MicroRNA is one of the hottest research fields even in KSHV. This kind of small RNA molecule seems to have profound effects on cellular processes and then viral activities but their details have not been elucidated totally. KSHV lytic and latent phases are regulated by viral but also cellular microRNAs. Two specialists; Liang et al. (2011) and Gottwein (2012) reveals the microRNA world of KSHV.

And finally, we have to think about treatment of KSHV-associated tumors such as KS, PEL and a lympho-proliferative disease, multicentric Castleman's disease. It should be very hard to treat these tumors in the immunodeficient setting. It will be desirable if KSHV specific strategy is designed, since these tumors are very tightly linked with KSHV infection. Dittmer et al. (2012) contribute for this theme and discuss about it.

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I would like to thank all contributors, and hope that this topic will be useful for the future study of KSHV.

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Editorial Open Access

Kaposi's Sarcoma-Associated Herpesvirus Induced Tumorigenesis; How Viral Oncogenic Insults are Evaded

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Some viral infections in human are strongly related to cancer formation. Apart from retrovirus induced cancer formation seen in rodents and avian, virus induced cancer formation in human seems to be very complicated. In human, mainly DNA viruses such as papillomavirus, hepatitis B virus (HBV), Epstien-Barr virus (EBV), and Kaposi's sarcoma-associated herpes virus (KSHV) are etiological agents and some RNA viruses such human T-cell leukemia virus and hepatitis C virus (HCV) are involved in their specific cancer formation. It takes long time for the viruses to cause cancers and we do not have good systems to observe how the viral infection leads to cancer formation.

KSHV is belonging to gamma-herpesviridae and an agent involved in the formation of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castelman's disease (MCD).

The virus' infection has a very strong link with these cancers. The mechanism how the virus causes such cancers is, however, still enigmatic and remains to be elucidated. KSHV latent infection should be important in terms that this type of infection provides with an origin of the related cancers. But, many genes with oncogenic activity of this virus are lytic genes, which are expressed only in the lytic phase.

As mentioned above, virus induced carcinogenesis is very complicated and is attractive to take an insight how the virus causes related cancers [1].

KSHV expresses an extremely limited number of viral genes such as latency-associated nuclear antigen (LANA), viral cyclin (v-cyc), viral FLICE inhibitory protein (vFLIP), kaposin and viral interferon regulatory factor-3 (vIRF-3) and 17 viral microRNAs in latency. The genes build an active gene locus in the KSHV genome in latency.

Among them, v-CYC, a homolog of cellular D-type cyclins, functions as an oncogene to deregulate cellular proliferation which leads to DNA damage response (DDR) and p53 induced apoptosis. Normal cells respond to oncogenic insults and cannot be easily transformed by choosing suicide pathway through p53 [2]. If the virus survives this situation, there must be a mechanism and this is one of ways how KSHV causes cancers.

In this point a recent report from Leidal et al. [1] is attractive for an insight to link the v-CYC induced oncogenic insult with subversion of this activity by vFLIP and how the virus causes related cancers. They found that v-CYC caused autophagy induced senescence and/or apoptosis. On the other hand, vFLIP is known for an autophagy inhibitor as well as an NF-kB activator [3,4]. And thus, vFLIP functions to evade from v-CYC induced oncogenic insult/senescence and make a direction of KSHV induced carcinogenesis.

However, we should be careful whether such a pathway happens in the natural infection course, since this kind of experiment is usually performed in over-expression system. Actually, vFLIP expression at the protein level has not been confirmed even in KSHV infected PEL cell lines and thus it is unclear whether such vFLIP activity is seen in the native situation.

In summary, a report from Liang seems to be very important to explain how KSHV causes cancers by connecting oncogene (v-CYC in this case) induced apoptosis and/or senescence [5]. Although there is no related report about the other human virus induced carcinogenesis, similar mechanisms might be stealthing.

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Overexpression of HGF attenuates the degeneration of Purkinje cells and Bergmann glia in a knockin mouse model of spinocerebellar ataxia type 7

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ABSTRACT

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant disorder associated with cerebellar neurodegeneration caused by expansion of a CAG repeat in the ataxin-7 gene. Hepatocyte growth factor (HGF), a pleiotrophic growth factor, displays highly potent neurotrophic activities on cerebellar neurons. A mutant c-met/HGF receptor knockin mouse model has revealed a role for HGF in the postnatal development of the cerebellum. The present study was designed to elucidate the effect of HGF on cerebellar neurodegeneration in a knockin mouse model of SCA7 (SCA7-KI mouse). SCA7-KI mice were crossed with transgenic mice overexpressing HGF (HGF-Tg mice) to produce SCA7-KI/HGF-Tg mice that were used to examine the phenotypic differences following HGF overexpression. The Purkinje cellular degeneration is thought to occur *via* cell-autonomous and non-cell autonomous mechanisms mediated by a reduction of glutamate transporter levels in Bergmann glia. The Purkinje cellular degeneration and reduced expression of glutamate transporters in the cerebellum of SCA7-KI mice were largely attenuated in the SCA7-KI/HGF-Tg mice. Moreover, phenotypic impairments exhibited by SCA7-KI mice during rotarod tests were alleviated in SCA7-KI/HGF-Tg mice. The bifunctional nature of HGF on both Purkinje cells and Bergmann glia highlight the potential therapeutic utility of this molecule for the treatment of SCA7 and related disorders.

1. Introduction

Spinocerebellar ataxia type 7 (SCA7) is a progressive inherited disorder characterized by ataxia and neurodegeneration of the cerebellum and retina (Ieraci et al., 2002). The disease is the result of an abnormal CAG repeat expansion in the ataxin-7 gene. SCA7 patients display ataxia in addition to neurodegeneration and neuronal death of Purkinje cells (Ieraci et al., 2002). SCA7 knockin mice also show a neurodegeneration of Purkinje cells (Yoo et al., 2003). Therefore, it is thought that the protection of Purkinje cells may represent a therapeutic strategy to combat SCA7.

Additionally, following findings suggest that Bergmann glial cells have been considered as another therapeutic target of the disease. Bergmann glia are cerebellum-specific astrocytes that are located around synapses between Purkinje cells and cerebellar granule cells or climbing fibers. The glial cells play a role in removing excess glutamate from synapses *via* two primary glutamate transporters, glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) (Huang and Bordey, 2004). A mouse model that expresses expanded ataxin-7 specifically in Bergmann glia displays a neurodegeneration of Purkinje cells, indicating that a dysfunction of Bergmann glia contributes to the degeneration of Purkinje cells in SCA7 mice and thereby progression of the disease in a non-cell autonomous manner (Custer et al., 2006). Therefore, prevention of cell degeneration and concomitant increase of glutamate transporter function may represent a valid therapeutic strategy for SCA7.

Hepatocyte growth factor (HGF), which was first identified as a potent mitogen for mature hepatocytes (Nakamura et al., 1984, 1989), exhibits neurotrophic activities in a wide variety of neurons in the hippocampus, the cerebral cortex, the cerebellum, the brainstem (midbrain dopaminergic neurons) and the spinal cord (sensory and motor neurons) (Funakoshi and Nakamura, 2011). Recent experiments have indicated that HGF exerts neuroprotective effects on various neurons in animal models of cerebral ischemia, amyotrophic lateral sclerosis (ALS) and spinal cord injury (Funakoshi and Nakamura, 2011; Sun et al., 2002; Ishigaki et al., 2007; Kitamura et al., 2011; Miyazawa et al., 1998). In the cerebellum, HGF is expressed in Purkinje cells and granular cells, and plays a role in the cerebellum during both developmental and adult

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stages (Honda et al., 1995; Ieraci et al., 2002). In a mutant with a partial loss of Met function, the cerebellum was smaller than in controls and showed abnormal foliation (Ieraci et al., 2002). In addition to the cell growth and development, HGF exhibits neuroprotective effects for mature granule cells in primary cerebellar neuron culture (Zhang et al., 2000; Hossain et al., 2002). Moreover, overexpression of HGF not only attenuates the degeneration of motor neurons as a neurotrophic factor but also maintains adequate levels of the astrocytic glutamate transporter GLT-1 in a transgenic mouse model of ALS (Sun et al., 2002). This evidence led us to hypothesize that HGF may have a therapeutic potential on cerebellar neurons and Bergmann glia, cerebellar astrocyte subpopulations, in a valid mouse model of SCA7 in which a targeted 266 CAG repeat segment (a length known to cause infantile disease onset) of ataxin-7 is knocked into the mouse *ATXN7/Sca7* locus. These mice show features, which resemble those observed in an infantile SCA7 patient (Yoo et al., 2003).

The purpose of this study was to examine the effect of HGF on the Purkinje cells and Bergman glia of SCA7-KI mice. For this purpose, transgenic mice overexpressing HGF in a neuron-specific manner (HGF-Tg mice; Sun et al., 2002) were crossed with SCA7 knockin mice (SCA7-KI mice; Yoo et al., 2003) and phenotypic comparisons were made in wild-type (WT), HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice. Overexpression of HGF attenuated the shrinkage of Purkinje cells and prevented reduction of glutamate transporters in Bergmann glia and improved motor performance during the rotarod test in SCA7-KI mice.

2. Materials and methods

2.1. Animals

The SCA7 knockin ($Sca7^{266Q/5Q}$: SCA7-KI) mouse is a knockin mouse, which is a valid model of SCA7 that contains a targeted insertion of 266 CAG repeats (a number that causes infantile-onset disease) into the mouse *Sca7* locus. The mice were generously provided by Dr. Huda Zoghbi from the Baylor College of Medicine, Houston, TX (Yoo et al., 2003). Neuron-specific enolase (NSE)-promoter driven HGF transgenic (HGF-Tg) mice were generated and maintained as previously described (Sun et al., 2002). Heterozygous SCA7-KI male mice were crossed with heterozygous HGF-Tg female mice, which had been backcrossed with C57BL/6J mice for more than seven generations, to generate WT, heterozygous HGF-Tg, heterozygous SCA7-KI, and heterozygous SCA7-KI/HGF-Tg mice. Mouse genotypes were determined by dot blot hybridization or by polymerase chain reaction (PCR) using forward (5′-TTGTAGGAGCGGAAAGAATGTC-3′) and reverse (5′-CCACCCACAGATTCCACGAC-3′) primers for SCA7-KI and with forward (5′-CCAAACATCCGAGTTGGTTACT-3′) and reverse (5′-ATTACAACTTGTATGTCAAAAT-3′) primers for HGF-Tg mice. Experimental protocols were approved by the Animal Experimentation Ethics Committee of Asahikawa Medical University and Osaka University Graduate School of Medicine. All efforts were made to minimize animal discomfort and the number of animals used.

2.2. Cerebellar neuronal culture

Sixteen-day-old mouse embryos (E16) were obtained from timed pregnant C57BL/6J females (Japan SLC, Hamamatsu, Japan) that had been deeply anesthetized with isoflurane and euthanized *via* decapitation. Routinely, two pregnant females were processed in parallel. Immediately after euthanasia, uteri containing the embryos were removed and transferred into a sterile 100 mm tissue culture dish that was kept on ice and filled with ~20 ml ice-cold Leibovitz's L-15 medium. The cerebella were dissected using a stereomicroscope. After removing the meninges, the isolated cerebellar primordia were minced and transferred to a 15-ml Falcon tube containing L-15 medium. The supernatant was replaced with a pre-warmed 0.25% trypsin solution and the cerebella were incubated for 4–5 min at 37 °C with gentle shaking. Incubation was terminated by the addition of fetal bovine serum (JRH Biosciences, Brooklyn, Australia). Following the addition of DNase I and centrifugation, cells were dissociated by repeated pipetting and separated from non-dissociated tissue by sedimentation. The cells were seeded in plates precoated with poly-L-ornithine (500 μg/ml) at 2.5×10^5 cells/cm^2. Cultures were grown in neurobasal medium (Gibco Invitrogen, Grand Island, NY) supplemented with B27 (Gibco Invitrogen), 2 mM GlutaMaxI (Gibco Invitrogen), 1 mM adenine, 3 mM KCl, 1% heat-inactivated horse serum (Gibco Invitrogen), and a mixture of penicillin–streptomycin (100 U/ml and 100 μg/ml; Nacalai Tesque, Kyoto, Japan). From 2 days after seeding, 10 μM triiodothyronine (T3) and 1 μM Ara-C were added in order to mature Purkinje cells and to prevent the proliferation of non-neuronal cells. Half of the medium was replaced with fresh medium every 2 days. The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air. The cells cultured for 14 days were washed with phosphate buffered saline (PBS) and fixed with 10% formalin in PBS.

2.3. Tissue preparation

Animals (WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice) at 10 weeks of age ($n = 3$ each) were deeply anesthetized with sodium pentobarbital and transcardially perfused with ice-cold PBS followed by ice-cold 4% paraformaldehyde in PBS. The cerebella were excised and immersed in the same fixative for several hours at 4 °C. Fixed tissues were immersed in 10% sucrose in PBS overnight at 4 °C, followed by 20% sucrose in PBS for 6 h at 4 °C, after which they were subsequently frozen in powdered dry ice or CO_2 gas. Frozen tissues were cut into either 16-μm or 40-μm thick sagittal sections using a Leica CM3050 S or CM1900 cryostat (Leica Microsystems GmbH, Wetziar, Germany).

2.4. Immunocytochemistry and immunohistochemistry

Formalin-fixed cerebellar neurons or cryosections were incubated in blocking buffer consisting of 10% normal goat serum (S26-100 mL, CHEMICON, Temecula, CA) and 0.3% Triton X-100 in PBS for an hour at room temperature followed by one or two of the following primary antibodies for 20 h at 4 °C: (1) mouse monoclonal anti-calbindin D28K antibody (1:250; 300, Swant, Marly, Switzerland); (2) mouse monoclonal anti-GFAP (glial fibrillary acidic protein) antibody (1:250; MAB3402, CHEMICON); (3) rabbit polyclonal anti-GFAP antibody (1:10; N150687, DAKO, Glostrup. Denmark); (4) rabbit polyclonal anti-c-Met antibody (1:50; SP260, Santa Cruz Biotechnology, Santa Cruz, CA); (5) rabbit polyclonal anti-rat HGF antibody (Ohya et al., 2007; Yamada et al., 1995) (6) guinea-pig polyclonal anti-GLAST antibody (1:500; AB1782, CHEMICON); (7) guinea-pig polyclonal anti-GLT-1 antibody (1:600; AB1783, CHEMICON). For immunostaining of phospho-c-Met, sections were incubated with Blocking One Histo (Nacalai Tesque) for an hour at room temperature, and then immunoreacted with rabbit polyclonal anti-phosphorylated c-Met antibody (1:200; C7240, Sigma, St. Louis, MO) in Signal Enhancer HIKARI B Solution (Nakalai Tesque) for 20 h at 4 °C. After washing the sections with PBS, immunoreactivity was visualized by incubating them further for 20 min at room temperature with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 546 or Alexa Fluor 647 diluted 1:600 (Invitrogen, Carlsbad, CA). Fluorescence-immunostained sections were observed under an Olympus FV1000

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microscope (Olympus, Tokyo, Japan) or an All-in-One BZ-9000 Fluorescence microscope (KEYENCE, Osaka, Japan). Digital image Z-stacks were created, and projections were made from them using an Olympus FV1000 microscope and FV10-ASW software (Olympus). Fluorescence intensity was measured using the NIH Image J Program.

2.5. Quantification of cellular Purkinje cell size

Sixteen-micrometer stacks of cerebellar optical sections were collected using an All-in-One BZ-9000 Fluorescence microscope and Z-stack images were joined into sagittal images using BZ-II software (KEYENCE). These data were transferred into Neurolucida software (MBF Bioscience, Japan, Inc., Chiba, Japan). Four stacks (each 0.09 μm^2 in area) for each of three sections from WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice ($n=3$ each) were used to measure the cell sizes of calbindin-positive Purkinje cells. Quantification of cell size was performed as previously described with slight modifications (Yoo et al., 2003). Briefly, each cell surface was outlined manually. The perikaryon area of each cell was estimated to be the approximately circular area enclosed by the cell perimeter and the extension of the cell perimeter toward the initial point of dendrite extension. Partial cells and binary cell images were excluded based upon cell shape and relative fluorescence intensity.

2.6. Enzyme-linked immunosorbent assay (ELISA)

After the mice were placed under deep anesthesia with an overdose of sodium pentobarbital, the cerebella of WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice ($n=4$ each) were collected, quickly frozen and stored at −80 °C until used. Frozen tissue samples were homogenized, subsequently sonicated using a Bioruptor UCD-250 (Cosmo Bio Co., Ltd., Tokyo, Japan), and centrifuged at 4 °C. The supernatants were then used to quantify HGF protein levels using ELISA (Institute of Immunology Co., Ltd., Tokyo, Japan) as previously described (Kadoyama et al., 2007; Sun et al., 2002; Yamada et al., 1995).

2.7. Assessment of motor performance

A rotarod apparatus (Bioseb, Paris, France) was used to assess the ability of an animal to balance on an elevating rotating metal rod (Carter et al., 2001). Rotarod tests are a common tool of studies of mouse models of spinocerebellar ataxia (Yoo et al., 2003; Custer et al., 2006). In this study, 10-week-old mice (WT; $n=13$, HGF-Tg; $n=12$, SCA7-KI; $n=12$, SCA7-KI/HGF-Tg; $n=8$) were placed on the rotarod, the speed of which was set at 5 rpm initially and accelerated until reaching a speed of 20 rpm for 5 min. After taking a rest for more than 5 min, the latency to fall from the rotarod was measured for 5 min using the machine mode (initial rate of 5 rpm; acceleration until reaching 40 rpm for 10 min). The average latency to fall for each genotype was calculated.

2.8. Statistical analyses

Statistical analyses were carried out using StatView software version 5.0.1 (SAS institute, Cary, NC). Differences in the total intensities of immunostaining for HGF, GLAST, GLT1, size of Purkinje cells, and regional HGF levels in the cerebellum among the above animal models were all determined by one-way ANOVA with Fisher's PLSD tests. Latency to fall in rotarod tests was analyzed using the Student's t-test. The results are presented as the mean ± S.E.M. A value of $P<0.05$ was considered statistically significant.

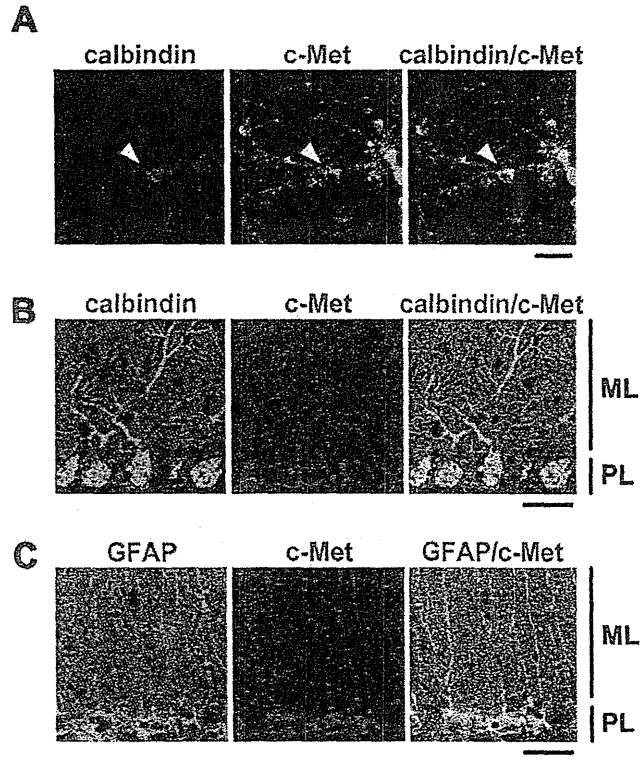

Fig. 1. Immunocytochemical and immunohistochemical localization of c-Met in cerebellar cells *in vitro* and *in vivo*. HGF receptor (c-Met) is expressed in Purkinje cells of the mouse cerebellum *in vitro* and *in vivo*. (A) Immunocytochemistry in primary neuronal cultures of the cerebellum from E16 mouse embryos. Purkinje cells show double immunoreactivity (white arrowheads) for calbindin (red) and c-Met (green) in culture. Bar, 30 μm. (B and C) Immunohistochemistry in 10-week-old wild-type (WT) mice. (B) Purkinje cells show double immunoreactivity against calbindin (green) and c-Met (red). PL, Purkinje cell layer; ML, molecular layer. Bar, 30 μm. (C) Bergmann glia show double immunoreactivity against GFAP (green) and c-Met (red). Bar, 30 μm. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

3. Results

3.1. c-Met is expressed in the cerebellar Purkinje cells in vitro

To assess whether cerebellar Purkinje cells are potential target cells of HGF, we first performed double immunostaining of c-Met (green) and calbindin (red), a marker for Purkinje cells, in primary cultures of embryonic mouse cerebellar neurons *in vitro*. c-Met-immunoreactivity (IR) was observed in a large number of cerebellar neurons, with most of these presumably being granular cells. In addition to these neurons, c-Met-IR was indeed detected in calbindin-positive Purkinje cells (Fig. 1A).

3.2. c-Met is expressed in the cerebellar Purkinje cells and Bergmann glia in vivo

We next assessed whether c-Met is expressed in Purkinje cells *in vivo* using double immunostaining for c-Met (red) and calbindin (green) in the cerebella of WT mice. c-Met-IR was detected in Purkinje cells of the cerebella of WT mice. In addition, c-Met-IR was detected in other cells surrounding to the Purkinje cells (Fig. 1B). These cells that were closely apposed to Purkinje cells resembled Bergmann glia. To determine if these non-Purkinje cells were Bergmann glia, double immunostaining for c-Met (red) and GFAP (green), a marker for Bergmann glia, was performed. As shown in Fig. 1C, c-Met-IR was detected in Bergmann glia. These findings

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indicate that both Purkinje cells and Bergmann glia are potential target cells of HGF *in vivo*.

3.3. Immunohistochemical analyses of HGF and phospho-c-Met in the cerebellum of WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice

To explore whether HGF can modify the degeneration of Purkinje cells in SCA7-KI mice, we used neuron-specific enolase (NSE)-driven HGF overexpression transgenic mice (HGF-Tg) to introduce HGF into the Purkinje cells of SCA7-KI mice. Crossing SCA7-KI mice with HGF-Tg mice generated the following four mouse models: (1) wild-type littermates (WT), (2) HGF-Tg, (3) SCA7-KI, and (4) SCA7-KI/HGF-Tg mice. This approach allowed the stable introduction of the HGF gene directly into the cerebellar neurons of SCA7-KI mice. Confirmation that HGF was introduced into the cerebellum of SCA7-KI/HGF-Tg mice was accomplished with immunostaining. HGF-IR was faintly detected in calbindin-positive Purkinje cells and GFAP-positive Bergman glia of the cerebellum in WT and SCA7-KI mice (Fig. 2A, upper panel), while more intense staining of HGF-IR was detected in Purkinje cells of HGF-Tg mice as well as in SCA7-KI/HGF-Tg mice (Fig. 2A, bottom panel). In addition to Purkinje cells, HGF-IR was detected in cells surrounding the Purkinje cells, *i.e.* GFAP-positive Bergmann glia. These findings were further confirmed by quantitative analyses of the immunofluorescent intensity of HGF-IR in sections of the cerebellum (Fig. 2B) and HGF content using ELISA analyses (Fig. 2C). These findings suggest that overexpressed HGF in cerebellar neurons is released into the extracellular space, and is in turn distributed to Bergmann glia.

3.4. c-Met is tyrosine-phosphorylated in Purkinje cells and Bergmann glia in SCA7-KI/HGF-Tg mice

Further attempts were made to determine if overexpression of HGF contributes to tyrosine-phosphorylation, and thereby activation, of c-Met in SCA7-KI/HGF-Tg mice. The level of phospho-c-Met-IR was much higher in both the Purkinje cells and Bergmann glia of SCA7-KI/HGF-Tg mice compared to those of SCA7-KI mice. These findings demonstrate that overexpression of HGF in SCA7-KI/HGF-Tg mice contributes to the activation of c-Met in Purkinje cells and Bergmann glia, prompting an examination of the role of HGF in the modulation of these cells in SCA7-KI mice (Fig. 2D).

3.5. Overexpression of HGF attenuates the degeneration of Purkinje cells of the cerebellum in SCA7-KI mice

To elucidate whether HGF plays a role in attenuating the degeneration of Purkinje cells in SCA7-KI mice, we next compared the morphology of Purkinje cells from the cerebella of SCA7-KI and SCA7-KI/HGF-Tg mice. Calbindin immunohistochemistry revealed that Purkinje cells appeared to have large spherical cell bodies in both WT and HGF-Tg mice, while a number of Purkinje cells with reduced cellular size (degenerated neurons, white arrowheads), were present in SCA7-KI mice (Fig. 3A). In contrast, many more large spherical neurons were observed in SCA7-KI/HGF-Tg mice compared to SCA7-KI mice (Fig. 3A). The size distribution of Purkinje cells in WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice is shown in Fig. 3B. Quantitative analyses showed that the size of Purkinje cells in SCA7-KI mice was varied from small (<150 μm^2) to large (>150 μm^2; healthy), while the quantity of small Purkinje cells (<150 μm^2) was reduced in SCA7-KI/HGF-Tg mice (Fig. 3B). Namely, the fraction of small Purkinje cells was significantly lower in SCA7-KI/HGF-Tg mice than in SCA7-KI mice. These findings demonstrate that overexpression of HGF attenuates the degeneration of Purkinje cells in SCA7-KI mice.

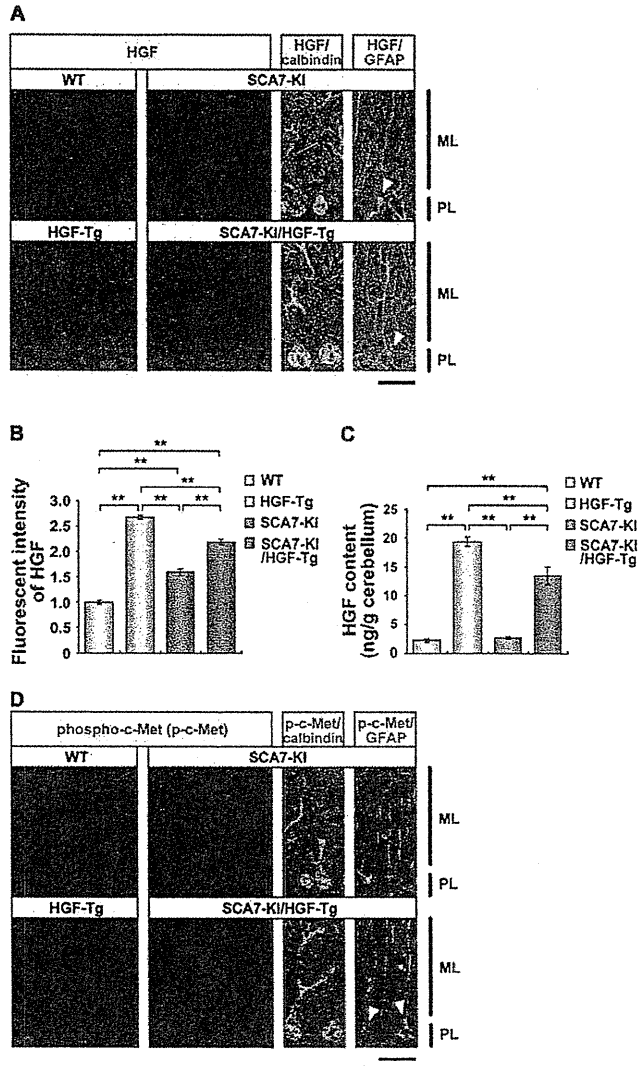

Fig. 2. Immunohistochemical localization of HGF and phospho-c-Met in cerebellar cells *in vivo*. Comparison of HGF levels by immunohistochemistry (A and B) and HGF ELISA (C) in the cerebellum of 10-week-old WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice using anti-rat HGF that detects both endogenous and exogenous (overexpressed) HGF proteins. (A) HGF-IR is elevated in HGF-Tg and SCA7-KI/HGF-Tg mice. PL, Purkinje cell layer; ML, molecular layer. Bar, 30 μm. White arrowhead indicates GFAP/HGF double-positive cells. (B) Fluorescent intensity ($n = 6$ per group) of HGF in each group. Mean HGF signal intensity was significantly elevated compared to WT (**$P < 0.01$, Fisher's PLSD test). Error bars indicate S.E.M. (C) HGF protein levels in the whole cerebellum are elevated in HGF-Tg and SCA7-KI/HGF-Tg mice ($n = 4$ per group, **$P < 0.01$, Fisher's PLSD test). Error bars indicate S.E.M. (D) Immunohistochemistry of tyrosine phosphorylation at positions 1230, 1234, and 1235 of c-Met (phospho-c-Met, red) in the cerebellum in 10-week-old mice. Phospho-c-Met staining is shown in the cerebellum in all mice groups. Phospho-c-Met-IR is elevated in both Purkinje cells (lower left panel, green) and Bergmann glia (lower right panel, green) of SCA7-KI/HGF-Tg mice compared to SCA7-KI mice (upper panel, green). White arrowheads indicate GFAP/phospho-c-Met double-positive cells. Bar, 30 μm. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

3.6. Overexpression of HGF maintains the levels of the glutamate transporters (GLAST and GLT-1) in the cerebellum of SCA7-KI mice

Bergmann glia are responsible for glutamate uptake (removal) from the Purkinje cell synaptic cleft. It has been suggested that polyglutamine-expanded ataxin-7 induces Purkinje cell excitotoxicity by interfering with Bergmann glia-mediated glutamate uptake. This is due to the fact that the expression of GLAST, a

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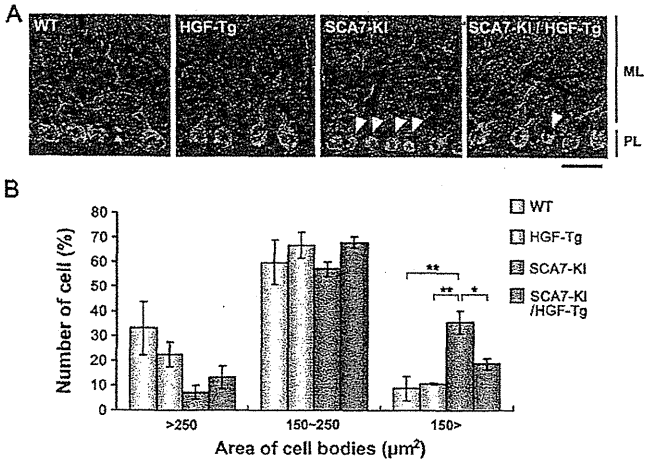

Fig. 3. HGF attenuates degeneration of Purkinje cell bodies. (A) Immunohistochemistry of calbindin (green) in the cerebellum of 10-week-old mice. SCA7-KI mice displayed smaller Purkinje cell body size than WT and HGF-Tg mice. SCA7-KI/HGF-Tg mice showed an attenuation of shrinkage of Purkinje cell bodies. PL, Purkinje cell layer; ML, molecular layer. Bar, 30 μm. White arrowhead indicates degenerative Purkinje cell changes. (B) Quantification of cell numbers with different Purkinje cellular body size (>250; 150–250; <150 μm^2) of each group ($n=3$ per group). The number of small cells (area are less than 150 μm^2) in SCA7-KI mice is significantly greater *versus* WT and HGF-Tg mice (**$P<0.01$, Fisher's PLSD test), SCA7-KI/HGF-Tg mice exhibit significantly fewer small cells compared to SCA7-KI mice (*$P<0.05$). Error bars indicate S.E.M. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

glutamate transporter in the cerebellum, is confined to Bergmann glia and marked reductions in GLAST expression (and glutamate uptake) have been observed in presymptomatic Gfa2-SCA7-92Q mice (Custer et al., 2006). As c-Met is expressed in the Bergmann glia of WT mice and is phosphorylated (*i.e.* activated) in Bergmann glia in SCA7-KI/HGF-Tg mice (Figs. 1C and 2D), we next examined whether HGF affects the morphology and function of Bergmann glia. Immunostaining for GFAP revealed that obvious morphological difference of Bergmann glia was not detected between WT mice and SCA7-KI/HGF-Tg mice (Fig. 4A). We then examined whether HGF modulates the down-regulation of GLAST levels in SCA7-KI mice. Immunostaining for GLAST revealed that GLAST levels were decreased in SCA7-KI mice compared to WT mice, while the levels were generally maintained in SCA7-KI/HGF-Tg mice (Fig. 4B and C). These findings demonstrate that HGF supports GLAST levels in SCA7-KI mice. We then examined HGF regulation of GLT-1, another glutamate transporter that is also abundant in the cerebellum, by a similar mechanism. Immunostaining for GLT-1 revealed that the levels of GLT-1 were markedly decreased in SCA7-KI mice compared to WT mice, while the level was maintained or even increased in SCA7-KI/HGF-Tg mice (Fig. 4D and E). These findings demonstrate that HGF maintains or even increases the levels of GLT-1 in SCA7-KI mice.

3.7. Overexpression of HGF improves rotarod performance in SCA7-KI mice

Data obtained so far suggested that SCA7 could be improved by HGF *via* the attenuation of Purkinje cellular degeneration and reduction of glutamate transporters in Bergmann glia. Therefore, we examined whether these improvements were reflected by

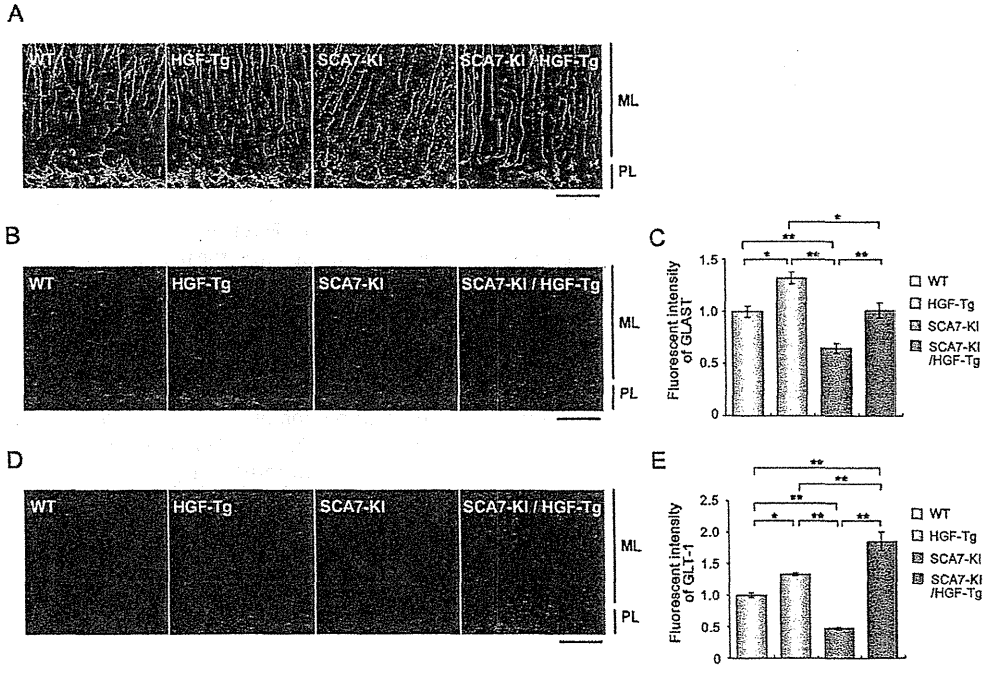

Fig. 4. HGF maintains the levels of glutamate transporters (GLAST and GLT-1) in Bergmann glia in the cerebellum of SCA7-KI mice. (A) Immunohistochemistry for Bergmann glia (GFAP, green) in the cerebellum in 10-week-old mice. No significant alterations were detected in the morphology of Bergmann glia. PL, Purkinje cell layer; ML, molecular layer. Bar, 30 μm. (B and C) Comparison of GLAST levels in 10-week-old mice. (B) Immunohistochemistry for GLAST in the cerebellum. GLAST staining is reduced in the SCA7-KI mouse cerebellum and is significantly rescued in the SCA7-KI/HGF-Tg cerebellum. Bar, 30 μm. (C) Quantification of fluorescent intensity ($n=3$ per group) of PL. Mean GLAST signal intensity is significantly elevated in SCA7-KI/HGF-Tg cerebellum *versus* SCA7-KI cerebellum (*$P<0.05$, **$P<0.01$, Fisher's PLSD test). Error bars indicate S.E.M. (D and E) Comparison of GLT-1 level at 10-week-old mice. (D) Immunohistochemistry for GLT-1 in the cerebellum. GLT-1 staining is reduced in the cerebellum of SCA7-KI mice, while significantly elevated in the cerebellum of SCA7-KI/HGF-Tg mice. Bar, 30 μm. (E) Mean fluorescent intensity ($n=3$ per group) of PL. Mean GLT-1 signal intensity is significantly elevated in the cerebellum of SCA7-KI/HGF-Tg mice (*$P<0.05$, **$P<0.01$, Fisher's PLSD test). Error bars indicate S.E.M. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

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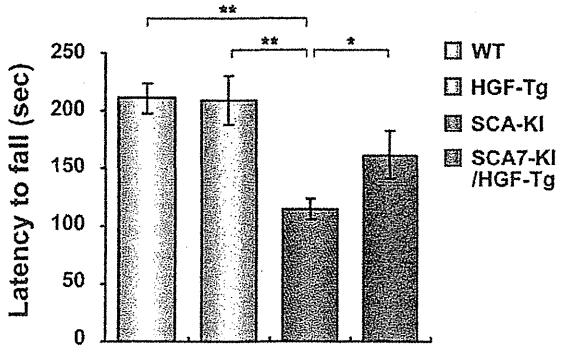

Fig. 5. HGF improves coordinated motor behavior of SCA7-KI mice. Comparison of motor coordination in 10-week-old WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice using the rotarod test. SCA7-KI/HGF-Tg mice display improved rotarod performance compared to SCA7-KI mice ($n = 8–12$ per group; $^*P < 0.05$, $^{**}P < 0.01$, Student's t test). Error bars indicate S.E.M.

motor performance of WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice. To examine the ability of an animal to balance on a rotating rod, rotarod tests were applied on each animal at 10 weeks of age. There was a marked reduction in the latency to fall in SCA7-KI mice compared to WT and HGF-Tg mice. However, the latency to fall of SCA7-KI/HGF-Tg mice was significantly longer than that of SCA7-KI mice (Fig. 5), suggesting that overexpression of HGF contributes to the amelioration of rotarod performance impairments in SCA7-KI mice.

4. Discussion

In the present study, we examined whether overexpression of HGF, a pleiotrophic growth factor with highly potent neurotrophic activities, exhibits a beneficial function in SCA7-KI mice. By crossing SCA7-KI mice with HGF-Tg mice that overexpress HGF under the NSE promoter, four groups of mice (WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice) were generated. The results indicate that overexpression of HGF attenuates the degeneration of Purkinje cells, maintains the levels of the glutamate transporters GLAST and GLT-1 in Bergmann glia and improves rotarod performance deficits observed in SCA7-KI mice.

The molecular mechanisms responsible for these events have not yet been clarified in detail. However, because HGF protein is expressed and distributed in Purkinje cells and Bergmann glia in SCA7-KI/HGF-Tg mice at much higher levels than in SCA7-KI mice, and because the expression and phosphorylation (activation) of c-Met was observed at much higher levels in both the Purkinje cells and Bergmann glia of SCA7-KI/HGF-Tg mice, it seems likely that HGF functions directly on Purkinje cells as well as Bergmann glia. If there is a direct interaction, the ability of HGF to function not only on Purkinje cells but also on Bergmann glia might represent a therapeutic opportunity for attenuating the degeneration of Purkinje cells, since recent genetic approaches suggest that an important mutual interaction of Purkinje cells and Bergmann glia in SCA7 might, at least in part, be involved in the degeneration of these cells in this disease (Custer et al., 2006; Furrer et al., 2011). Furthermore, Bergmann glia are also shown to secrete neurotrophic factors that support Purkinje cells (Mount et al., 1995).

Purkinje cells are integrated into a complex neural network and receive glutamatergic input from axons projecting from the inferior olive and cerebellar granule cells. Hence, in addition to Purkinje cells and Bergmann glia, which we focused on the present study, other cells and their neural networks in the cerebellum may also play a role in the pathogenesis of disease models of SCA7 and related diseases (Gatchel et al., 2007; Furrer et al., 2011). For example, transcriptional down-regulation of insulin-like growth factor binding protein 5 (*igfbp5*) in cerebellar granule cells is proposed to be involved in non-cell-autonomous degeneration of Purkinje cells in SCA7-KI mice (Gatchel et al., 2007). It has not yet been determined whether HGF could alleviate reduction of *igfbp5*, and this possibility is worth examining in a future study. Given that HGF elicits neurotrophic activity on cerebellar granular cells both *in vitro* and *in vivo* (Zhang et al., 2000; Ieraci et al., 2002), we cannot exclude the possibility that HGF functions on granular cells and alleviates the down-regulation of *igfbp5*. Therefore, HGF may also contribute to attenuation of Purkinje cell degeneration *via* cerebellar granular cells.

It has not yet been determined whether HGF alleviates the degeneration of the retina, the other region associated with phenotypic changes appearing in SCA7-KI mice. HGF and c-Met are expressed in various populations of rat retinal neurons during development as well as in the adult, and neuroprotective effects of HGF on rat retinal photoreceptors have been reported (Machida et al., 2004; Ohtaka et al., 2006; Shibuki et al., 2002; Sun et al., 1999).

The rotarod test is used to analyze motor phenotype, in the aspect of motor balance and/or its coordination (Carter et al., 2001; Custer et al., 2006). Hence, the ability of HGF to improve rotarod performance raises the potential utility of HGF for the improvement of motor impairment of affected individuals. However, further experiments are required to address the relationship between the outcome of rotarod tests in the present study and the clinical ataxic phenotype of SCA7.

Cvetanovic et al. (2011) recently reported that genetic overexpression or pharmacologic infusion of recombinant vascular endothelial growth factor (VEGF) ameliorates the ataxic phenotype and degeneration of Purkinje cells in a mouse model of another type of spinocerebellar ataxia, spinocerebellar ataxia type 1 (SCA1). Given that HGF promotes angiogenesis in a variety of disease models (Funakoshi and Nakamura, 2003, 2011) and that c-Met is not only expressed in Purkinje cells and Bergmann glia but also in other types of cells including vascular cells and neural progenitor populations in WT mice (Funakoshi and Nakamura, 2011; Noma et al., unpublished results), it would be interesting to know how HGF plays a role in SCA1-model mice and whether HGF promotes angiogenesis and neurogenesis in SCA7-KI mice. It should be noted that exercise produces beneficial effects in alleviating SCA1 symptoms in mice (Fryer et al., 2011). Exercise is known to promote HGF production in some patients (Yasuda et al., 2004) and that HGF improves the phenotype of SCA7-KI as shown in the present study. Hence, it would also be interesting to examine whether exercise plays a role in the attenuation of the progression of the course of SCA7-KI pathology and if HGF is involved in the process.

In summary, the present study provided the first evidence that overexpression of HGF is beneficial for attenuating the degeneration of both Purkinje cells and Bergmann glia. Considered with the notion that intrathecal injection of recombinant human HGF protein has been shown to be effective in several disease models, such as a transgenic rat model of ALS (Ishigaki et al., 2007) and a primate model of spinal cord injury (Kitamura et al., 2011), our findings may raise the possibility of a therapeutic use of HGF in SCA7 and related disorders.

Acknowledgements

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ウイルス肝炎のすべて

Ⅲ 血液感染するウイルス肝炎

1．B型肝炎

（1）B型肝炎のウイルス学

上田 啓次*

1960年代にBlumbergがオーストラリア抗原と肝炎との関連を示してから半世紀が迫ろうとしている。この抗原こそがB型肝炎ウイルス(hepatitis B virus：HBV)の遺伝子産物であった。分子生物学の発展とともに，HBVの塩基配列の決定，遺伝子同定，遺伝子機能の解析は進み，ワクチンの開発にも成功した。しかしながら，簡便な感染系が存在しないことからHBVのウイルス学はまったく進展しておらず，HBVの真のライフサイクルのみならず，急性あるいは慢性肝炎，肝硬変，肝がんといった深刻な病態の発症機構は不明な点が多い。問題解決にはHBV感染受容体を分離・同定し，確固とした，かつ簡便な感染系を樹立することが不可欠である。ウイルス学最大の難問を解決する糸口を模索する。

Key Words：hepatitis B virus，HBV，逆転写，感染受容体，感染系

Ⅰ はじめに

Blumbergが白血病患者血清中に新規の抗原(オーストラリア抗原)を発見し報告したのは1965年[1]，その後，数年で輸血後の血清肝炎との関連が確立された[2]。この感染性因子がB型肝炎ウイルス（hepatitis B virus：HBV)である。わが国では大河内らが最初に，日本人における本抗原の保有率を報告している[3]。疾患概念の確立は，慢性B型肝炎から肝硬変，肝がんへの病態進行や母体からの垂直感染の存在を明らかにした。

1970年代後半～80年代前半にかけて，分子生物学的研究手法の確立と相まって，次々とウイルスゲノムのクローニングと塩基配列が決定され，げっ歯類のウッドチャック(woodchuck hepatitis virus：WHV)や地リス（ground squirrel hepatitis virus：GSHV)，鳥類ではアヒル（duck hepatitis B virus：DHBV）や鷺（heron hepatitis B virus）にも同族のウイルスが存在することが明らかにされた。哺乳類では類人猿であるチ

Viorology of the hepatitis B virus

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ンパンジー (chimpanzee hepatitis B virus) やオランウータン (orangutan hepatitis B virus) などにも同様のヘパドナウイルスが蔓延していることが知られている[4) 5)]。

また, HBVは従来, サブタイプとしてadr, adw, aywのように分類されていたが, 最近ではゲノタイプA～Hとして分類されている[4)]。

ヘパドナウイルスの特徴は, ゲノムサイズがきわめて小さく, およそ3.2kb前後の部分的2本鎖の環状DNAウイルスであること, 遺伝子は大まかに言って, コア遺伝子, pol遺伝子, S遺伝子とX遺伝子のたった4つである (鳥類のヘパドナウイルスにはX遺伝子は存在しないとされている) ことや, DNAウイルスでありながら複製に逆転写過程が存在することである[4) 5)]。

分子生物学的手法により各遺伝子の機能や複製機構, 組み込み体の解析から肝がん発生との関連が研究されてきた。しかしながら, ヘパドナウイルス学を論じる上で解決されていない決定的な問題が2つあり, その問題が解決されない限り, ヘパドナウイルスの真のウイルス学, 病態論やウイルスの本質に則した治療法の開発はないものと考えている。その問題点とは, 有用かつ簡便な感染系が存在しないこと, ヘパドナウイルス複製酵素polの*in vitro*アッセイ系が存在しないことである。

本稿ではHBVを概説した後, 前述の解決されない主要2問題について概説したい。

Ⅱ HBV粒子構造, ゲノム, 遺伝子, 転写産物

HBVの感染性粒子はDane粒子と呼ばれる膜粒子とその内部のコア粒子(キャプシド)で構成され, キャプシド内にウイルスゲノムを内在する (図1A)。前述のごとく, HBVのゲノムはきわめてコンパクトに組織されている。また, 単純な2本鎖DNAゲノムではなく, 部分的に2本鎖の環状DNAである。さらに, ⊖鎖DNAの5'端に末端タンパクが付着し, ⊕鎖DNAの5'にはプライマーRNAがついているという独特の構造をしている (図1B)。また, レトロウイルスゲノム末端の非翻訳領域 (untranslated region : UTR) に機能的に相同と考えられるdirect repeat 1および2 (DR1, DR2) 配列がある。DR1はプレゲノムRNAの5'に, 3'にはDR2およびDR1が存在している[4)]。

このきわめてコンパクトなゲノムにウイルスとしての営みに必要な最小限の遺伝子がコードされている。それらの遺伝子は, コア (C), pol, SとX遺伝子の4つである。コア遺伝子とS遺伝子は読み取り枠の違いにより, コア遺伝子はpreC-CとCの2つに, また, S遺伝子はlarge S (LS), middle S (MS), small S (SS) の3つに分けられる (図1)。

Ⅲ preC-C (プレコアーコア) 遺伝子

ウイルスのキャプシド形成にかかわる構造遺伝子産物を供給する。2つのin-frame翻訳コドンにより, preC (29アミノ酸 [aa])-C (185aa) の読み取り枠となるか, Cのみの読み取り枠となるかが決定される。PreC-CあるいはC遺伝子に固有の転写産物はないが, preC-Cの読み取り枠になるにはpreC-C翻訳開始コドンATGより上流から転写が開始される必要がある。また, Cの翻訳にかかわる転写産物は, いわゆるプレゲノムRNAと考えられる (図2A)。

PreC-C産物はN末に疎水性アミノ酸配列からなる分泌シグナルがあり, また, C末のアルギニンに富む領域の前で切断されてHBe抗原として分泌される[4)]。HBe抗原産生のウイルスにとっての生理的な意義はよくわからないが, C遺伝子産物とほとんどオー

HBV (hepatitis B virus ; B型肝炎ウイルス)　UTR (untranslated region ; 非翻訳領域)

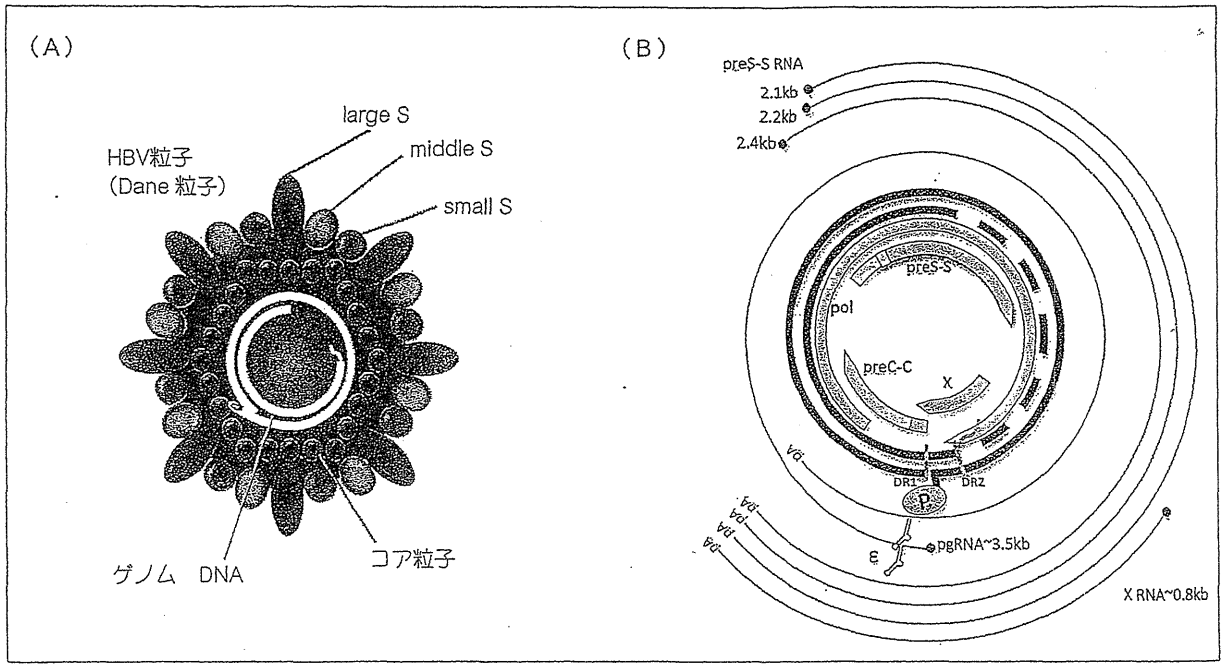

図1 HBV粒子とゲノム構造

(A) HBVの感染性粒子構造。HBVは部分2重鎖DNA環状構造をもつゲノムを内在するコア粒子（キャプシッド）と，それを被う外膜構造をもつ。外膜は，LS，MS，SSで構成される。(B) HBVゲノム（太い実線，部分2重鎖は破線）の構造，関連転写産物（外枠：細い実線），遺伝子（タンパク読み取り枠）（内側：ブロック矢印）を示す。ゲノムは⊖鎖5'に末端タンパク（P）（HBV pol）が付着している。複製に重要なDR（direct repeat）1，DR2の位置が示されている。転写産物の5'はcap構造を取り（●）ポリAで終わる。3.5kb mRNAは正確にはpreC-C産生にかかわるpreC ATGを含むものと，含まないプレゲノムRNA（pgRNA）に分けられる。pgRNAについてのみ，εの位置，構造を示してある。preS-S産生にかかわるmRNAは，2.4kb（LS），2.2kb（MS），2.1kb（SS）である。また，0.8kb mRNAはX遺伝子固有のmRNAである。

HBV：B型肝炎ウイルス （文献4より）

バーラップしていることから，キャリア化や慢性肝炎化などの持続性HBV感染に起因している可能性があると思われる。

血中HBe抗原の量はHBV慢性肝炎の活動性ともよく関連し，HBV増殖状況をよく反映している。HBe抗原陰性化とHBe抗体の陽性化というseroconversionにpreC領域内の終始コドンへの変異をともなうことが知られており，これにともない，肝炎も鎮静化することが多い[6]。

C遺伝子産物はHBVのコア粒子形成に不可欠なタンパク因子である。2量体が集合して最終的なコア粒子を形成すると考えられている。単一構成因子であるため，T（T；triangulation number）＝1のもっとも単純な正二十面体キャプシドを形成すると予測されるが，cryoelectronmicroscopyによる粒子結晶構造解析ではT＝3～4で，約180～240分子からなる正二十面体構造をとっていることがわかっている[7]。

コア粒子の形成過程でパッケージングシグナルεを介してプレゲノムRNAが粒子に取り込まれ，あるいはコアタンパクの分子集合に付随しつつ，プレゲノムRNAから逆転写により⊖鎖DNA合成，そして⊕鎖DNA合成が進行するものと考えられる[8]。コアタンパクが核内に存在することは報告されているが，コア粒子そのものがどこで形成され，どのような過程で膜粒子に取込まれるかなど，ウイルス粒子形成のダイナミックな分子集合プロセスには不明な点が多い。

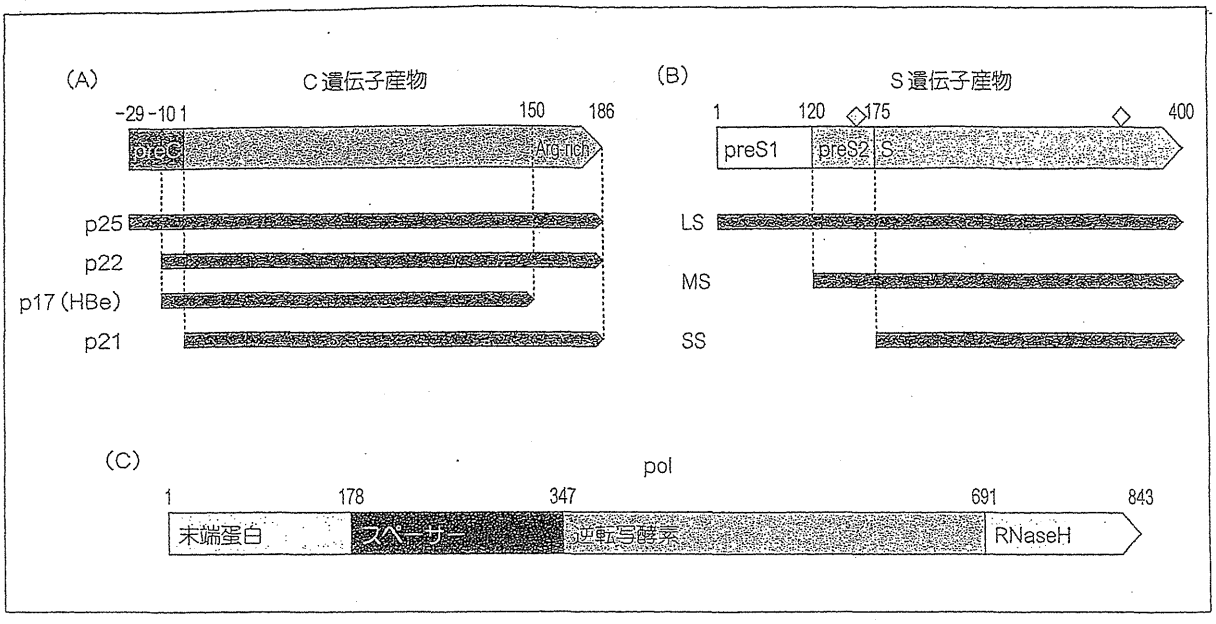

図2 コア (C), S, pol の産物と機能領域

(A) C 遺伝子産物。読み取り枠は preC-C と C の2つであるが，実際発現されるタンパクは最低4種類あると考えられている。p25 は preC-C の前駆体として発現し，N 端の分泌シグナル (−29 ～−9aa) (p22) と 150aa 以降のアルギニンに富む領域がプロセスされて p17 として分泌される。コア粒子の産生には p21 がかかわると考えられる。(B) S 遺伝子産物。SS 部分を共通として preS2 が加わった MS，さらに preS1 が加わった LS が産生される。preS2 と SS の C 端の◇は糖鎖付加部分を示す。(C) HBV pol タンパクの機能構造。N 端からタンパクプライミング逆転写にかかわる末端タンパク，機能領域を連結させるスペーサー，酵素活性を担う逆転写酵素領域，RNA 分解反応にかかわる RNaseH 領域からなる。

(文献5より)

IV pol

HBV ゲノム複製の根幹的役割を果たす因子である。845aa 前後からなり，末端タンパク領域，tethering 領域（もしくは spacer 領域），逆転写酵素活性領域，RNaseH 領域という各ドメインから構成されると考えられる（図2B）。前述のコアタンパクにはレトロウイルスの gag-pol 融合タンパクのようにプロテアーゼの活性を担う領域はなく，HBV pol がプロセスを受けて各ドメインに分解され，機能を発揮する可能性はないと考えられる[5) 9)]。

HBV は逆転写過程をもつ DNA ウイルスであり，逆転写過程をもつ RNA ウイルスであるレトロウイルスの生活環と根本的な違いがある。すなわち，レトロウイルスは感染後，細胞内侵入後に逆転写過程が始まり，最終的には2本鎖 DNA となり宿主染色体に組み込まれ，遺伝子発現，複製過程が進行する。

一方，HBV では感染後，転写産物として合成されたプレゲノム RNA を鋳型に粒子形成過程で逆転写が進行する。また，HBV の生活環には宿主染色体に組み込まれる過程は本質的にはない（感染肝細胞の増殖過程で非特異的に組み込みが起こることは知られており，初期段階ではレトロウイルスゲノムの末端部位の LTR (long terminal region) に相当する DR (direct repeat) が 5', 3' の両端に位置するほぼ完全な形で頻繁に組み込みが起こることが知られている）[10)]。

LTR (long terminal region)　DR (direct repeat)

V preS-S遺伝子産物

HBV粒子外殻を構成する膜タンパクである。Small S（SSもしくはHBs）を共通部分として，preS1（108～120aa）翻訳開始コドンから翻訳されるとlarge S（LS，389～401aa），preS2（55aa）翻訳開始コドンから翻訳されるとmiddle S（MS），SS（226aa）翻訳開始コドンから翻訳されるとSSが産生される（図2C）。これらの違いは転写の開始がどこから始まるかで決定されると考えられているが，SSをコードする転写産物が圧倒的に多い。SSにはそれ自体で粒子形成・分泌能があり，感染能をもつDane粒子に比し約1,000倍の量でSS粒子（もしくはHBs粒子）として大量に分泌される。

Dane粒子の形成にはSSに加え，LSが必要であり，コア粒子を内包する過程で粒子サイズや機能の点からも重要な役割を担っていると思われる。MSはDane粒子形成に必ずしも必要ではないと思われるが，分泌を促進する過程で機能していると考えられ，やはり，LS，MS，SSの量比はDane粒子形成を促進するか，subviral粒子としてのみ分泌されるかの点で鍵になっているものと考えられる[11,12]。

VI X遺伝子産物

HBVゲノムの塩基配列が決定されたとき，機能未知の翻訳読み取り枠（ORF：open reading frame）として同定された。約0.8kbの固有の転写産物から翻訳されると考えられる（図1B）細胞内シグナル伝達や，ウイルス宿主遺伝子の転写活性を修飾する機能が数々報告されている。こういった機能面での重要ポイントは，DHBVにはX遺伝子が存在せず，DHBV感染アヒルでは肝がんが発症しない点に着目したX遺伝子機能と肝発がんとの関連である。実際，HBV Xトランスジェニックマウスで肝発がんが促進される[13]。哺乳類ヘパドナウイルスでは感染能に影響を与えるとの報告もあるが，感染そのものに影響があるのか，転写活性化に原因があるのかなど不明な点も多い。

Xタンパクはさまざまな機能をもつが，培養細胞を用いた大量かつ一過性発現系から得られたものである[14]。Xタンパクは哺乳類細胞系でも難溶性であり，実際の生活環の中でどの程度発現し，その発現程度に見合った機能は何なのか，また，大量に発現される事態があるとすると，それは一体どういう局面なのか，実際何が起こるのか，疑問は尽きない。

VII HBVの生活環（図3）と複製（図4）

HBVの生活環はおもに，DHBVの初代培養肝細胞感染系や個体レベルでの感染系を用いた解析で一般的には次のように考えられている[5]。

まず，細胞表面の特異的感染受容体を介して付着・侵入し，感染を成立させる。HBVのゲノムは前述のごとく，⊖鎖DNAの5'に末端タンパク（pol）が共有結合しているが，これが取り除かれ，また，末端タンパク付着部位近傍の重複配列部分もトリミングされる。⊕鎖DNAのRNAプライマーも取り除かれ，完全長が複製される。最終的にはギャップはすべて埋め尽くされ結合されて，プラスミド型の環状2本鎖DNA（covalently closed circular DNA：cccDNA）となる。この過程にHBV polが必要かどうかいまだ議論のあるところである。

cccDNAはエピゲノムとなり，HBV関連mRNA（3.5kb mRNA：プレゲノムRNA〔C，pol〕，preC-プレゲノムRNA〔preC-C，pol？〕，2.4kb mRNA〔LS〕，2.2kb mRNA〔MS〕，2.1kb mRNA〔SS〕，0.8kb mRNA〔X〕）が転写される（図1B，3）。この他にも，

ORF（open reading frame）　cccDNA（covalently closed circular DNA）

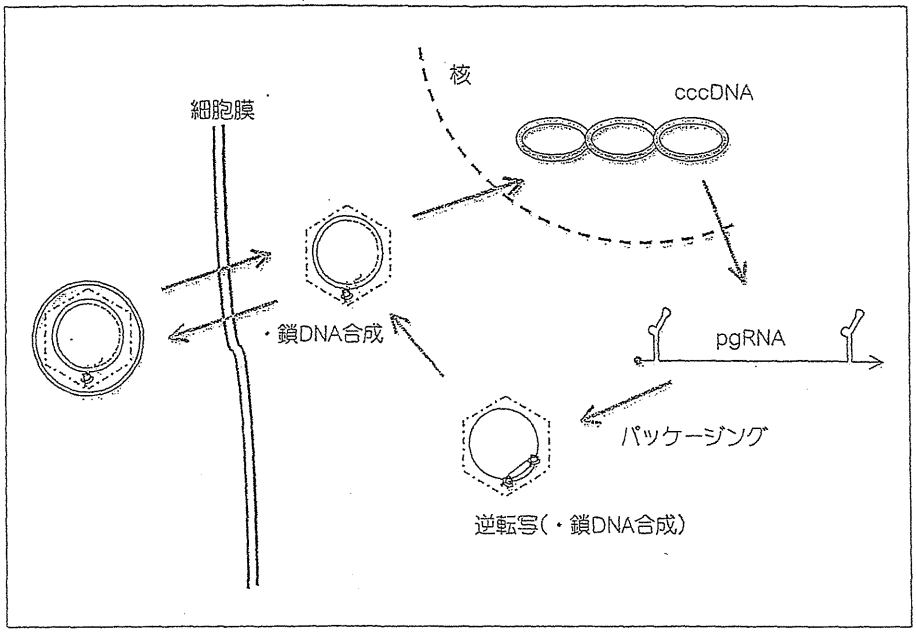

図3 HBVの生活環

HBVの生活環の概略を示した。HBV感染粒子は想定される受容体に結合後，細胞膜上もしくはエンドゾームで膜融合を起こし，コア粒子が細胞質へ取り込まれる。コア粒子は核へ移送されると仮定されているが，このプロセスでゲノムDNAのギャップは埋められ，末端タンパクは取り外されて，核内ではプラスミド型の閉環状cccDNAとなる。このエピゾームは被転写競合型であり，HBV関連転写物が発現される。ゲノム合成に供与されるRNAはpgRNAであり，コア粒子のアセンブリーと共役して，パッケージング，逆転写反応が進行する。分泌された感染性HBV粒子のゲノムは完全なHBVゲノム構造をとっており，⊕鎖DNA合成は膜粒子に取り込まれるまでに完了していると考えられている。

HBV：B型肝炎ウイルス

(文献9より改変)

C遺伝子の後半からpreSにかけてスプライスされた2.3kb前後のmRNA(機能不明)も産生される[15]。これらのmRNAはすべて⊕鎖極性をもつもので，⊖鎖極性をもつ転写産物は基本的には知られていない。プレゲノムRNAはゲノムサイズより長く，5'と3'が重複配列になっていることが特徴であり，また，逆転写複製に不可欠である。

プレゲノムRNAから翻訳されたpolはプレゲノムRNAのパッケージングシグナルεに結合し，タンパクプライミングによって⊖鎖DNAの合成を開始する(HBVの場合は63番目のチロシンからチミンが合成開始の発端になる)(図4A)。εからDR1，5'の配列はプレゲノムRNAの3'にも存在するため，ここまで合成された⊖鎖DNAはプレゲノムRNA3'と同様の相補的な配列に転座して，さらに，5'までの両端の重複した完全長の⊖鎖DNAを完成する。プレゲノムRNA自体は⊖鎖DNA合成が進むに連れてpol自身のRNaseH活性により分解されるが，5'のDR1を含む短い配列部分が最終的に⊖鎖DNAのDR2に相補的に結合することが⊕鎖DNAのプライミングになる。⊖鎖DNAの5'のDR1辺りまで合成された⊕鎖DNAは3'のDR1に転座して，さらに⊕鎖DNA合成を進める。この⊕鎖DNA合成はS遺伝子ORFとX遺伝子のORF辺りで停止している。粒子内での基質が枯渇してくることが理由のひとつかもしれない[9](図4B)。

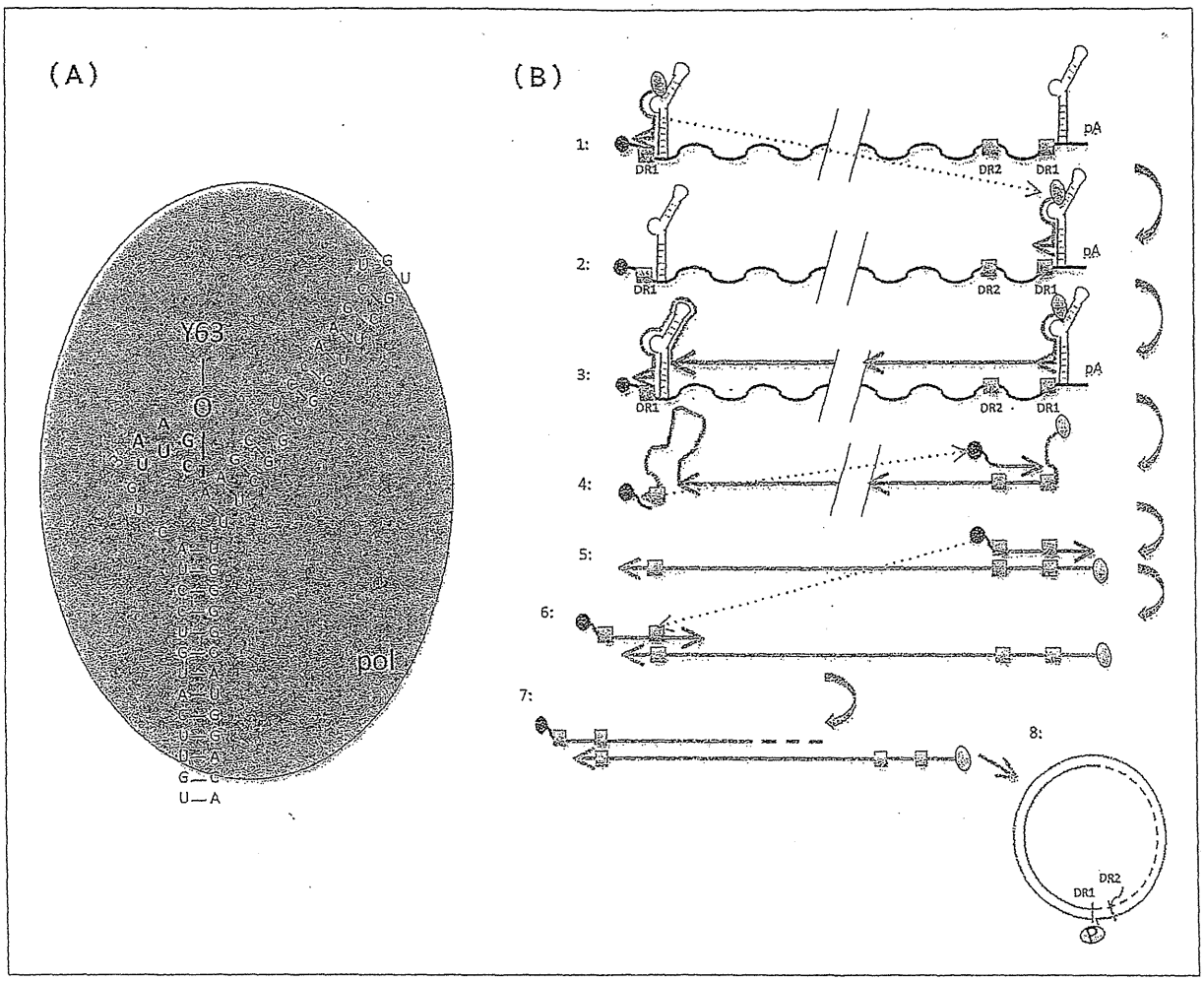

図4 HBVパッケージングシグナル（ε）と逆転写複製メカニズム

(A) HBV HBVパッケージングシグナル（ε）。RNAによくみられるpseudo-not構造をとると考えられている。HBV pol末端タンパク領域で認識されると考えられていて，部分的に1本鎖となる膨らみ (bulge) の根元のアデニンと相補的にHBV polの63番目のチロシンからプライミングされる。(B) HBV複製メカニズム。① εから開始した逆転写産物は5' DR1まで合成されると，② 3' DR1に転座することで全長⊖鎖DNA合成が可能となる。③ 5' DR 1まで⊖鎖DNA合成が進行するにともない，④ 鋳型pgRNAはHBV polのRNaseH活性により順次分解される。キャップ構造～ DR1をもつ5'の短いpgRNA部分は完全なDNA-RNAハイブリッドにならないため分解を免れ，この部分のRNAが3'に近いDR2領域に相補的に転座することで⊕鎖DNA合成のプライマーとして機能する（レトロウイルスの⊕鎖DNA合成のプライマーとなるpolypurine tract〔ppt〕に相当する）。⑤ ⊕鎖DNA合成は⊖鎖DNA5'まで進み，⑥ その後，3' DR1に転座することで，⑦ さらに⊕鎖DNA合成が進む。この反応過程で，⑧ DR領域の相補的な配列をもとに環状化構造をとる。⊕鎖DNA合成は確固とした理由は定かではないが，50～80%くらいのところで合成が停止している。本図では末端タンパクを独立した因子として描いているが，HBV polは機能構造に分解されないため，末端タンパク～合成酵素活性はひとつのタンパクとして機能している。

HBV：B型肝炎ウイルス

（文献9より）

HBVの逆転写（⊖鎖DNA合成）から⊕鎖DNA合成といった一連のHBVゲノム複製がコア粒子内で行われると考えられており，ゲノムを含むコア粒子はERに分子集合している膜タンパクに内包されてERを経由して細胞外に分泌されると考えられる。コア粒子の

ERでの内包から分泌の過程にどのような宿主因子がかかわり，ウイルス因子がどのように機能しているのか，受動的なものなのか，能動的な移送・分子集合なのか，いまだ謎だらけである。

Ⅷ HBV研究の将来

HBV感染者は世界に2～3億人，わが国でもいまだ150万人存在すると考えられている。このような巨大感染症はワクチン回避変異体を生み，慢性化率の高いゲノタイプが蔓延しつつある。HBV慢性肝炎の治療はインターフェロンによるseroconversionを目指した治療が最良であるが，効果は限定的である。したがって，HBV増殖を抑えることに主眼がおかれるが，抗HBV剤は抗HIV剤のたらい回し的利用で変異株とのいたちごっこである。

HBVの研究は分子生物学的手法を主体になされ，各遺伝子の機能が明らかにされて来た。しかし，これまでの報告がHBVの本質的なウイルス学をどこまで正確に記述しているのかを検討する必要がある。すなわち，少なくとも培養細胞を利用した*in vitro*感染系で，可能であればマウス等を利用した個体感染モデルでの実証が必要である。これらの感染系の樹立はHBVのウイルス学を発展させるに留まらず，病態発症機構の解明や，その事実に則した治療法の開発へと発展することを約束する。

そこで，今後のHBV研究を進める上でもっとも大きな問題点を2つあげておきたい。①HBV感染受容体を分離・同定し，その性質を明らかにしつつ，有用かつ簡便な*in vitro*，*in vivo*感染系を樹立すること，②HBV polの簡便なアッセイ系によるhigh-throughput抗HBV剤スクリーニングシステムを確立することの2点である。これらの問題はウイルス学に託された最難問である。

Ⅸ HBV感染受容体の分離・同定と感染系の確立

ウイルスの発見からすでに半世紀足らず，HBVの感染受容体はそのかけらもわかっていないに等しい。本来の感染宿主実質肝細胞に感染増殖するが，肝実質細胞由来の培養肝がん細胞にはほとんど感染しない。もちろん，培養肝がん細胞が元々の正常の肝実質細胞の性質を維持していないことは想定される。初代培養ヒト肝実質細胞の系では確かにHBV感染が確認されるが，倫理的な側面，調達の煩雑さを考えると，研究室での使用にはまったく耐えない。

C型肝炎ウイルスに起因すると思われる肝がんから樹立されたHepaRGという培養肝がん細胞がHBVにも感染感受性を示すことが示された[16]。しかし，感受性を得るために2～3週間程度の分化誘導が必要なこと，感染効率は最大で30%程度で，しかもディッシュ1枚当たり10万円以上する高価な細胞株であることなどから汎用性に耐えない（増殖維持は特許の関連で制限されている）。iPSから分化させた肝細胞の利用もひとつのアイデアではあるかもしれないが，その誘導から維持の手間を考えると，HBV研究者が低コストで簡便・自由に使用できる状況にはない。

そこでやはり，HBV感染受容体の分離・同定とその応用としての感染系の樹立が必要不可欠と考えている。とは言っても，半世紀近い研究でまったく成果が出なかった対象である。ウイルス感染機構の概念を根本から変える非常識的なアイデアが必要となると，かえってかなり厄介であるが，斬新なスクリーニング方法を考案しつつ，ウイルス感染の基本に立って地道な同定作業を続けている。

Ⅹ HBV polアッセイシステム

タンパクプライミング逆転写が，パッケージングシグナルε，コア粒子のアセンブリー

と共役して活性化するなどの特殊性や，発現タンパクの可溶化が困難であるなど，試験管内反応系を拒む問題点が立ちはだかる。High-throughput/mass screeningシステムの構築，立体構造から *in silico* 解析を行うにしても純度の高い評品が大量に必要である。いかに活性を維持した状態でこういった評品を得るか，種々のアイデアをもとに試行錯誤を続けている。

XI おわりに

抗HBVワクチンが開発されて，わが国では特段HBV感染症に対する意識が下がったのか，わが国におけるHBVの基礎研究者はほとんど影を潜めてしまった。わが国の150万人強の患者，世界に数億人の感染者をもってして，容易に減少していく感染症ではないと考える。HBVウイルス学もHBV肝炎，肝がんもよくわからないことだらけである。一見きわめて単純なウイルスのようにみえるが，逆に抗ウイルス標的材料が少なく，種々の側面からの治療薬の開発も難しい。本ウイルスの基本的な問題を解決することが，いろいろな意味で新たな展開を生むかもしれない。

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遺伝子挿入HBVを用いた感染レセプターの探索

上田啓次*

索引用語：HBVベクター，HBV psuedotype，HBVレセプター，HBV感染系

要旨：B型肝炎ウイルス(hepatitis B virus, HBV)が同定されてから，約半世紀が過ぎようとしている．日本には今なお130万人前後，世界的には3.6億人の感染患者が存在するが，本感染症の抜本的な克服手段は今もって存在しないといっても過言ではない．その根本的な理由のひとつが，本ウイルスには簡便な*in vitro*あるいは*in vivo*感染系が存在しないことによると思われる．このことは逆にHBV感染レセプターの同定を阻んでいると思われるが，世界中で展開されている多くのデータの積み重ねにも関わらず謎は深まるばかりである．ウイルス学的にもまたその感染による病態発症機構の解明，治療法やその評価系の考案には，*in vitro*あるいは*in vivo*感染系の構築が必須であると考える．しかしながら，このような経緯を克服し，HBV感染レセプターを同定，感染系を樹立するにはかなりの創意工夫が必要と思われる．本稿では，これまで進展してきたHBV *in vitro*感染系，HBV感染レセプターに関する研究を総括し，今後のHBV感染レセプター同定と感染系樹立へ向けた遺伝子挿入HBVの作製とそれを用いたレセプターの同定戦略を概説する．

1 はじめに

1964年にBlumbergがオーストラリア抗原として，世界で初めてHBVの存在を示してから[1]，約半世紀が迫ろうとしている．HBVは肝炎，肝硬変，肝癌の発症に関わる社会的にも重要視されるウイルスであり，その克服のため，さまざまな観点からウイルス学的，臨床医学的研究が展開されてきた．

HBVは，複製に逆転写過程をもち，3.2 kbの部分二重鎖DNAにコードされたたった4つの遺伝子で，ほぼ特異的に肝実質細胞において生活環を全うするウイルス学的にも特異なウイルスである[2]．分子生物学的手法の開眼と相まって，ゲノムクローニング，塩基配列解析，HBV関連遺伝子解析，組込みと病態解析などが展開され，ワクチンの開発やインターフェロン，抗HIV剤の流用による治療法の進展もあったことも事実である．こういった経緯を背景にし，基礎ウイルス学的に

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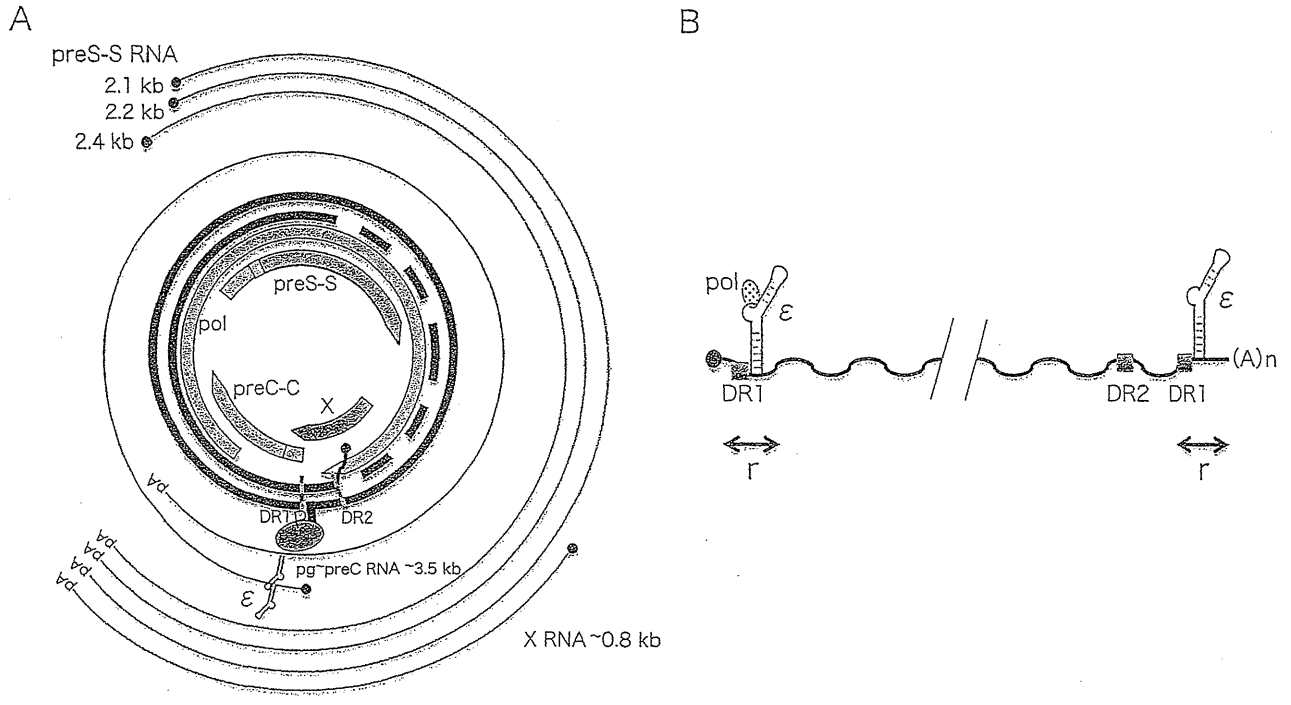

図1　HBVゲノム構造と遺伝子転写産物

A：HBVのゲノム構造を太線，太破線で示す．⊖鎖DNA末端にpolが付着する．DR1およびDR2の位置を示した．その内側にHBV関連遺伝子産物の読取り枠を示している．遺伝子は基本的にpreCore-Core (preC-C)，preS-S，pol，Xの4つである．3.5 kb mRNAがpreCのATGを含む場合，preCore-Coreとして翻訳され，分泌過程でN末の分泌シグナルおよびC末が切断されてHBe抗原(p17)となる．preCのATGを含まない場合がpregenome RNA (pgRNA)である．pgRNAは逆転写の鋳型となる重要な機能をもつと同時にcore蛋白，polの翻訳に利用される．preS-S遺伝子はmRNAの開始部位の違いにより，2.4 kb mRNAからlarge S (preS1-preS2-small S [SS]) (LS)，2.2 kb mRNAからmiddle S (preS2-SS) (MS)，2.1 kb mRNAからSSが翻訳される．この内最も産生量として多いのはSSである．X遺伝子は固有のmRNA (0.8 kb)から翻訳される．●はキャップ構造を示す．

B：pgRNAの構造．両端は重複した配列(r)となる．DR配列，パッケージングシグナル(ε)，polのプライミングする位置を示す．

はもはややり尽された感があったのか，ここ10年間，日本のHBVの基礎研究は暗黒の時代であった．しかし，現在なお日本国内に約130万人，世界的には3.6億人の巨大感染症を形成する人類史上最も危惧すべき感染症のひとつであり，その克服にはHBVの本質を詳細に解析できる*in vitro*あるいは*in vivo*感染系が何としても必要であり，感染系を土台とした病態発症機構の解明とそれらに基づくウイルス排除へ向けた抜本的な治療法の開発が重要であると考えている．

HBVは本来の宿主であるヒト若しくはチンパンジーなどのヒト近縁類人猿にしか感染しない．これらの初代培養肝細胞はHBV感染系としての効率は高いが，その調達は倫理的にも不可能に近く，できたとしてもその手間を考えると，とても日々の実験に容易に用いられる感染系とはいえない．

類人猿に感染するHBVを除いたanimal hepadnaviruses（アヒルB型肝炎ウイルス[duck hepatitis B virus = DHBV][3]，ウッドチャック肝炎ウイルス[woodchuck hepatitis virus = WHV][4]，地リス肝炎ウイルス[ground squirrel hepatitis virus = GSHV]）を用いた分子ウイルス学的アプローチは，確かに多くの事実を明らかにしてきたと思うが，それに