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INFORMATION

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The titles of theses and other information are as follows:

Development of therapeutic strategy for infectious diseases using the antimicrobial peptide or receptor antagonist

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Infectious diseases are still one of the most important causes of morbidity and mortality of people and animals all over the world. Epidemiological research and development of vaccine are important as primary prevention for the infectious diseases. Because the primary prevention is not sufficient to overcome the infectious diseases, secondary prevention, including development of prophylactic and therapeutic strategy is also important. However, the therapeutic strategy is not well established for many infectious diseases, therefore it is necessary to establish effective therapeutic approach for the infectious diseases.

In the chapter I, I examined the efficacy of antimicrobial peptides (AMPs) against bacterial infection. AMPs play pivotal role in innate immunity that is non-specific and fast-acting defense system against infection and bind to bacterial membrane, resulting in bactericidal activity. For this non-specific defense activity, it is suggested that the emergence of AMPs-resistant bacteria rarely occur and AMPs may be useful for therapeutic strategy for infectious diseases.

I isolated a novel gene that is similar to mouse β-defensins which are one of the family of AMPs, and observed that the location of this gene in mouse genome and the characteristics of its gene structure are very common to that of β-defensins. Moreover, I predicted the region corresponding to the mature peptide of this gene, synthesized a small peptide based on this sequence, designated as K17, and analyzed structure and function of the small peptide. This peptide has positive charge as same as known AMPs. Circular dichroism (CD) spectroscopy analysis of K17 demonstrated that K17 presents random coil conformation in aqueous solution, but adopts α-helical conformation in a membrane-mimicking environment. K17 exhibited bactericidal activity against *Salmonella enterica* serovar Typhimurium (Gram negative) and *Staphylococcus aureus* (Gram positive), but it was not cytotoxic in cultures of mammalian cells or hemolytic in cultures of erythrocytes. From these results, it is suggested that the peptide (K17) which I synthesized in this study may be a candidate therapeutic for the treatment of infectious diseases.

In the chapter II, I aimed at the host response occurred after the development of Adult T-cell Leukemia (ATL). ATL is the T-cell malignancy caused by Human T-cell leukemia virus type-I (HTLV-I) that belongs to retrovirus. Because there is no effective ATL therapy, the development of therapy is needed. A characteristic manifestation of ATL is extensive infiltration of

leukemic cells into various organs, including lymph nodes, liver, spleen, lungs, and skin. The molecular mechanisms associated with ATL cell infiltration are poorly understood. Therefore, the investigation of the infiltration mechanism may be important for development of ATL therapy. Because tumor cells are attracted to several tissues by chemokine expressing in those tissues, I examined the response of ATL cells to several chemokines and found that ATL cells show markedly response to stromal-cell derived factor-1α (SDF-1α). AMD3100, the antagonist against CXCR4 that is receptor for SDF-1α, suppressed the responsibility and the infiltration of ATL cells to tissues in mouse model. These results suggest the SDF-1α/CXCR4 interaction involves in leukemic cell migration and this pathway may become a novel candidate target for ATL therapy.

In the chapter I and II, I demonstrated development of therapeutic approaches for infectious diseases.

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A study on contrast-enhanced ultrasonography with second-generation contrast agent Sonazoid® for diagnosis of liver and splenic nodules in dogs

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Ultrasonography is one of the first modalities that has high sensitivity for detection of the abdominal mass lesion. In many patients, however, ultrasonographic characterization of masses is not possible because of poor specificity. In human medicine, contrast-enhanced ultrasonography with Sonazoid®, a second-generation contrast agent incorporated by the reticuloendothelial system, allows an accurate diagnosis of hepatic tumors. In veterinary medicine, although contrast-enhanced ultrasonography has been performed for many organs, the utility of Sonazoid® has been uncertain. The principal aim of this thesis was to investigate the clinical utility of Sonazoid®-enhanced ultrasonography in veterinary medicine. Firstly, the author evaluated the usefulness of Sonazoid®-enhanced ultrasonography in differentiating between malignant and benign hepatic nodules with clinical patients (Chapter 1). Secondly, the author evaluated the usefulness of Sonazoid®-enhanced ultrasonography in differentiating between malignant and benign splenic nodules with clinical patients (Chapter 2).

In chapter 1, contrast-enhanced ultrasonography using Sonazoid® was performed in 6 normal beagles and 27 dogs with 28 hepatic nodules. An appropriate protocol for the evaluation of all 3 phases (arterial, portal, and parenchymal) was established based on the results for normal beagles. By evaluation of the echogenicity of hepatic nodules during the arterial and

parenchymal phases it was possible to differentiate malignant tumors from benign nodules with very high accuracy. In 15 of 16 nodules diagnosed as malignant tumors, nodules were clearly hypoechoic to the surrounding normal liver during the parenchymal phase. Additionally, malignant tumors had different echogenicity compared to the surrounding normal liver during the arterial phase in 14 of 15 nodules. In the portal phase, there were no characteristic findings.

In chapter 2, contrast-enhanced ultrasonography using Sonazoid® was performed in 6 normal beagles and 29 dogs with spontaneous focal or multifocal splenic lesions. An appropriate protocol for the evaluation of all 3 phases (early vascular, late vascular, and parenchymal) was established based on the results for normal beagles. In 29 dogs with splenic lesions, qualitative assessment of the enhancement pattern was performed in the early vascular, late vascular, and parenchymal phase. In the early vascular phase, a hypoechoic pattern was significantly associated with malignancy ($P = 0.02$) with sensitivity of 38% [95% CI, 25–38%], and specificity of 100% [95% CI, 84–100%]. In the late vascular phase, a hypoechoic pattern was significantly associated with malignancy ($P = 0.001$) with sensitivity of 81% [95% CI, 66–90%], and specificity of 85% [95% CI, 65–95%]. There was no significant difference between malignant and benign lesions during the parenchymal phase.

In conclusion, the result of the present study demonstrated that Sonazoid®-enhanced ultrasonography could differentiate benignancy and malignancy of liver and splenic lesions in dogs. For liver lesions, the arterial and parenchymal phase imaging could be useful for the differentiation. For the splenic lesions, the early vascular and late vascular phase imaging could be useful for the differentiation. This thesis could be a huge first step towards making definitive diagnosis with this noninvasive diagnostic method, Sonazoid®-enhanced ultrasonography.

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The original papers of this thesis appeared in *Vet. Radiol. Ultrasound*, **50**: 104–108 (2009). *Vet. Radiol. Ultrasound*, **51**: 79–85 (2010) and *J. Vet. Intern. Med.*, **24**: 1290–1297 (2010).

Antigenic and genetic analyses of avian influenza viruses isolated in Japan

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H5 and H9 influenza virus isolates in Japan were genetically and antigenically characterized and the birds infected with the virus were pathologically examined.

In 2005, H5N2 influenza viruses were isolated form layer chickens in Ibaraki Prefecture, Japan and the Ibaraki prefectural governor declared the end of the H5N2 outbreak in June, 2006, 1 year after the first detection. Sixteen H5N2 viruses were isolated from 9 farms of the affected 41 farms. Phylogenetic and antigenic analysis of the isolates showed that these isolates were closely related to the H5N2 strains prevalent in Central America that have

been circulating since 1994. Experimental infection of chickens with the index isolate (A/chicken/Ibaraki/1/2005 (H5N2)) demonstrated that this virus replicated efficiently in the respiratory tract without clinical signs. Pathological findings of the chickens indicated that the virus efficiently replicated in salivary epithelial cells. The virus was transmitted among the chickens in separated cage. This result indicated that the infection to chickens spread by droplet transmission in the outbreaks.

In 2008, H5N1 highly pathogenic avian influenza virus was isolated from whooper swans (*Cygnus cygnus*) found dead in Hokkaido, Japan. Pathological findings indicate that the swan died due to severe congestive edema in the lungs. Phylogenetic analysis of the HA genes of the isolates revealed that these are the progeny viruses of the isolates Clade 2.3.2 from poultry and wild birds in China, Russia, Korea, and Hong Kong. Antigenic analyses indicated that the viruses are different from the H5N1 viruses isolated from wild birds and poultry before 2007. The chickens vaccinated with A/duck/Hokkaido/Vac-1/2004 (H5N1) survived 14 days after challenge with the isolate, although small amount of the challenge virus was recovered from the tissues of the birds. These findings indicate that the H5N1 highly pathogenic avian influenza virus are circulating in wild birds in addition to domestic poultry in Asia and showing antigenic variation that may be due to vaccination.

Recently, H9N2 influenza viruses were isolated from poultry and mammals in Asian countries. The hemagglutinins (HAs) of H9 influenza viruses isolated from birds and mammals of different species were antigenically and genetically analyzed. Based on the reactivity patterns with the panel of 8 monoclonal antibodies, 21 H9N2 virus strains isolated from birds and mammals were divided into 7 antigenically distinct groups. The present findings indicate that H9N2 viruses are genetically and antigenically varied.

The vaccinations to poultry in some countries resulted in antigenic variation of H5 and H9 influenza viruses isolated in Asia. These result indicate that the stamping-out policy is most important to control for avian influenza.

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Asian lineage H5N1 highly pathogenic avian influenza virus replication in feathers of waterfowl

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Asian lineage H5N1 highly pathogenic avian influenza (HPAI) virus has spread from Asia to Europe, the Middle East, and Africa, causing profound economic losses in the poultry industry. Several reports of virus detection in wild and domestic waterfowl indicate that waterfowl can serve as carriers and amplifying hosts of this virus. Therefore, the author hypothesized that the detailed analysis on the pathogenesis and viral replication in waterfowl would provide information to reduce the risk of viral transmission from infected waterfowl. In

particular, the author focused on the viral replication in feathers, and discussed the possible role of waterfowl feathers in viral transmission from infected birds

In chapter I, the author examined the pathogenesis in domestic ducks inoculated with Japanese H5N1 HPAI virus. The virus caused mortality, neurologic symptoms, and pathologic changes such as the encephalitis, pancreatic necrosis, corneal opacity and epidermal necrosis of the feathers. High mortality in ducklings indicates that the age of birds is a factor influencing on the viral replication. The feather lesions caused by virus replication raise the potential of feathers as new route of virus shedding from waterfowl infected with H5N1 HPAI virus.

In chapter II, the experimental infection was performed to examine whether the feather lesion observed in the previous chapter is common to other H5N1 HPAI virus strains or other waterfowl species. Two different clades of the virus replicated in the feather epidermis of domestic ducks and geese in experimental infection. Electron microscopic examination provided the direct evidence of viral replication in feather epidermal cells. Viral replication in the feather tissue was also found in whooper swans naturally infected with the virus. In addition, the viral transmission was confirmed in domestic ducks by oral inoculation of down feathers plucked from a duck infected with the virus.

In chapter III, it was reported that feathers of infected domestic ducks can be useful samples for virus detection as well as their swabs. In the experimental infection using 2 H5N1 HPAI viruses and domestic ducks, larger amounts of viruses were isolated for longer period from feathers than from the swabs, indicating that feathers are considered to be useful clinical samples for surveillance or diagnostic examination of H5N1 HPAI virus in domestic ducks. The author also investigated the viral persistence in feathers detached from bodies of domestic ducks infected with H5N1 HPAI viruses to evaluate the possible environmental contamination by infective feathers. Feathers plucked from experimentally infected domestic ducks were examined by virus isolation. Infectious viruses persisted for the longest period in feather calami compared with drinking water and feces. Viral infectivity persisted in the feathers for 160 days at 4°C and for 15 days at 20°C. These results indicate that feathers detached from domestic ducks infected with H5N1 HPAI virus can function as fomites containing high viral loads in the environment.

In conclusion, the author presented a new viewpoint on an epidemiological role of waterfowl feathers in Asian lineage H5N1 HPAI virus transmission.

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