. Therefore, T1R1, T1R2, and T1R3 are likely to function in the perception of taste in cattle. Furthermore, because gustducin, which is G protein of the T1R family is reported in cattle, it is believed that cattle taste sweet and umami through T1R in taste buds.

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Studies on the behavior of myofibrillar ATPase activities and actin in chicken meats heated at a low temperature

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Introduction

When animal and fish meats which have the muscle structure are processed by vacuum cooking, *i.e.*, heating at a low temperature under 68 °C for a long time, their texture is shown to be softer than that of meats cooked at a higher temperature for a shorter time. Hence, vacuum cooking is in the limelight as a new cooking method. Changes in the texture, water holding capacity and microstructure of the beef, pork and chicken meats when they are vacuum cooked or heated at a low temperature, have been investigated.

However, mechanism of softening meats has not been fully elucidated. In particular, there are few reports on the changes in the binding of actin and myosin in muscle fibers.

However, it is considered that the change of the bond state of both proteins is related greatly to a change of the texture. Hence, our studies have been focused on this. We clarified that heating meat at a low temperature, 60-65 °C, caused the liberation of actin from myofibril. The liberation of actin is estimated to be one of the causes of softening meat through vacuum cooking. In addition, it has been estimated that actin liberation is caused by IMP responsible for dissociating the acto-myosin (the complex of actin and myosin, abbreviated as AM).

In this study, in order to know more closely the behavior of actin and myosin in the meat heated at a low temperature, myofibrillar ATPase activities and actin liberation in chicken meats heated at 50 °C where changes proceed relatively slowly were investigated. Moreover, the effect of IMP on the ATPase activities and actin liberation were examined.

Materials and Methods

Minced meat, minced meat homogenate, myofibrillar fraction (abbreviated as MFF), sarcoplasmic fraction (abbreviated as SPF) and AM were prepared from chicken breast meat. SPF was fractionated into a low-molecular mass sarcoplasmic fraction (abbreviated as LMS) which contains substances with molecular mass lower than 10,000, and a high-molecular mass sarcoplasmic fraction (abbreviated as HMS) which contains substances with molecular mass higher than 10,000. LMS was prepared from SPF in which IMP would disappear by keeping at 30 °C. Each of these samples was heated at 50 °C up to 1 h in 0.2M KCl. The Ca-ATPase and Mg-ATPase activities of myofibrils were measured in 0.6 M KCl and 0.05 M KCl, respectively. Amount of the liberated actin was investigated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Results and Discussion

The Mg-ATPase activity decreased faster than the Ca-ATPase activity, when minced meat and minced meat homogenate were heated at 50 °C. This result revealed that the actin denatured faster than the myosin when the minced meat was heated. On the other hand, when the MFF was heated, the Ca-ATPase activity decreased faster than the Mg-ATPase activity, which was the result contrary to the case of the minced meat. The addition of IMP repressed the inactivation of the Mg-ATPase, suggesting that promoters of actin denaturation, other than IMP, are included in minced meat, particularly in SPF. In order to determine whether the promoters are high or low molecular mass substances, each of LMS and HMS was heated with MFF, or both of them were heated simultaneously with MFF. As a result, the inacti-

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vation rate of the Ca-ATPase was almost the same in any case, while that of the Mg-ATPase was largest in addition of both LMS and HMS, and then the following order, LMS, HMS and no addition. The addition of IMP to the samples unchanged the order of the inactivation rate. These results suggested that some promoters of inactivation of the Mg-ATPase, *i.e.*, denaturation of the actin, is present in sarcoplasm, and that the main responsible substances are the components other than IMP present in the LMS.

When minced meat and minced meat homogenate were heated, actin liberation was observed in first 20 minutes when 90% of the Mg-ATPase was inactivated. The actin liberation did not occur in the heated MFF, but did in the heated MFF with IMP. Thus, it was demonstrated that IMP has the ability to liberate actin from MFF. When MFF was heated with HMS or LMS, the actin liberation was observed only in the presence of LMS. Moreover, it was found that the change in the actin liberation with the elapse of time is different between LMS addition and LMS + IMP addition. The difference indicated that the substance to have the ability of actin liberation, other than IMP, is contained in LMS. In order to know the properties of the promoters of actin denaturation, LMS was boiled or hydrolyzed. As a result, the promotion ability was not lost in boiling, and increased by hydrolysis. However, we

could not find the factor in the examination so far. When AM was heated, the Ca-ATPase activity decreased faster than the Mg-ATPase, which was the same result of heated MFF. Therefore, it became clear that this phenomenon is not specific to myofibril and occurs in common with the complex of actin and myosin.

From the all results obtained by the present study, the reaction to proceed in myofibrils of chicken meat heated at 50 °C is presumed as follows : When only MFF was heated at 50 °C, myosin denatures faster than actin. The heat treatment was estimated to release immediately at least one of a few bonds between the both proteins. Of myosin and actin, whose bond was dissociated, only actin denatures remarkably fast by the action of low-molecular mass compounds present in the sarcoplasm. The compounds are unknown substances other than IMP. Because the denatured actin is not liberate immediately, it is presumed to bind to myosin through the linkage that was not dissociated at 50 °C, and is estimated to be liberated after the compounds in LMS would act on the different linkage.

The compounds are presumed to be IMP and other unknown substances. The bonds between actin and myosin are damaged through the above mechanism, which could contribute to softening meat by heating at a low temperature.

Studies on changes of imidazole dipeptide contents in chicken meat

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〈Introduction〉

Imidazole dipeptides are dipeptides which consist of β -alanine and the amino acid possessing imidazole ring. This contains carnosine (Car), anserine (Ans) and ophidine. Car consists of β -alanine and histidine, and anserine consists of β -alanine and 1-methyl histidine. These peptides are predominantly contained in muscle, brain and olfactory bulb. Especially, chicken muscle contains lots of imidazole dipeptides. Although Car have been found over 100 years ago, the real physiological function was not still clear. Recently, imidazole dipeptides were shown to have physiological functions such as anti-oxidant and anti-fatigue activities and were focused as material for functional foods.

In this research, the contents of Ans and Car in muscle of the different fowls were measured, and the amount of m-RNA of proteins related to metabolism of Ans and Car were also measured. Then, the factor involved in differences of the contents of both dipeptides in chicken muscle was speculated.

〈Materials and Methods〉

After slaughter of broilers and Japanese native fowls (Awaodori), muscles such as breast and thigh, livers and hearts were taken from their carcasses, immediately. The contents of Ans and Car in muscles and tissues were measured by amino acid analyzer. The mRNA was prepared from fresh muscles, and the contents of gene of carnosine synthase 1 (CARNS1) and carnosinase (CNDF2) were also measured by Real Time-PCR.

〈Results and Discussion〉

1. Contents of Ans and Car in muscles, liver and hearts of chicken

The content of Ans in breast and thigh muscles of male broilers (8 weeks old) were $28.3\mu\text{mol/g}$ and $9.4\mu\text{mol/g}$ meats, respectively. On the other hands, the content of Car in breast and thigh muscles of male broilers (8 weeks old) were $15.8\mu\text{mol/g}$ and $5.7\mu\text{mol/g}$ meats, respectively. The contents of Ans and Car in both muscles of female broilers were almost same as those of male ones.

In liver and heart, there were few contents of Ans and Car.

2. The contents of Ans and Car in broilers and Japanese native fowl (Awaodori)

The contents of Ans and Car in muscles of 8weeks old broilers and 12 weeks of Awaodori were measured.

The Ans content in breast of Awaodori was $42.5\mu\text{mol/g}$ meat and 1.4 times higher than that of broilers. There were no significant differences in the content of Car in muscles between Awaodori and broilers.

The Ans content in thigh of Awaodori was $10.9\mu\text{mol/g}$ meat and significantly higher than that of broilers. On the other hand, the content of Car was significantly lower than that of broilers.

Effect of feeding on their amount in both meat was also examined by addition of β -alanine and Histidine to feed of fowls.

Feeding the diets by addition of β -alanine and histidine did not affect with the amounts of Ans and Car in breast meat of broiler. On the other hand, this diet increased the amount of Car in thigh meat. Feeding the diet by addition of only β -alanine decreased the amount of Car in thigh meat. Although feeding the diet by addi-

tion of β -alanine and histidine gave no effect on the mRNA level of carnosine synthase I (CARSN I) in thigh meat, it increased the mRNA level of carnosinase. From these results, the changes in the amount of carnosine in thigh meat seemed to be caused by the change in mRNA level of carnosinase.

3. The contents of Ans and Car in muscles of broiler and Awaodori at the same weeks old

The contents of Ans and Car in muscles of 3 weeks of broiler and Awaodori were measured.

The Car content in breast meat of Awaodori and broiler was 7.9 and 9.8 $\mu\text{mol/g}$ meat, respectively. There was no difference in the Car content in breast meat between Awaodori and broiler. On the other hand, the Ans con-

tent in breast of Awaodori and broiler was 33.6 and 26.4 $\mu\text{mol/g}$ meat, respectively. The former was 1.3 times higher than that of the latter.

In the 5 and 7 weeks olds as well as 3 weeks olds, the content of imidazole dipeptides, that is, the sum of Ans and Car, in Awaodori was also higher than that in broiler.

The gene expression of carnosinase (CNDP2) in breast muscle of Awaodori was significant lower than of broiler in the whole ages such as 3, 5 and 7 weeks olds.

From these results, the content of imidazole dipeptides in muscles of Awaodori was shown to be higher than that of broiler. This difference seems to be caused by the differences in carnosinase level in muscles.

Production of offspring from Japanese field vole, *Microtus montebelli*, via various assisted reproductive technologies

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Microtus genus is a herbivorous animal with double stomachs, and some of them possess a mating system similar to human. So, these species have been expected as a model animal for the large herbivory and mating system model. In addition, ten of sixty-four species have been classified into the endangered category in Red List, and the preservation of such species have been required from viewpoint of biological diversity protection. From above reasons, it is important to maintain *Microtus* as an animal genetic resource. Our final goal is to establish the procedures of individual regeneration and preservation in this genus. To accomplish this objective, we examined at first whether the mouse sperm cryopreservation system was applicable to spermatozoa of *Microtus montebelli*. Secondly, we used non-invasive artificial insemination to attempt production of offspring to perform the final evaluation of sperm cryopreservation technology on *Microtus montebelli*. Finally, we examined experimental condition and production of offspring by using *in-vitro* fertilization (IVF) and/or intra-cytoplasmic sperm injection (ICSI) to establish more reliable technologies of individual regeneration.

Microtus montebelli has been maintained in our laboratory, and 3- to 62- week-old females and 9- to 49- week-old males were used. The cauda epididymis was collected and suspended in freeze protecting agent which was constituted with 18% raffinose and 3% skim milk. Then, sperm suspension was dispensed into cryotube and saved in liquid nitrogen until experiments. The rate of sperm motility, viability and DNA integrity were assessed by visual observation, nigrosin-eosin staining and alkaline comet assay, respectively. For evaluation of fertilizing capacity, a cryopreserved vole spermatozoa was micro-inseminated into a mouse oocyte by ICSI with piezo drive. For artificial insemination, female voles were

superovulated by intraperitoneal injection of 30 IU eCG followed by 30 IU hCG 46–48h later. Next, 2×10^6 cells ($20 \mu\text{l}$ / an uterine horn) of frozen-thawed (FT) sperm was transferred to uterine horn of female which had been mated with vasectomized vole males at 4–6 hours after copulation. With an otoscope to see externaluterineos, the injector was inserted to uterine cervix and sperm suspension was transferred. Moreover, in order to improve litter size with FT, we performed artificial insemination at 7–9 hours after copulation (delayed transfer time) and examined influence of reagent (1 mM hypotaurine) for improving sperm motility. For IVF, 3 week- old female voles were superovulated and *in-vivo* mature oocytes were collected. Then, we examined effect of hypotaurine on IVF with FT sperm. Furthermore, we performed IVF using fresh and FT sperm and transferred zygotes into ampulla of uterine tube of recipient. For ICSI, we performed ICSI with fresh and FT sperm in a manner similar to mouse procedure, and transferred zygotes in a manner similar to IVF.

Total motility of vole sperm before (fresh) and after freezing (FT) was 79% and 67%, respectively. After FT, the rate of vole sperm motility trended to be lower than fresh. The average viability of vole fresh and FT spermatozoa was 87% and 67%, respectively, and rate of vole FT sperm viability was significantly lower than fresh ($P < 0.05$). The rate of sperm DNA with a comet tail (this means damaged DNA) was 2.0% and 2.5%, respectively ($P > 0.05$). Then, all of mouse oocyte injected resumed meiosis and formed pronucleus, when they were injected with a single vole fresh or FT spermatozoon. Average litter size by artificial insemination using fresh and FT sperm was 7.2 ± 1.5 ($n = 6$) and 1.7 ± 0.3 ($n = 3$) neonates, respectively. Although litter size derived artificial insemination using FT sperm was low, we demonstrated that

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the pups could be produce from FT sperm. These results showed that FT sperm of *microtus montebelli* sufficiently maintained the fertilizing capacity. Furthermore, the litter size derived from FT sperm was increased by delayed transfer time (at 7–9 hours after copulation) or hypotaurine treatment. Regardless of hypotaurine treatment, IVF with fresh sperm showed a high fertility rate (83.6–86.0%). Also, IVF with FT sperm showed a high fertility rate (97.1%). When we transferred IVF embryos derived from fresh (n= 8) or FT (n= 6) spermatozoon, birth rates were 25% (2 / 8) and 16.7% (1 / 6), respec-

tively. Oocytes micro-injected with fresh or FT sperm oocytes resumed meiosis and formed pronucleus (91.2% and 89.2%, respectively). After transfer of ICSI embryos derived from fresh and FT spermatozoon, there was no offspring in both groups. Overall, we revealed that vole sperm could be successfully cryopreserved with mouse system and the pups could be produced from fresh and FT sperm by artificial insemination. On the other hand, it was suggested that improvement of IVF and ICSI procedures was needed to perform stable production of vole offspring, since IVF and ICSI couldn't produce the pups.

Use of dogs to reduce crop damage by wild animals in Tokyo

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1. Background and purpose

There is over \20 billion a year in the amount of crop damage by wildlife in Japan. The amount of wildlife damage in Tokyo is only 0.2% of all over Japan, but understory vegetation has been devastated through the predation of deer and that causes large scale soil erosion, agricultural and forestry damage in Okutama region.

The present studies revealed that use of dogs, which was introduced in Nagano prefecture for the first time in 2007, has been effective to reduce crop damage in many parts of Japan. It is supposed that this measure may be effective in Okutama region. This paper tried to verify that the monkey dog is effective to wildlife damage control in this region.

2. Methods

First, conducted some interviews at the areas where introduced it earlier in order to verify the present condition, administrative support systems and future prospects of monkey dog :

- ①Omachi city, Nagano prefecture
- ②Fujiyoshida city, Yamanashi prefecture
- ③Nabari city, Mie prefecture and Uda city Nara prefecture
- ④Nagiso town, Nagano prefecture
- ⑤Yosano town, Kyoto prefecture

Second, conducted some interviews to government officials about present conditions of wildlife damage and subsidies for farmers at two geographically different areas, Okutama town and Hachioji city, in order to verify the possibility of monkey dog in Tokyo. In addition, conducted the questionnaire survey about interest on monkey dog, the condition of wildlife damage and damage control to farmers in each area.

3. Results

Early introducing areas

Through the research at early introducing areas, it is revealed that there are two ways of dog training. One is depositing dogs to a school, such as a police dog training school. An advantage of this way is high degree of training by professional trainer for longer term. On the other hand, a disadvantage is higher cost to train.

Another one is inviting trainers and holding a training workshop in each area. An advantage of this way is that the owners of dogs can learn how to train dogs so they can continue to train. On the other hand, it is difficult to combine schedule of participants and a term of training may be longer.

From survey, there are two method of operating monkey dogs. One, "Omachi method", has been adopted in areas where farmers are principal of dogs. In this method, dogs drive away the target wildlife when they haunt near the mooring location. On another one, "Fujiyoshida method", a resident move with the dogs to the site where wildlife haunts and make dogs drive away when they receive information.

Survey in Tokyo

Against damage of \40 million per year, Tokyo is hitting various measures taken to plan. In Tokyo, wildlife damage is caused by mainly wild boar, monkey and deer.

In Okutama town, wildlife damage of about \5 million a year has occurred by mainly wild boar, monkey and deer. In recent years damage caused by palm civet has been prominence.

According to the results of a survey of farmers in Okutama town, there were many reports of damage by wild boar and monkey. In addition, in the question about the monkey dog, farmers have less awareness. But 70% of respondents are interested in monkey dog.

In Hachioji city, wildlife damage of about \3.4 million a

year has occurred and damage of wild boar is the largest.

According to the results of farmers in Hachioji city, 30% of respondents are interested in monkey dogs.

4 . Discussions and Conclusion

It is essential for introduction of monkey dog to work together as many researchers pointed out about wildlife damage control.

Considering this point, it is difficult to introduce monkey dog in Hachioji city because the proportion of damage in agricultural input and tendency for the victim of an interest is both low.

In Okutama town, about 50% of farmers are practicing

wildlife damage control in local community so it is supposed that introduction of monkey dog is in succeed there. But there are many climbers compared with other areas so it is necessary to tell them practice of monkey dog to prevent accidents.

Therefore, as expected support for the introduction of the monkey dog, government some financial aid, the grouping of operators, like education and public relations activities.

Through this study, a possibility of introducing monkey dog against wildlife damage in Tokyo is suggested. It would be a good opportunity to inform the conditions of wildlife damage for general public not only farmers.

Studies on influence of growth performance and fattening management on carcass price of Japanese beef cattle

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The physical properties of stock cattle at the time of purchase greatly affect the characteristics and price of the carcass. However, the information available at the time of purchase is limited to such as breed strain and the appearance of the animal, so that the appraisal of the animal and fattening management for details rests solely with the experience of the buyer and the farmer. There has been no reported research dealing with information to clarify how body structure at the time of purchase and subsequent fattening management affects the characteristics and price of the carcass. Therefore, it is necessary to consider the effect of the differences in growth and fattening management for the fattening period. The relationships between the body structure, biochemical profile of blood and carcass price were investigated using Japanese Black steers. Furthermore, the factors that affect the differences between carcass price and fattening management in terms of the design of the trough, feeding methods, herd size and percentage of the number of accidents etc. using crossbred steers of Japanese Black and Holstein were investigated.

For the appearance of the animal and carcass unit price, test animals were purchased in March and April 2010 at the Motomiya Market, Fukushima prefecture and 13 Japanese Black steers aged 9.8 months. The cattle were fattened on a farm in Oyama-city, Tochigi prefecture. The animals used were measured six times from the age of 10 months to 30 months when they were shipped from the farm. The animal used was measured once four months. The 13 measurements physical structure were done for the following : in addition to regular measurements of weight, withers height, etc., ratio of chest depth, chest depth divided by the height of the chest depth point, and the ratio of width of chest divided by the width of forearm which the farmer might evalu-

ate (Katatsuki). Analysis of blood taken from the jugular vein measured vitamin A, vitamin E, β -carotene, total cholesterol and β -hydroxybutyrate acid to reveal the state of fattening management. Coefficient of correlation between measured values and carcass characteristics and carcass price was undertaken at each of the six instances of measurement.

The fattening management technologies using 2860 head shipped from the nine fattening farms in Iwate prefecture from October 2010 to August 2008. The nine farmers equally divided three classes based on the carcass price, high, medium and low class farms. The relationships between the 13 items in terms of the design of the trough, feeding methods, herd size, and percentage of accidents, etc., the carcass grade and carcass price were investigated. Furthermore, the difference of the 13 items among the farms was investigated.

Through the study of body structure at 10 months of age, there was a significant positive correlation coefficient between the girth of chest and the carcass weight ($r=0.799$), as well as rib thickness ($r=0.862$). The chest depth significantly correlated to the subcutaneous fat thickness ($r=0.818$), the ventral girth between carcass weight ($r=0.776$) and the longissimus thoracis muscle area ($r=0.734$). In addition, a significant negative correlation was observed in the ratio of chest depth between subcutaneous fat thickness ($r=0.919$) and Katatuki between rib thickness ($r=0.718$). These findings indicate the strong possibility that the value for girth of chest, chest depth, ventral girth, width of chest and width of forearm at 10 months of age has a correlation to later carcass characteristics. In addition, a positive correlation was observed in total cholesterol concentration in the blood of 10 months of age between BMS (No.) ($r=0.718$) and carcass unit price ($r=0.758$) and also in the concen-

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tration of β -hydroxybutyrate acid between the longissimus thoracis muscle area ($r=0.628$). These findings indicate the strong possibility suggesting that selected breeding cattle have a developed rumen and have a high total cholesterol level and that vitamin A value may affect the carcass characteristics and the carcass unit price.

Through investigations of fattening management in carcass weight, the medium and low class farms were significantly higher than the high class farm ($p<0.05$). In longissimus thoracis muscle area, the high class farm was significantly higher than the medium and low class farm ($p<0.05$). In rib thickness, there were no significant differences among the three farms. In addition, no significant difference among the three farms was found in difference of feeding time between evening and following

morning, the trough height from the floor, trough area, the depth of the trough, and head management per person and pen area per head. In the incidence of accidents and carcass damage, no significant difference was found among the three farms. A negative correlation was found between the trough height from the floor ($r=-0.710$). The incidence of carcass damage was found to be a positively correlated to the number of workers, ($r=0.750$).

Although there was no statistically significant difference in the feeding time between the evening and the following morning tended to increase linearly from the low to the high farmers. These results suggest that the physical properties of stock cattle at the time of purchase and the subsequent fattening technologies affect the fattening management

Studies on enzymatic synthesis of poly-L-cysteine and its application to protein cross-linking

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Gel-forming ability of proteins is responsible for the physical properties of foods and edible and biodegradable film formation. One of the predominant gelation mechanisms is disulfide bond formation between the thiol groups of cysteine residues in protein molecules. For example, wheat gluten is used as an effective improving agent for preparing viscous rice bread dough through disulfide bond formation. In this study, enzymatic synthesis of poly-L-cysteine (pC) was demonstrated, and the feasibility of the synthesized product as a protein cross-linker was investigated from the standpoint of protein chemistry.

Enzymatic peptide synthesis offers an attractive alternative to chemical methods. As characteristics of enzymes, high specificity and high reactivity under mild conditions are expected to reduce the operation steps requisite for protection-deprotection and to avoid side reactions. In the reaction, the acyl group of a substrate specific to the S1 subsite (specific substrate-binding site) in the catalyst is transferred to an amino component with a high affinity to the S1' subsite through the acyl-enzyme intermediate. This deacylation of the acyl-enzyme intermediate giving a peptide product is called aminolysis. To suppress the hydrolysis occurring competitively with aminolysis, freezing the reaction media is expected as an effective way of avoiding the hydrolysis and oxidation of SH groups in the substrate and the product. Therefore, we performed α -chymotrypsin-catalyzed peptide synthesis under frozen states.

1. Enzymatic synthesis of poly-L-cysteine

α -Chymotrypsin-catalyzed polymerization of Cys-OEt (L-cysteine ethyl ester) was performed at -20°C and pH 8.0 for 3 days. As a result, nearly 90% of the substrate was used in the synthesis of precipitable peptide.

It was found that the precipitated reaction product mainly contained pC of degree of polymerization ranging from 6 to 11 confirmed by MALDI-TOFMS analysis. In addition, about 70% of all Cys residues of pC were ascertained to be in the form of free SH group.

To elucidate aminolytic activity of Cys-OEt in the pC synthesis, ATEE (N-acetyl-L-tyrosine ethyl ester) was used as a highly specific acyl-donor substrate for α -chymotrypsin, and the aminolytic activity of Cys-OEt was compared with Arg-OEt (L-arginine ethyl ester) that is known to have high affinity for S1' subsite of this enzyme. Arg-OEt showed high aminolytic activity at 10°C , but at -20°C Cys-OEt exhibited the higher activity than Arg-OEt. In addition, the presence of high concentrations of Cys-OEt depressed the acylation step, indicating that Cys-OEt competed with ATEE in the interaction with the S1 subsite of α -chymotrypsin and functioned as the acyl-donor substrate. The efficient enzyme-catalyzed synthesis of pC was successfully achieved by using a frozen reaction system in this manner.

Next, the stability of free SH groups in pC was compared with Cys, Cys-OEt and GSH (glutathione). pC and reference standards were suspended in the buffer ranging from pH 2.0 to 8.0, and then heated at 90°C or stored at 0°C for 30min. The SH group of every sample was stable at 0°C , but when heated at 90°C the amount of the free SH groups of Cys-OEt, Cys and GSH was significantly decreased in this order at alkali pH regions. In contrast, free SH groups in pC were quite stable under the same conditions. The stability of SH group is presumed to be related to their pK values. When the pK value is high, free SH group is stably maintained. Since it is reported that the pK value of free SH group is affected by juxtaposed $-\text{NH}_3^+$ and $-\text{COO}^-$ groups, the pK value of SH group in pC is deduced to be considerably high at

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C terminal and comparatively low at N terminal.

2. Analysis of pC and lysozyme interaction

The lysozyme solution was mixed with pC in the presence of 4M urea at pH 7.0, and was incubated at 30 ° C and 60 ° C for 30min, and then the residual free SH groups in heated lysozyme mixtures were alkylated by IAA (iodoacetamide) to prevent further side reactions. The treated samples were analyzed by SDS-PAGE under both non-reducing and reducing conditions. SDS-PAGE analysis under the non-reducing condition revealed that any lysozyme oligomer bands were not observed with or without the presence of pC when incubated at 30 ° C. But at 60 ° C, lysozyme oligomer bands were markedly observed only in the samples treated with pC. These oligomer bands were converted into a lysozyme monomer band when reduced by DTT (dithiothreitol). Therefore, it was deduced that the addition of pC promoted disulfide-mediated polymerization of lysozyme.

Moreover, these samples were separated into low and high molecular fractions by ultrafiltration. Then, these fractions were analyzed by RP-HPLC after reduced by DTT. A number of pC peaks were detected in both

fraction samples. As a result of analyzing these peaks by MALDI-TOFMS, partially-alkylated pC was a major component in the low molecular fraction and non-alkylated pC was mainly detected in the high molecular fraction. Hence, it was interpreted that an inter-molecular disulfide bond formation between pC and lysozyme through thiol/disulfide exchange reaction was predominant in the low molecular fraction. Following this step, the pC-conjugated lysozyme must have been joined together to the high molecular aggregation via disulfide bond formation.

3. Application of pC to other food proteins

BSA (bovine serum albumin), OVA (ovalbumin) and rice flour solutions were treated with pC and then analyzed by SDS-PAGE. All of these protein samples formed disulfide-linked aggregates by the addition of pC, suggesting that the pC acted as a reducing agent and/or a cross-linking agent. From these results, the pC can be expected to be utilized for creating new proteinous materials and for improving physical properties of foods through reducing action and effective cross-linking formation provided by pC.

Basic research on development of objective evaluation system for bitter taste using cultured STC-1 cells

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〈Introduction〉

Bitterness is well known to be dislike taste for human beings because of its signal for prevention from poison. Many compounds possessing physical function for keeping our health show bitterness. Therefore, it is an important problem to be resolved to decrease bitterness of functional foods and to develop bitterness-suppressers in foods.

In general, sensory evaluation is used in order to evaluate bitterness of foods. However, this method is also thought to be less objective, and the taste sensor was developed. Furthermore, the new method by taste cell model such as STC-1 cell or the HEK cells expressed human taste receptor is being developed. STC-1 cells are thought to be useful taste cell model which responds to bitterness.

In this research, the response of bitter taste substances and bitterness suppressers to STC-1 cells was evaluated. These substances were also evaluated by sensory evaluation. By the comparison between cell and sensory evaluation, the usefulness of the system using cultured STC-1 cells was evaluated.

〈Materials and Methods〉

Caffeine, quinine hydrogen chloride salt, Denatonium benzoate, L-leucine, Tryptophan and Phenylurea were used as bitterness substances. STC-1 cells were used to evaluate bitterness of bitter solution. After culture of STC-1 cells, fluo8-AM was added to culture solution and induced into the cells. The evaluation of bitterness of bitter substances was carried out by calcium-imaging method with microscopy (LSM710) using cultured STC-1 cells. Responses by substances were measured in 5 seconds for 1 min and calculated as the changes of $[\Delta F/F =$

$(F - F_0)/F_0]$.

Xanthan gum (XG), β - and χ -cyclodextrins (CD) were used as suppresser for caffeine solution. Emulsified caffeine solution with soy oil was also evaluated by sensory evaluation and STC-1 cells.

〈Results and Discussion〉

1. Responses of cultured STC-1 cells for bitter substances

The cultured STC-1 cells responded by addition of caffeine and quinine hydrogen chloride salt (QHD). The strength of responses was increased by the increase of concentration of substances. STC-1 cells responded to caffeine solution quickly after addition, while they did to QHD slowly and also lasted in responses after the maximum response. These differences seemed to be caused by the differences in the affinity between substances and receptor on the STC-1 cells.

There were no responses of STC-1 cells for denatonium benzoate, L-Leu, L-Trp and phenylurea. This indicated that STC-1 cells had no receptor for these compounds, although they had receptors for caffeine and QHD.

2. Effect of addition of bitterness-suppressers and emulsion on the responses of bitterness in sensory evaluation and STC-1 cells

1) Effect of addition of bitterness-suppressers on bitterness

Addition of 5 or 10 mM XG to caffeine solution suppressed its bitterness in sensory evaluation. On the other hand, the addition of XG did not give no effect on the responses by bitter substances in STC-1 cells, suggesting that XG had no interaction with bitter substances as well as receptors in STC-1 cells.

Addition of 5 or 10 mM β - and χ -cyclodextrins (CDs) to caffeine solution also suppressed its bitterness by sen-

sory evaluation. The responses of STC-1 cells by addition of 5 or 10 mM β - and χ -CDs were significantly lower than those of only caffeine solution.

2) Effect of the emulsified solution on bitterness

The response by emulsified 5 mM caffeine solution was significantly weaker than that by the non-emulsified 5 mM caffeine solution in sensory evaluation. The response of STC-1 stimulated by the former solution was

also lower than that by the latter solution, indicating that the result in the evaluation by STC-1 cells corresponded to that by sensory evaluation.

From these results, STC-1 cells are shown that to be useful for the evaluation of the suppression of bitterness by CDs and emulsified solution. The mechanism for suppression of bitterness by XG was clarified to be different from that by CDs and emulsified solution.

Analysis of amylin expression in the brain area essential for mouse maternal behavior

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The medial preoptic area (MPOA) of the hypothalamus is considered to be a critical area for the parental behavior. Neurons in the MPOA express receptors for various hormones such as oxytocin, prolactin, and estrogen, which are involved in regulation of parental behavior. Amylin is a hormone secreted in the β -cells in the pancreas. It has been recently reported that mRNA and protein of amylin are expressed in the MPOA of lactating rats. In the brain, amylin binds to a calcitonin receptor associating with a regulatory protein. The calcitonin receptor is abundantly present in the MPOA, and therefore, it is suggested that the amylin-calcitonin receptor system participates in the regulation of parental behavior. In this study, the relationship between amylin-calcitonin receptor system and parental behavior was investigated.

Brain sections for the MPOA were prepared from C57BL/6 mice of 3-4 month-old. Expression of mRNA for amylin and calcitonin receptor was examined by in situ hybridization and c-Fos protein was detected by immunostaining. The mRNA and protein were detected by double-staining on the same brain section. The number of positive-staining neurons was determined by NeuroLucida (Micro Bright Field).

The number of c-Fos-immunoreactive cells among calcitonin receptor-expressing cells in the MPOA significantly increased 2 hours after the beginning of parental behavior. The number of c-Fos-positive cells among amylin-expressing cells also significantly increased. These results suggest that amylin is produced during parental behavior to activate calcitonin-receptor positive cells. The number of amylin-positive cells in the MPOA of female mice significantly increased at day 19 pregnancy and the day of parturition as compared with nulliparous mice. The number temporarily decreased 7 day after parturition, then markedly increased to maximum level at 21 day after parturition. The number was significantly reduced by removal of pups. In nulliparous mice, the number of amylin-positive cells was decreased by single housing and was recovered by group housing. In male mice, the number of amylin-positive cells was very low at any housing states.

These results indicate that amylin expression shows marked sex-difference and reproductive cycle-dependent change. It is suggested that amylin expression in the female MPOA is mainly regulated by developmental stages of pups and cohabitation with other mice rather than by lactation or parental behavior.

Association of mastitis and bovine immune genes in Japanese Holstein cows

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Mastitis is a disease caused by microorganisms invading the teat. In the mammary gland, the invaded bacteria are recognized to be foreign by the major histocompatibility complex (MHC) of bovine (bovine leucocyte antigen ; *BoLA*) which exists in the macrophages in milk. *BoLA* is involved in antigen presentation as well. Simultaneously, Toll-like receptor 2 (*TLR2*) recognizes the peptidoglycan in gram-positive bacteria, and *TLR4* recognizes lipopolysaccharides in gram-negative bacteria. When these TLRs recognize the invading bacteria, IL-8, a kind of cytokine, is secreted from the mammary epithelial cells. When IL-8 binds to the receptor called *CXCR1* which present on the surface of neutrophils, neutrophils migrate from blood and are secreted into milk. Thus, the immune system in the mammary gland functions in a complex manner. Studies on the association of mastitis with genes related to mammary immune system have focused on the resistance and susceptibility to mastitis. The association of *DRB3* locus with a high occurrence of polymorphisms in the *BoLA* gene in mastitis has been investigated. Recently, it was reported that some single nucleotide polymorphisms (SNPs) in *TLR2* and *TLR4* genes are involved in inducing resistance to mastitis. Furthermore, the *CXCR1* gene has a large number of SNPs, and the haplotypes of this gene have been reported to be related to mastitis. However, studies on these genes have resulted in contradictory findings, and therefore, their consistent association with mastitis has not been clearly established. Moreover, mastitis is a multifactorial disorder, and therefore, a genome-wide analysis of the association of the genes with mastitis is required.

Therefore in the present study, we analyzed *BoLA-DRB3* gene and 5 SNPs (*TLR2* +385, *TLR4* -226, *TLR4*+1656, *CXCR1*+777, and *CXCR1*-1768) that have been suggested to be related to mastitis development.

We also performed an association analysis to determine the association between these polymorphisms and mastitis in cattle ; we evaluated whether these combinations induce mastitis resistance or susceptibility to mastitis.

Two hundred and ninety two Holstein cows from 20 farms in the Tama area of Tokyo were involved in the study. These cows were classified as healthy cows and mastitis cows on the basis of long-term data from 1st to 5th lactations of the monthly official herd tests of somatic cell count (SCC). Furthermore, 226 Holstein cows from other dairy herds in Chiba Prefecture who had been infected with mastitis pathogens were used. Pathogens such as *Streptococci*, *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CNS) and *Escherichia coli* causing mastitis were detected in the milk of the cattle. We extracted genomic DNA from the blood samples of the cows. *BoLA-DRB3* alleles were identified by performing PCR-SBT, and were classified in 3 groups (Type1, Type2, and Type3) on the basis of the amino acid motifs of the alleles. Moreover, 1 SNP in bovine *TLR2* gene and 2 SNPs in the *CXCR1* gene were genotyped using the PCR-RFLP method, and 2 SNPs in the bovine *TLR4* gene were genotyped using the Tetra-primer ARMS-PCR method. The frequencies of presence of the *TLR2* and *CXCR1* genotypes and their alleles and the combinations of *BoLA-DRB3* genotypes with *CXCR1* or *TLRs* genotypes in healthy cows and those with mastitis were compared. Statistical analyses were performed by the chi-square test or Fisher's exact test by using the *FREQ* procedure of SAS.

DRB3 gene analysis showed the frequency of presence of the type1 homozygote is higher in healthy cows than in those with mastitis, and the frequency of presence of the type 2 homozygote is higher in the mastitis cows than in the healthy cows. The frequency of presence of

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the genotype GT of *TLR2+385* was slightly higher in the healthy cows than in those with mastitis, and the frequency of presence of the genotype TT was slightly higher in the cows with mastitis than in the healthy ones. The frequency of the genotype GG and GC of *TLR4-226* was slightly higher in the healthy cows than in those with mastitis, while that of genotype CT of *TLR4+1656* was slightly higher in the healthy cows than in those with mastitis, and the frequency of presence of the genotype CC was higher in the mastitis cows than in the healthy ones. In case of *CXCR1+777*, the frequency of presence of the genotype GG was higher in the healthy cows than in those with mastitis, and there were differences in the frequencies of presence of the genotype GC and CC between the mastitis cows defined by SCC and the mastitis cows identified mastitis pathogen. The frequency of presence of the genotype TT was higher in the in the healthy cows than in those with mastitis. Moreover, we analyzed combinations of genes to clearly determine about the association of the genes with mastitis. In the gene combinations of *DRB3/TLR2/TLR4*, the frequency of type 1 homo/GT/GC/CT was

significantly higher in healthy cows than in those with mastitis. The frequency of type 2 homo/TT/GG/CC was slightly higher in the cows with mastitis than in the healthy cows. However, these combinations showed the same patterns as those observed for each genotype when they were analyzed separately. In the combination of *DRB3/CXCR1* genotype, when *DRB3* genotype was a type 1 homo type, *CXCR1* CC/TT genotype showed significantly higher frequency in cows with mastitis than in the healthy cows, and when *DRB3* genotype was a type 2 homo type, the frequency of *CXCR1* GG/TT genotype combination was slightly higher in the healthy cows than those with mastitis. Even if the cows carried the *DRB3* genes that are believed to induce resistance to mastitis, a combination of *DRB3* with *CXCR1* gene showed susceptibility to mastitis. These results show that a combination of *DRB3/CXCR1* is probably associated with resistance and susceptibility to mastitis. Therefore, analyzing the association of 2 or more genes with resistance or susceptibility to mastitis appeared to be a useful technique, and determining gene combinations will help selecting cows with resistance to mastitis.

The basal secretion of appetite regulating hormones in Tsumura, Suzuki, Obese Diabetes male mice and the effects of pair-feeding on those secretion

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In recent years, obesity has been increasingly cited as a major health issue in developed countries. Obesity or metabolic syndrome is considered to be a major risk factor for chronic diseases such as hypertension, cardiovascular disease and type II diabetes, and researches of these areas have been increasing. Selecting animal models to the study of obesity is important, and the most characteristic examples of obese models are ob/ob and db/db mice with monogenic background. These mice develop obesity due to mutations in leptin (ob/ob) or leptin receptors (db/db) gene. On the other hand, Tsumura Suzuki Obese Diabetes (TSOD) mice were newly established as multi-gene obese diabetes model, which showed hyperphagia, hyperglycemia, hyperinsulinemia and insulin resistance. TSOD mice have been reported that the abnormality of insulin secretion, but another endocrine problem in TSOD mice is not clear. In the present study, we examined the basal secretion of feeding-related hormones, leptin, peptide YY (PYY) and glucagon-like peptide1 (GLP-1) in TSOD mice. In addition, leptin was injected to TSOD mice to examine the leptin resistance, and the effects of pair-feeding on insulin and leptin secretions in TSOD mice were also investigated.

Male TSOD and Tsumura Suzuki NonObesity (TSNO) as control mice were used in this experiment. Body weight and food consumption of all mice were measured during the period of the experiment. All mice were sacrificed by decapitation at 4, 8, 12, 16 weeks of age and the weight of epididymal fat pad was measured. Intraperitoneal injection of leptin (1 or 2mg/kg) to the mice at 12 weeks old was performed at 1 hour before light-out (6 : 00 p.m.), and food consumption of all mice were measured at 6, 12 or 24 hour after the injection of leptin. In the pair-feeding study, TSOD mice were given individu-

ally pair-fed each day (between 3-12 or 3-16 weeks of age) to the amount of food consumed daily on TSNO as control mice. TSNO mice (control group), TSOD mice fed ad libitum (AL group) and TSOD mice pair-fed to TSNO (PF group) were sacrificed by decapitation at 12 or 16 weeks of age.

As a result, food consumption in TSOD mice increased from 8 weeks of age, and the weight of body and epididymal fat pad in TSOD mice increased from 4 weeks of age compared with TSNO mice ($p < 0.05$). In TSOD mice, plasma concentrations of insulin and leptin increased from 8 weeks of age, PYY increased from 12 weeks of age and GLP-1 increased at 16 weeks of age compared with TSNO mice ($p < 0.05$). In the leptin administration test, food consumption of TSNO mice was suppressed ($p < 0.05$) at 12 and 24 h after the injection of leptin (2mg/kg), but administration of leptin was not effective in TSOD. In the pair-feeding test, the weight of body and intraperitoneal fat pad, and plasma concentrations of insulin and leptin showed higher values in the AL group, intermediate values in the PF group and lower values in the control group.

The present study confirmed that TSOD mice showed hyperphagia, obesity and hyperinsulinemia as previous reports mentioned. In addition to these phenomenons, the present study clearly demonstrated that leptin secretions in TSOD mice increased as a result of decreased the sensitivity of leptin. Another anorexigenic hormone including PYY or GLP-1 secretion also increased in TSOD mice, and resistance of PYY and GLP-1 might be existed in TSOD mice. To clarify whether the obesity in TSOD mice can be explained simply by hyperphagia, pair-feeding test was performed in the present study, and the results suggest that hyperphagia is not the sole

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cause of obesity in TSOD mice. In addition to hyperphagia, energy metabolisms or spontaneous locomotions may also be responsible for obesity in TSOD mice. TSOD mice showed characteristics similar to human obesity, and the present study suggests that TSOD mice estab-

lished as a multi-gene obese animal model, might be more useful for the study of human obesity and type II diabetes than ob/ob or db/db mice as models of single-gene defect.