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INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy (Ph.D) in Veterinary Medicine on September 25, 2007 to 2 recipients and December 25, 2007 to 1 recipient. The titles of theses and other information are as follows:

## Toxicological evaluation of chlorinated hydrocarbons exposure on wildlife

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Original paper of this thesis appeared in *Environ. Toxicol. Chem.,* 21: 842-847 (2002) and *Chemosphere,* 51: 491-500 (2003).

## Establishment of murine model of latent infection with pseudorabies virus

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Mice model latently infecting with psesudorabies virus (PrV) and virus reactivation system by using acetylcholine (Ach) were established.

1. Ach was utilized to activate PrV in a latent state in pigs. Pigs treated with Ach showed some side effects just after inoculation and PrV was isolated from nasal swabs. In contrast, pigs inoculated with dexamethasone (Dex) highly depressed and virus was not isolated from these pigs. Ach also could activate PrV in cultured trigeminal ganglia (TGs) of latently infected pigs. These results demonstrate that Ach is an effective material to activate latently infected PrV.
2. Small laboratory animal model for PrV latent infection was established by pre-treating mice with anti-PrV porcine serum followed by the challenge of YS-81, a PrV wild strain. The latent PrV was reactivated from TGs with Ach *in vitro.* It was found that this model was useful to analyze the mechanism of latent PrV reactivation by Ach.
3. A latency model of PrV infection in mice was utilized for the reactivation of latent PrV *in vivo.* Reactivated virus with Ach or Dex was detected in nasal swabs, and neutralizing antibody against PrV was detected in serum. The results showed that Ach was possible to use as inducer to reactivate latent PrV in this model as well as in pigs.
4. The effects of mild stress treatment to PrV latently infected mice were examined in order to demonstrate that the mice model simulated natural latent infection. Latently infected mice excreted PrV from the nasal cavity under stress treatments. The present findings

demonstrate that these kinds of mild stress reactivated the virus in murine latent infection models in a manner similar to the induction of latent infection in pigs in the field.

5. The kinetics of cytokines related to stress was analyzed to clarify the relationship between virus reactivation by Ach and immune system. Interleukin-1$\beta$ (IL-1$\beta$) and Interleukin-6 (IL-6) were detected after stimulation by Ach, which means that Ach induced physiological stress conditions. However, no relationship between the kinetics of the cytokine levels and PrV excretion was observed and neither IL-1$\beta$ nor IL-6 could reactivate latently infecting PrV by itself.

In conclusion, the mice PrV latency model reactivating latent virus with Ach is a useful system to investigate not only mechanisms of latency and reactivation of herpesviruses including PrV but also stress mechanism. According to this system, it is possible to analyze the mechanism of latent infection and reactivation of herpesviruses more in details. It is not clear yet how Ach reactivate latently infecting PrV. It is suspected that a lot of factors are related to the mechanism of latently infection and reactivation of herpesviruses. The mouse model described here may help to analyze this complicated phenomenon by step-by-step investigations.

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Original papers of this thesis appeared in *Arch. Virol.,* 141: 161-166 (1996), *J. Virol. Meth.,* 70: 103-106 (1998), *Exp. Anim.,* 51: 407-409 (2002), *Exp. Anim.,* 52: 383-386 (2003) and *Exp. Anim.,* 53: 457-461 (2004).

# The Studies on the usefulness of a novel ultrasound contrast agent, Sonazoid, in diagnosis of hepatic tumor and mechanism of the contrast effect

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Ultrasonography (US) is a useful and convenient modality for the diagnosis of hepatic focal lesions, which exhibit characteristic gray-scale morphologic features based on their distinctive echogenicities. Some kinds of malignant tumors, however, especially small ones, have a low echogenic difference in comparison with the surrounding healthy tissue, so that the detection of these tumors is not always possible. Therefore, detection and characterization of tumor vascularity are generally important for the differential diagnosis of a focal lesion.

Because the backscattering echo signal is particularly strong at gas-liquids interface, microbubbles in liquids enhance the echo signal at the region. Microbubbles that are enough small to go through capillaries exhibit similar behavior to red blood cells, thus they act as blood flow tracer and visualize vessels. First-generation of US contrast agents which utilize such property of microbubbles to ultrasound consisted of easily soluble air-microbubble, thus the contrast effect was not enough for diagnostic satisfaction due to instability of the microbubbles *in vivo.* From the viewpoint of US imaging machine technology, harmonic imaging technology has been developed to visualize the harmonic echo from the microbubbles: backscatter signal (echo) from the microbubbles contains more harmonic component than that from tissues. With harmonic imaging mode, first-generation agents can visualize not only large vessels but also tissue perfusion.

Meanwhile, a second-generation agent, Sonazoid (NC100100), comprising a suspension of mi-

crobubbles of a low solubility inert gas is being developed recently. In addition to showing high contrast effect in vessels in the early phase, which is immediate after injection of the agent, this agent is known to demonstrate parenchyma-specific contrast in the liver imaging in the delayed phase, which starts after the decay of the vascular contrast.

In this study, therefore, characteristic contrast effects of the agent and its time-response was compared with histological observation, and, diagnostic significance and clinical meaning of both the early and delayed phases of Sonazoid in the liver imaging was investigated by using healthy and disease-model animals. In addition, the mechanism of parenchymal contrast effect, which Sonazoid provides in the liver imaging, was also investigated through observation of dynamics of Sonazoid microbubbles in the liver using the newly developed technique by which localization of microbubbles *in vivo* could be visualized.

At first, the liver contrast effects of this agent were evaluated by quantitative measurement of videodensity and visual analysis in conventional B-mode and harmonic imaging mode. In normal rabbits, contrast enhancement of vessels and parenchyma peaked immediately (1 minute) after injection in both modes, although signal enhancement in the parenchyma lasted for 120 minutes compared with rapid decay in vessels, in which the signal intensity returned to the levels before injection. Therefore, the contrast effects in the early phase was set 1 minute after injection, when the signal enhancement in the vessels was high, and those in the delayed phase was evaluated at approximately 10 minutes after injection, when the vascular contrast had decreased to baseline and the parenchyma was specifically enhanced, respectively. When Sonazoid was intravenously injected into metastatic carcinoma-model (VX-2) rabbits, all hepatic tumors showed ring enhancement in the early phase followed by clear contrast-defects in the delayed phase, because signal enhancement remained only in normal parenchyma. Visual analysis scores for the diagnosis of tumors were improved by Sonazoid injection, and the videodensitometric differences between tumor and normal tissues were significantly greater after injection. In this first study, Sonazoid exhibited potential for the detection of undifferentiated tumors in the liver by visualization of neovascularity in the early phase and clear contrast defects in the delayed phase, not only in the harmonic but also in the conventional B-mode by visual and quantitative analysis.

Next, the author compared the contrast US by Sonazoid with phase (pulse) inversion imaging (PII) and histopathology of the tumor by taking advantage of high resolution of PII technique, which substantially improves resolution and signal-to-noise ratio. Histopathologic observation revealed that the ring-enhancement was caused by neovascularity in the tumor, and the contrast defects corresponded to living and dead parts of tumors, which lack Kupffer cells. In addition, transmission electron microscopy (TEM) showed microbubble-like structures in Kupffer cells, which indicated that Sonazoid microbubbles were taken up by these cells. However, a mechanical slowdown of the microbubbles of another second-generation contrast agent in the sinusoids was observed, and the slowdown was reported as a mechanism for the parenchymal contrast effect in the liver. Since the liver vessels were perfusion-fixed to prepare the section in the study of Sonazoid by TEM, it was impossible to observe Sonazoid microbubbles in the sinusoidal space.

In the last section, therefore, a microscopic technique using intravital microfluoroscopy superimposed on phase-contrast transillumination image was developed to observe distribution of microbubbles in hepatic microcirculation in real-time. This technique demonstrated freely flowing microbubbles in the sinusoid and some microbubbles which was likely to be taken up by Kupffer cells. Then, in order to verify internalization of the microbubbles in Kupffer cells, confocal laser scanning microscopy (CLSM) with very thin focal plane was used. CLSM of primary cultured rat Kupffer cells *in vitro* revealed that reflected light spots from

microbubbles were observed in Kupffer cell cytoplasm after addition of Sonazoid to the culture medium; these results indicate that microbubbles actually exist within the Kupffer cells. *In vivo* uptake of Sonazoid was observed in the liver of rats whose Kupffer cells were fluorescent-labeled with intravenous injection of fluorescently labeled liposomes. The conscious rat was intravenously injected with Sonazoid and the liver was excised 10 minutes later. When the liver was observed by CLSM, the percentage of Kupffer cells taking up microbubbles increased dose-dependently, and, at clinical dose, that was approximately 1%, which corresponded to that estimated from distribution data in rat.

In conclusion, an US contrast agent, Sonazoid, demonstrated vascularity in the early phase which was immediate after intravenous injection, and in the delayed phase, when hepatic parenchyma-specific contrast was observed, demonstrated Kupffer cell distribution in the liver. Sonazoid was considered to achieve homogenous enhancement of the hepatic parenchyma only through its minimal uptake into Kupffer cells. Therefore, Sonazoid might be useful for the detection of undifferentiated tumors in the liver by making it possible to visualize neovascularity in the early phase and show clear contrast defects at the location of tumors without Kupffer cells in the delayed phase. In the veterinary practice, Sonazoid was also expected to be useful for detection of hepatic tumor, especially metastasis, as well as for differentiation with benign lesions in the liver.

However, this study was based on only the specific image of a rabbit liver tumor model alone. As hepatic primary or metastatic tumors are of great variety, manner of survival and proliferation should be variable. Therefore, contrast effect by Sonazoid on such various tumors might have variety. Based on understanding of the mechanism and characteristics of contrast effect of this agent, the accumulation of specific imaging of various hepatic tumors is desirable for improvement of diagnostic accuracy on hepatic lesions using convenient modality, US, and this agent.

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Original papers of this thesis appeared in *Biol. Pharm. Bull.,* 26: 1272-1277 (2003), *Biol. Pharm. Bull.,* 28: 972-977 (2005), *Methods Find. Exp. Clin. Pharacol.,* 29: 321-327 (2007) and *Invest. Radiol.,* 42: 643-651 (2007).