the risk estimate was also adjusted for HCV infection status, although anti-HCV Ab prevalence is not significantly associated with radiation dose.[20] We therefore examined HBV and HCV infection status and concomitant radiation effects separately, excluding persons with one or the other viral infection.

RRs of HCC for radiation after excluding persons infected with HBV or HCV were generally higher than with the full data, but differed little depending on which virus was used for exclusion (Table 3). As with the full data, adjustment for HBV or HCV infection status reduced the statistical significance of the radiation effect but had little impact on the RR estimates themselves. The RR of HCC for radiation after excluding persons infected with HBV and HCV (i.e., the RR of non-B, non-C HCC for radiation) was significant with or without adjustment for alcohol consumption, BMI, and smoking habit. As there can be no viral mediation of the radiation risk in noninfected individuals, lower radiation risks estimated in infected individuals might be considered evidence of mediation, but mediation would imply that risk decreases with adjustment for viral infection status, which did not occur. The reduction in statistical significance with adjustment for HBV and HCV infection status might be due to loss of power when further parameters for the risks of HCC for hepatitis virus infection are estimated or the number of subjects is reduced by exclusion.

As with the results reported previously,[1] there is evidence that alcohol consumption of $\geq$40 g/day ethanol and BMI >25.0 kg/m^2 10 years before diagnosis are associated with non-B, non-C HCC risk (Table 4). However, the evidence is not as strong given the small amount of data after excluding persons infected with HBV and HCV. The current study demonstrates that smoking is significantly associated with non-B, non-C HCC risk, although lack of continuous data precluded estimation of the relationship to amount smoked. This finding is consistent with recent assessments by the International Agency for Research on Cancer (IARC) where HCC has been positioned as a smoking-related malignant disease.[39] Some studies have shown effects of smoking on risk of HCC, but few studies have incorporated, in a strict and in-depth manner, HBV and HCV infections.[11,40]

Cohort studies of atomic bomb survivors[13-16] and Mayak nuclear facility workers[22-24] have indicated beyond a doubt that radiation increases liver cancer risk, even though hepatitis virus infection was not taken into account. It is also well known that persistent long-term internal exposure to α particles from Thorotrast, a radioactive contrast agent, can induce hemangiosarcoma, cholangiocarcinoma, and HCC in humans.[41-43] Because a significant radiation effect is observed in a high proportion of HCC cases having a p53 mutation, it has been suggested that p53 is one of the intracellular targets of atomic bomb radiation and thus a cause of the increased HCC incidence among atomic bomb survivors.[44] A lifespan study in mice exposed to continuous low-dose-rate γ rays demonstrated that the incidence of HCC was significantly increased, especially in male mice.[25] Liver weights of irradiated mice were significantly greater than those of nonirradiated controls, and the lipid content was significantly increased in irradiated mouse livers.[45] It is considered that hepatic steatosis itself is a state conferring risk for high carcinogenicity, and that in steatohepatitis, oxidative stress due to fatty acid oxidation in hepatocytes may cause DNA injury and eventually lead to carcinogenesis.[46] There is a significant association of radiation dose with prevalence of fatty liver among Nagasaki AHS participants, although a significant association has not been found between obesity (BMI $\geq$26.0 kg/m^2) and radiation dose.[47] These findings may explain part of the mechanism of increased risks of HCC with radiation exposure.

The main strengths of our study include its prospective cohort-based, nested case-control design, which minimizes selection bias, the use of stored sera, and a wealth of epidemiological information obtained prior to HCC diagnosis. It is difficult and expensive to perform full cohort serum analyses, whereas the nested case-control design utilized here can provide substantial reductions in cost and effort with little loss of statistical efficiency.[36] Another major strength of our study is that it incorporated, in a strict and in-depth manner, hepatitis virus infection status and HCC cases were identified through the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registry, supplemented by additional cases detected by way of pathological review of related diseases.[26]

A limitation of our study is that the joint effects of radiation and hepatitis virus infection could not be estimated from the standpoint of causality. As discussed previously, HBV and possibly HCV infection may act as intermediate risk factors in radiation-associated HCC. Previous studies have consistently demonstrated that prevalence of HBsAg increases with radiation dose within the AHS,[17-19] although no dose response for anti-HCV Ab has been detected.[20] Therefore, when the risk of HCC for radiation is estimated while controlling for HBV infection, some of the radiation risk may be absorbed in the coefficient for HBV infection. In other words, the radiation risk coefficient

does not represent the radiation effect independent of mediation by HBV infection and the HCC risk for HBV infection itself is not correctly estimated, because the actual causal pathway is not explicitly modeled. In addition, we cannot easily disentangle the joint effects of radiation and HBV infection using standard regression models, because HBV infection is not a true confounding risk factor but an intermediate risk factor. Nevertheless, that the radiation risk did not decrease with concomitant adjustment for viral infection suggests that the practical extent of mediation may be small. We are currently developing methods of statistical analysis that jointly consider the dose response for the intermediate viral factor as well as the joint risk of HCC for both hepatitis virus infection and radiation in the countermatched, nested case-control design.

In conclusion, radiation exposure was associated with increased risk of HCC, even after adjusting for HBV or HCV infection, alcohol consumption, BMI, and smoking habit. Moreover, radiation exposure was an independent risk factor for non-B, non-C HCC with no apparent confounding by alcohol consumption, BMI, or smoking habit. The mechanistic form of joint effects of radiation and HBV or HCV infection on HCC risk could not be estimated, but the development of new statistical methods that jointly consider the dose response for the intermediate viral factor will make such an analysis possible in the future. In particular, in-depth understanding of the mechanisms by which radiation exposure as well as obesity, alcohol drinking, and smoking contribute to development of non-B, non-C HCC may lead to prevention, early detection, and better therapeutic strategies.

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Animal model for study of human hepatitis viruses

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Abstract

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) infect only chimpanzees and humans. Analysis of both viruses has long been hampered by the absence of a small animal model. The recent development of human hepatocyte chimeric mice has enabled us to carry out studies on viral replication and cellular changes induced by replication of human hepatitis viruses. Various therapeutic agents have also been tested using this model. In the present review, we summarize published studies using chimeric mice and discuss the merits and shortcomings of this model.

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are pathogens that cause chronic infection in humans. There are 360 million and 170 million people infected worldwide with HBV or HCV, respectively.[1,2] Infected individuals develop acute hepatitis, chronic hepatitis and liver cirrhosis. The viruses are also important causative agents of hepatocellular carcinoma, especially in the Asia–Pacific region.[3] Study of the biology and development of therapies for each virus has long been hampered by the lack of a small animal model that supports hepatitis virus infection. This is probably as a result of the lack of receptor molecules necessary for viral infection in animal liver cells.

Transgenic mice that express over-length HBV-DNA export viral particles into the serum,[4] and such animals can be used to evaluate antiviral agents,[5–7] as well as HBV-targeted siRNA[8]. However, the virus life cycle is not established in this model, and it is inappropriate for studying drug-resistant HBV strains. Accordingly, researchers attempted to transplant human hepatocytes into mice. The development of the trimera mouse was one such attempt, in which human hepatocytes were transplanted under the kidney capsule of immune-deficient mice after lethal irradiation.[9,10] However, the number of hepatocytes that could survive on the kidney capsule was small, and normal liver architecture was not present. Although 85% of HBV-inoculated animals developed HBV viremia, the titer was less than 10^5 virus particles or IU/mL.[9] Similarly, 85% of HCV-inoculated animals also developed viremia,[10] but the level of the viremia only reached 10^5/mL. Thus, the advent of human hepatocyte transplanted uPA/scid mice has provided the first really useful model for acute and chronic infections of human hepatitis virus.

Human liver cell transplanted uPA/scid mice

Transgenic mice in which the urokinase gene is driven by the human albumin promoter/enhancer were developed and shown to have accelerated hepatocyte death and consequent chronic stimulation of hepatocyte growth.[11] Transplanted rat hepatocytes proliferated and repopulated injured livers in immunodeficient uPA mice, which were produced by mating uPA transgenic mice with scid mice.[12] Human hepatocytes were then transplanted into uPA/scid mice; these cells proliferated and replaced the apoptotic mice liver cells (Fig. 1).

Such human hepatocyte chimeric mice have been shown to be susceptible to both HBV[16] and HCV[17] infections. Repopulation levels by human hepatocytes have been estimated by measuring human albumin levels in mouse serum. Replication levels of both HBV[13] and HCV[17] were higher in mice in which the repopulation index was higher. A unique attempt to remove mouse residual liver cells with the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) system failed to result in a higher repopulation rate as a result of damage to the transplanted human hepatocyte caused by bystander effects.[18] Despite this, mice with livers that have been highly repopulated with human hepatocytes

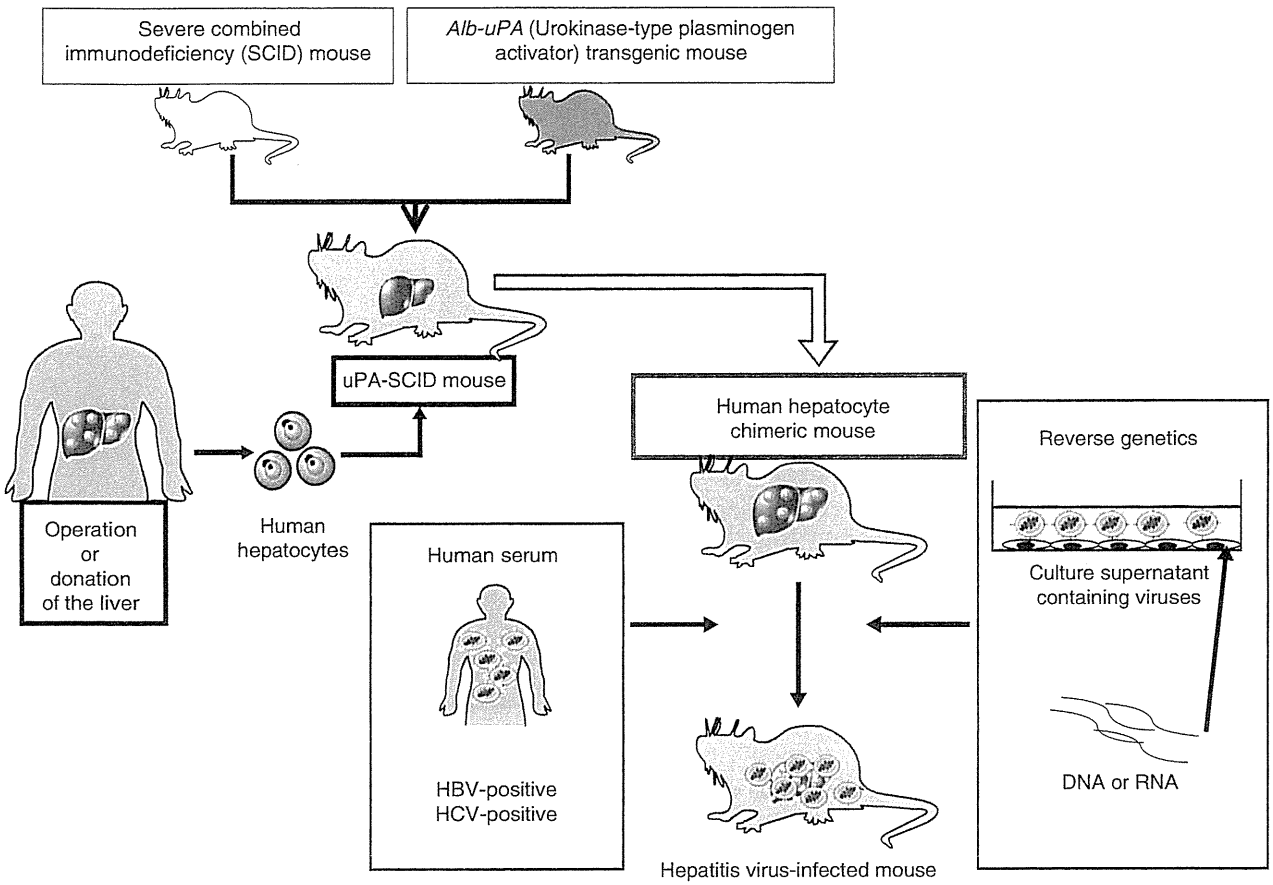

Figure 1 Generation of human hepatocyte chimeric mice and hepatitis virus infection model. A uPA/scid mouse was created by mating uPA transgenic mouse and scid mouse. Human hepatocytes obtained by surgical resection or donation were transplanted to newborn mice. The chimeric mice can be infected with hepatitis B virus (HBV) or hepatitis C (HBC) virus by injecting human serum containing these viruses. Alternatively, the mice can be infected by HBV[13] or HCV[14] created in cell culture or by injecting HCV RNA into the mouse liver.[15]

are susceptible to infection with both HBV and HCV, and as such comprised the most effective small animal model for chronic hepatitis so far developed.[19,20] An example of a highly repopulated mouse liver that we are using in experiments is shown in Figure 2.

Highly repopulated mice have been shown to be a valuable model for the study of drug metabolism.[21–29] Advances in technology for human hepatocyte transplantation have enabled serial passage of human hepatocytes in uPA/scid mice and have been shown to retain infectivity for HBV.[30]

This mouse model and other animal models for the study of hepatitis viruses have been summarized in reviews by Meuleman and Leroux-Roels,[31] Dandri *et al.*,[32,33] Barth *et al.*,[34] and Kneteman and Toso.[35] The present review will focus on key issues and updated information.

Study of hepatitis B virus infection using human hepatocyte chimeric mice

Since the initial reports of successful transmission of HBV to human hepatocyte chimeric mice in 2001 and 2004,[16,27] several researchers have reported transmission of HBV into similar mice.[13,36,37] In these studies, passage experiments studies show that HBV replicating in mice retain infectivity.[13,36] Further, the presence of viral proteins has been shown immunohistochemically in human hepatocytes transplanted into mouse livers, but these are not present in mouse hepatocytes.[13,36,37] Formation of viral particles in infected mouse livers can be shown by electron microscopy.[36,37] Genetically engineered viruses lacking HBe-antigen have also been shown to infect chimeric mice, proving that e antigen is dispensable for viral infection and replication.[13] In contrast, HBx protein has been shown to be indispensable for viral replication.[38] Transcomplementation of HBx protein with hydrodynamic injection restored HBV infectivity in mice. Interestingly, all revertant viruses show a restored ability to express HBx.[38]

By infecting chimeric mice with genotype A, B and C, differing proliferative capacity has been shown between HBV genotypes.[37] In mice infected for a relatively short time, there are no morphological changes in HBV infected mice livers in studies.[13,36] In contrast, the occurrence of liver cell damage has been reported after long-term infection of chimeric mice with HBV[39] or with specific strains of HBV;[40] these findings are consistent with direct cytopathic effects of HBV under certain conditions.

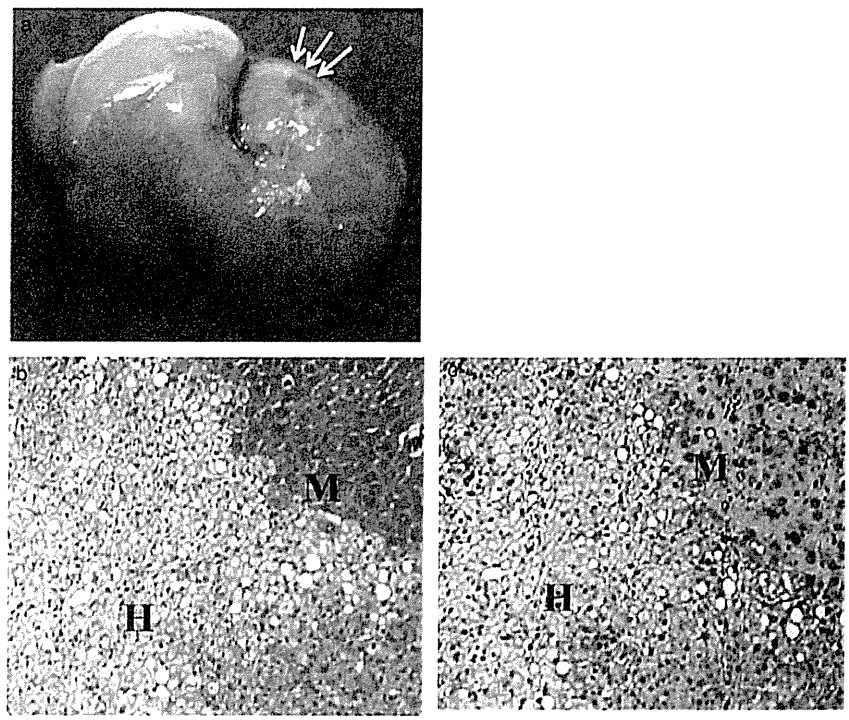

Figure 2 Representative uPA/scid mouse livers repopulated by human hepatocytes. (a) Mouse liver almost completely repopulated by human hepatocytes. Only a small portion of mouse hepatocytes are shown by arrows. (b) Microscopic figure of the mouse liver. M and H indicate regions consisting of mouse and human hepatocytes, respectively (Hematoxylin–eosin staining, magnification: ×100). (c) Microscopic figure of the mouse liver stained with antibody directed against human serum albumin.

The biological properties of a newly identified unique strain of HBV, genotype G, which replicates only in the presence of another genotype, were confirmed using the chimeric mouse.[41] Infectivity of another novel HBV strain, identified from a Japanese patient, that is divergent from known human and ape HBV has also been confirmed.[42] Titration of HBV infectivity, which previously could only be carried out using chimpanzees, can be carried out effectively using chimeric mice.[43]

Taking advantage of the absence of human immune cells in the chimeric mice, Noguchi *et al.*[44] showed that hypermutation of HBV increases in human hepatocytes under interferon treatment. Dandri *et al.* measured viral half-life in human and chimeric mice repopulated with wooly monkey hepatocytes.[45] The results clearly showed that viral half-life is shortened by immunological mechanisms in humans with low viral levels, but not in chimeric mice where functional immunity is absent. Hiraga *et al.*[46] showed an absence of interference between HBV and HCV.

Evaluation of therapeutic agents is the most important role for this mouse model. Tsuge *et al.*[13] assessed the effect of interferon and lamivudine using chimeric mice. Similarly, Dandri *et al.*[47] showed the effects of adefovir using uPA/scid mice repopulated with tupaia hepatocytes, which also support replication of human HBV. Oga *et al.*[48] identified a novel lamivudine-resistant variant that has an amino acid substitution outside of the YMDD motif. They showed that lamivudine was ineffective against the novel mutant strain. It is thus apparent that this mouse/human liver chimeric model is ideal to study the susceptibility of mutant strains to various drugs, because mutant viruses can easily be made and infected into chimeric mice.[13] The model has also been utilized to evaluate viral entry inhibitors derived from the large envelope protein.[49]

Study of hepatitis C virus using human hepatocyte chimeric mice

As observed in studies on HBV, HCV infection efficiency was poor and levels of viremia were low in mice where the repopulation rate of the mouse liver with human hepatocyte was low.[17,50] As shown in Figure 3, human albumin levels in mouse serum were significantly higher in mice in which measurable viremia developed (Hiraga *et al.* unpublished data). Recent studies have therefore been carried out using highly repopulated mice. The usefulness of a newly developed HCV assay,[51] and infectivity of a newly identified intergenotypic recombinant strain,[52] have been reported using the chimeric mice.

Using the remarkable replication ability of the JFH1 genotype 2a strain,[53] infectivity of JFH1 or intergenotypic chimeric viral particles, previously shown in cell culture, has now been shown to be infectious in chimeric mice.[54–56] Infectivity of viruses that were replicated in chimeric mice in cell culture has also been shown, and virus fitness has been studied.[55,56] The role of the HCV core+1 open reading frame and core *cis*-acting RNA elements has also been examined using the chimeric virus.[57] These elegant studies have the limitation that the non-structural part of the virus is limited to that of JFH1. Hiraga *et al.*[14] have shown that infectious clones of genotype 1a and JFH1 can be infected with direct injection of *in vitro* transcribed RNA into the mouse liver.[14] Similarly, Kimura *et al.*[15] reported the establishment of infectious clones of genotype 1b and ablation of RNA polymerase by site-directed mutagenesis abolish infectivity. These infectious clones will be useful for the study of drug-resistant strains.

The model of HCV infection has also been used to show that infection of the virus can be prevented by antibodies against

Table 1 New therapeutic strategies tested by human hepatocyte chimeric mice

n	Drug or cell	Strategy	Reference
1	Interferon alpha 2b	Activation of antiviral genes	Kneteman *et al.*[65]
	BILN-2061	NS3-4A protease inhibition	
	HCV371	NS5B polymerase inhibition	
2	Modified BID	Induction of apoptosis	Hsu *et al.*[66]
3	Serine palmitoyltransferase inhibitor	Disruption of lipid raft	Umehara *et al.*[67]
4	Lymphoblastoid interferon alpha	Activation of antiviral genes	Hiraga *et al.*[14]
5	Amphipathic DNA polymers	Blocking viral entry	Matsumura *et al.*[60]
6	Sec-butyl-analogue of HCV-371	NS5B polymerase inhibition	LaPorte *et al.*[68]
7	HCV796	NS5B polymerase inhibition	Kneteman *et al.*[69]
8	Liver allograft-derived lymphocyte	Adoptive immunotherapy	Ohira *et al.*[70]
9	Telaprevir	NS3-4A protease inhibition	Kamiya *et al.*[71]

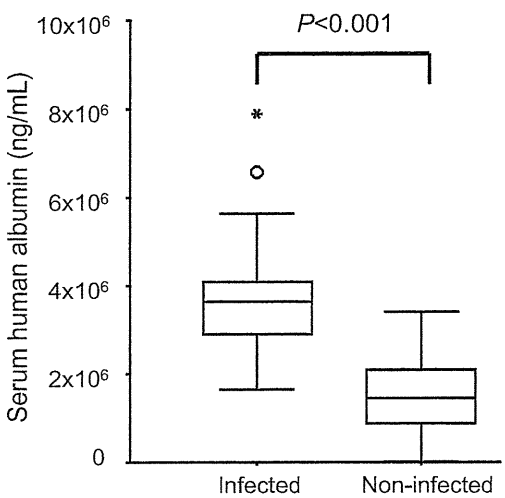

Figure 3 Human albumin levels in mice used in the hepatitis C virus (HCV) infection experiments. A total of 54 mice were injected with HCV positive serum samples containing 5×10^5 virus particles. A total of 24 mice became persistently positive for HCV-RNA, but 30 mice did not. Serum human albumin levels 2 weeks after human hepatocyte transplantation were compared between infected and non-infected mice.

CD81,[58] polyclonal human immunoglobulin directed to a similar strain,[59] and amphipathic DNA polymers.[60] Notably, the presence of broadly neutralizing antibodies to HCV that protect against heterologous viral infection has been reported, suggesting the possibility of a prophylactic vaccine against HCV.[61]

With respect to evasion of the virus against the innate immune response, altered intrahepatic expression profiles in the early phase of infection is of particular interest. The chimeric mice model is ideal for such studies; cross-hybridization of mouse and human can be avoided by careful experimental procedures.[62] Microarray analysis of livers of HCV infected and non-infected mice showed transcriptional activation of genes related to innate immune response, lipid metabolism, endoplasmic reticulum (ER) stress and apoptosis in HCV-infected mice.[63,64] The HCV infected mouse model is particularly useful for the study of newly developed HCV agents. The effect of recently developed chemicals and a unique therapy using intrahepatic lymphocytes have been shown using this model (Table 1). However, none of these therapies have yet been able to completely eradicate HCV from mice. It is noteworthy that ultra-rapid cardiotoxicity has been reported with the protease inhibitor BILN 2061 in the uPA/scid mice, but not in scid mice, implicating involvement of the uPA transgene.[72] Care should therefore be taken in interpreting the results obtained by this model.

Conclusion

Development of a small animal model using human hepatocyte chimeric mice has enabled us to study key aspects of HBV and HCV biology. The characteristic feature of the absence of human immune cells is suitable for studying viral replication and observing changes occurring in liver cells during viral infection, such as the innate immune response and cellular stress and metabolic responses. The model is also useful for studying the effect of drugs without the influence of cytokines and cytotoxic T lymphocytes. Nonetheless, the model is insufficient to study carcinogenesis of hepatitis viruses, because non-parenchymal cells in mouse liver are of mouse origin and do not support inflammation and fibrosis, which are probably closely related to carcinogenesis. The lack of human immune cells also limits the study of inflammation and immunity. Furthermore, the availability of human hepatocytes is limited. Despite these limitations, the current model shows great potential as a mouse model for the study of hepatitis viruses. Development of a small animal model with or without human immunity using stem cells or iPS cells would be an ideal model in the future.

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特集II B型肝炎に対する新治療戦略

B型慢性肝炎における核酸アナログ治療中止例の検討*

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Key Words：hepatitis B virus(HBV), nucleotide analogue, HBV replication, HBV RNA

はじめに

近年，核酸アナログ製剤の登場により，B型慢性肝疾患に対する治療は劇的に変化した．2000年以前は，インターフェロン(IFN)治療が抗ウイルス療法の主体であり，投与期間の制限や投与可能な症例が限られたことなどから，十分な治療効果が得られず，治療に難渋する症例がしばしば認められた．しかしながら，2000年11月以降，B型慢性肝疾患に対する治療薬としてラミブジン(ゼフィックス®，LMV)，アデフォビル(ヘプセラ®，ADV)，エンテカビル(バラクルード®，ETV)といった核酸アナログ製剤が認可され，良好な抗ウイルス効果と肝炎の改善が認められることから，現在のウイルス性肝疾患治療の標準化に関するガイドラインでは治療の主体となっている[1]．しかしながら，長期間の核酸アナログ製剤の使用は，薬剤耐性株の出現が懸念され，実際に核酸アナログ単剤に対する耐性株だけでなく，複数の核酸アナログ製剤を併用したことによる多剤耐性株の出現も報告されてきている[2)~8)]．核酸アナログに対する耐性株の出現率は，治療期間が長期化することにより増加することが報告されており[9]，2008年度以降に発表された「肝硬変を含めたウイルス性肝疾患治療の標準化に関するガイドライン」では，35歳未満の若年症例では，IFN単独療法やsequential therapy(ETV＋IFN連続療法)などを用いたdrug freeを目指した治療が基本となっている[1]．しかしながら，現在のところ，核酸アナログ投与中の症例に対していかに安全に核酸アナログ療法を中止し，drug freeとするかに関しては検討中である．

一方，当院では，以前より核酸アナログ療法中の患者血清中には，肝細胞内で逆転写反応が行われずに細胞外に放出されたHBV RNAを含むウイルス粒子が存在してきた[10)11)]．通常，B型肝炎ウイルス(HBV)は，肝細胞内で複製する際，pregenome RNAはコア粒子内にencapsidationされ，逆転写反応・(＋)鎖DNA合成の過程を経てHBV DNAへと変換され，envelope蛋白で被われた後に，完全なHBV粒子として細胞外へと放出される[12]．しかしながら，核酸アナログ投与下では，逆転写反応が著しく阻害されることから，pregenome RNAの状態のまま，envelope蛋白に被われ，放出される粒子が出現するものと考えられる．当院では，それらの粒子が，薬剤耐性株出現に関与することやIFN治療併用時に減少することを報告してきた[10)11)]．このHBV RNAを含むHBV粒子の出現は，細胞内におけるHBVの複製能に強く関与していることが予想され，ウイルスの複製能力が高いほど，血清HBV RNA量は

* The analysis of the chronic hepatitis B patients who discontinued the treatments with nucleotide analogues.

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表1 解析対象36症例の治療開始時の臨床背景

男女比	男：女＝23：3
HBV genotype	B：1例，C：33例，不明：2例
年齢*	41歳(22～66)
血小板数*	$17.5\times10^4/\mu l$(9.6～28.0)
ALT*	141IU/l(20～1277)
HBV DNA量*	6.8LogIU/ml(2.5～8.9)
HBs抗原量*	2,983IU/ml(66～40,967)
HBe抗原	陽性：21例，陰性：15例
HBコア関連抗原量*	6.3LogU/ml(3.4～8.8)
使用した核酸アナログ製剤	LMV：31例，LMV＋ADV：1例，ADV；1例，ETV：3例
Sequential therapyの併用	あり：28例，なし：8例
治療期間*	30週(19～304)
観察期間*	270週(73～490)
24週以内のHBV DNA再上昇	あり：22例，なし：14例
24週以内のALT再上昇	あり：15例，なし：21例

* 中央値(レンジ)

増加し，薬剤耐性変異の出現頻度も増加するものと考えられている．そのため，核酸アナログ療法中止後に生じるHBV DNAの再上昇には，HBVの複製能が強く関連していることが予想されたことから，本研究では，当院および関連施設にて経験した核酸アナログ治療中止36例を対象とした核酸アナログ療法中止後の臨床経過と治療前・治療中の血清HBV RNA量を含めた臨床背景との関連性を解析し，核酸アナログ療法の中止をより安全に行うための条件について検討した．

対　象

対象は，2010年9月までに，当院および関連施設にてB型慢性肝炎に対して核酸アナログ療法を施行され，核酸アナログ治療を中止した46例のうち，HBV RNAなどの解析が可能であった36例．表1に核酸アナログ療法開始時の臨床背景を示す．男女比は23：13，年齢の中央値は41歳．治療開始時のHBe抗原陽性例が18例存在した．また，治療開始時のHBV DNA量，HBcr抗原量はそれぞれ6.8Log IU/ml，6.3Log U/mlだった．

方　法

HBV関連マーカーの測定

各種HBV関連マーカーの測定は，各症例の保存血清を用いて行った．治療開始前のHBV DNA量は，コバスTaqMan HBV「オート」v2.0(ロシュ・ダイアグノスティクス)を用いて測定した．治療前，治療経過中のHBs抗原量は，Elecsys HBsAg II Quant(ロシュ・ダイアグノスティクス)，HBコア関連抗原に関しては，ルミパルス HBcrAg(富士レビオ)を用いて測定した．また，治療中のHBV DNA量，HBV DNA＋RNA量の測定は，既報に従って，当研究室にて行った[10)11)]．すなわち，保存血清より，SMY-TEST R & D にて核酸を抽出後，二分した．一部はそのままreal time PCRにてHBVを定量し，HBV DNA量(Log copies/ml)を測定．もう一方の核酸抽出物は，Random primer(TAKARA)，ReverTra Ace,(TOYOBO)を用いて逆転写反応を加えた後に，real time PCRにてHBVを定量することにより，HBV DNA＋RNA量(Log copies/ml)を測定した．また，HBV RNA＋DNAとHBV DNAの差をHBV RNA＋DNA/HBV DNA比として，以下の検討を行った．

核酸アナログ治療中止後の評価基準

「HBV DNA再上昇」の判定は，核酸アナログ中止時，HBV DNA陰性(＜2.6Log copies/ml)の場合，HBV DNA4.0Log copies/ml以上となった時点を，HBV DNA陽性の場合，HBV DNAが1.0Log copies/ml以上上昇した時点を，HBV DNA再上昇と判定した．一方，「ALT再上昇」は，核酸アナログ中止時，ALT正常(≦35IU/ml)の場合，ALT＞50IU/lとなった時点を，核酸アナログ中止

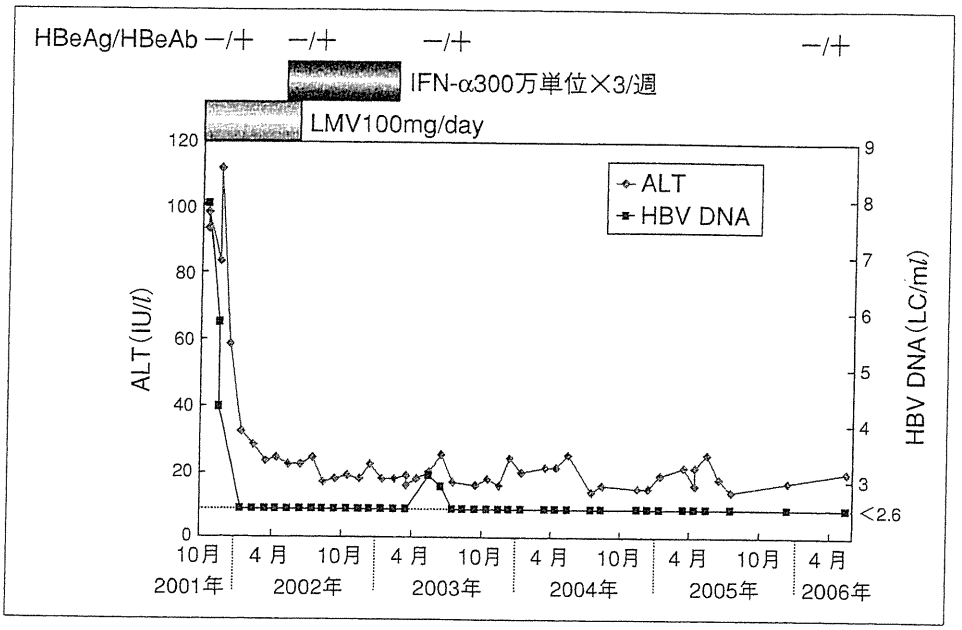

図1　Sequential therapyにて核酸アナログ療法を中止し，中止後も経過良好であった症例

47歳男性．HBV genotype C．B型慢性肝炎に対し，lamivudine100mg/dayにて治療開始．治療5か月後からIFN-α300万単位×3/週にてsequential therapyを施行．IFN開始1か月後から核酸アナログ治療を中止し，IFNも6か月間の投与で中止した．中止後，HBV DNAは一時的に3.1LC/m*l*程度までの増加は認められたものの，その後は，HBV DNA低値が持続しており，ALTも30IU/*l*以下で安定している．

時，ALT＞35IU/*l*の場合，ALT＞正常上限の2倍以上となった時点をALT再上昇と判定した．

結　果

核酸アナログ治療中止後の累積再燃率

B型慢性肝疾患に対する核酸アナログ治療は，生体内から完全にHBVを排除することはきわめて困難であり，核酸アナログ治療中止の目的の一つは，いかにdrug freeの状態で肝炎が鎮静化したまま保つことができるかにある．つまり，図1に示すように核酸アナログ中止後にHBV DNAやALTの再上昇を認めず，肝炎が鎮静化された状態が持続することが理想的な形と言える．図2は，当院における核酸アナログ中止例において中止後の肝炎再燃を検討したものであるが，核酸アナログ治療中止後のHBV DNAやALTの累積再上昇率はきわめて高く，24週後にはそれぞれ52.8%，33.3%，48週後にはそれぞれ83.3%，72.2%となっており，治療中止後7割以上の症例が再燃している結果となった．図3は，核酸アナログ療法中止後に肝炎再燃をきたした症例であるが，entecavir投与終了後より，HBV DNAの再上昇を認め，その後速やかにALTも上昇し，肝炎が再燃したことがわかる．このように，核酸アナログ療法中止後には高率に肝炎の再燃が認められることから，現在，核酸アナログ療法を安全に中止するための評価基準の確立が期待されている．

HBV DNA再上昇に関与する因子の検討

核酸アナログ療法中止後に，HBV DNAが再上昇する因子について検討するため，HBV DNAが24週以内に再上昇した22症例(DNA再上昇群)と再上昇しなかった14症例(DNA非上昇軍)に群別し，治療前の臨床背景を検討した．その結果，治療前のALTとHBV DNA量が再上昇に関与する因子として抽出されたのみで，治療期間や治療中止時のHBV関連マーカーに有意な相関は認められなかった(表2)．

一方で，HBV DNAの再上昇は，感染HBVの複製能力に強く関与していることが予想されたことから，治療前，治療開始1～6か月後，治療中止時のHBV DNAおよびHBV DNA＋RNAの変化について，2群間で検討を追加した．その結果，DNA非上昇軍では，治療開始後から中止時まで

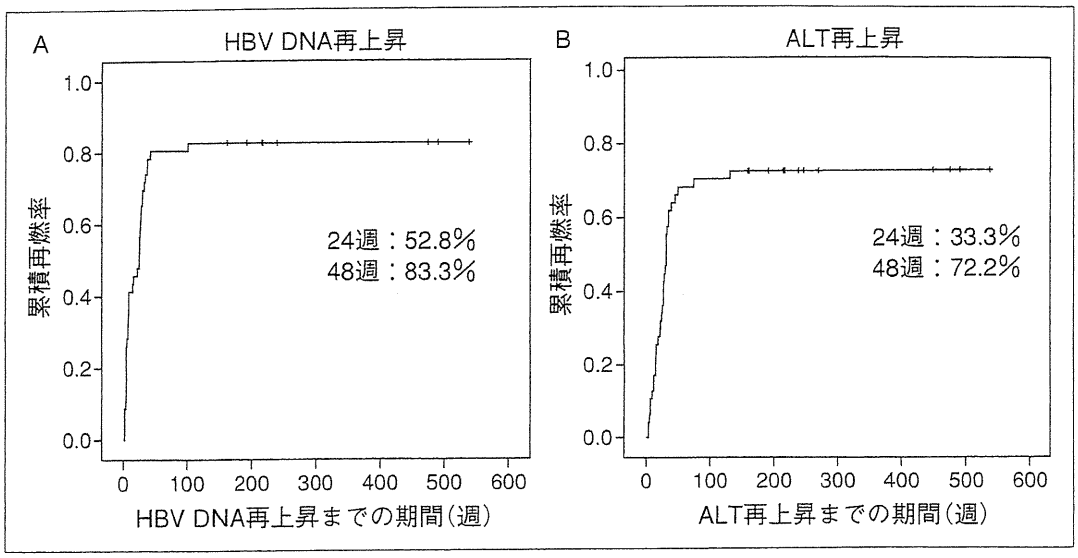

図2　核酸アナログ中止後のHBV DNA，ALTの累積再上昇率
核酸アナログ中止36症例を検討したところ，HBV DNAの累積再上昇率は，24週で52.8%，48週で83.3%だった(A)．一方，ALTの累積再上昇率は，24週で33.3%，48週で72.2%だった(B)．

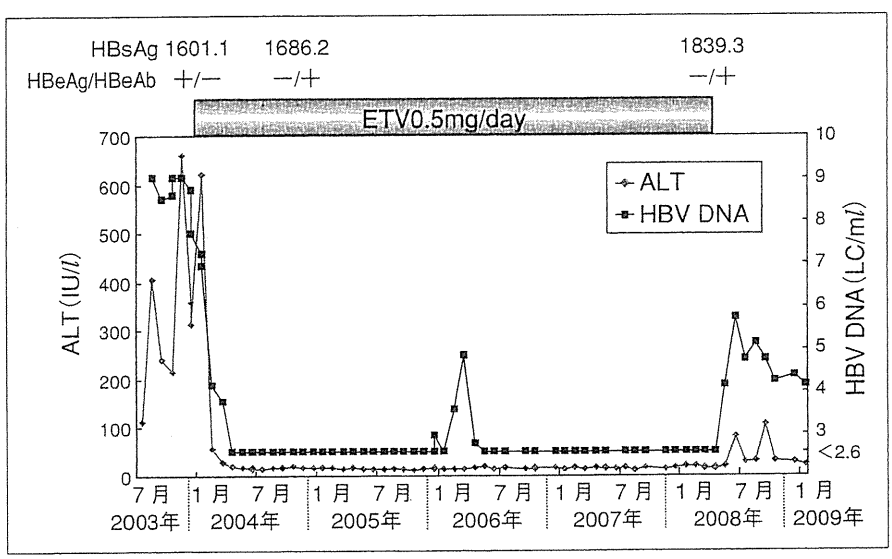

図3　核酸アナログ投与中にHBe seroconversionが得られたにもかかわらず，核酸アナログ中止後，肝炎再燃をきたした症例
29歳男性．HBV genotype C．B型慢性肝炎の急性増悪に対し，entecavir0.5mg/dayにて治療開始．治療中，HBe抗原のセロコンバージョンを認め，HBV DNAの持続陰性化，ALT正常化が得られた．約4年間のentecavir投与の後に治療を中止したところ，HBV DNAの再上昇とともに，ALTは100IU/l以上となり，肝炎の再燃を認めた．

ほとんどHBV DNA量とHBV DNA＋RNA量の値に乖離は認められなかったのに対し(図4)，DNA再上昇軍では，治療開始1か月後から，HBV DNA量とHBV DNA＋RNA量の値に約1 Log copies/ml程度の乖離を認め，治療終了時までその乖離は持続した(図5)．つまり，HBV DNA＋RNAとHBV DNAの間の乖離は，感染したHBVの複製能力を反映していると考えられ，複製能が低い状態では，HBV RNAを含むウイルス粒子の産生は減少することから両者の乖離はほとんど出現しない．一方，複製能が高い状態では，HBV DNAの再上昇をきたしやすく，両者の間に大きな乖離が出現したと考えられた．

以上の結果から，核酸アナログ療法中止後の

表2　核酸アナログ中止後24週以内のHBV再上昇に関与する因子の検討

	再上昇あり(N=22)	再上昇なし(N=14)	*P* value
性別(男：女)	13：9	10：4	0.501*
HBV genotype(B：C：ND)	0：20：2	1：13：0	1.000*
治療開始時			
年齢(歳)	40(25～59)	48(22～66)	0.104**
血小板数(×10⁴/μl)	18.1(9.6～28.0)	15.2(9.6～21.0)	0.265**
ALT(IU/l)	176(37～1277)	176(37～1277)	0.050**
HBs抗原量(IU/ml)	3,431(351～40,967)	1,524(66～10,109)	0.099**
HBe抗原(＋：－)	13：9	5：9	0.305*
HBコア関連抗原量(LogU/ml)	6.4(4.0～8.8)	6.3(3.4～7.9)	0.347**
HBV DNA(Log IU/ml)	8.2(3.7～8.9)	5.5(2.5～8.5)	0.029**
治療中止時			
HBs抗原量(IU/ml)	2,306(306～20,488)	817(5.5～10,313)	0.109**
HBコア関連抗原量(LogU/ml)	5.1(3.0～8.2)	4.7(3.1～6.6)	0.281**
HBV DNA(Log copies/ml)	3.7(1.8～9.2)	3.4(2.3～6.1)	0.761**
HBV DNA＋RNA(Log copies/ml)	4.0(2.3～8.7)	3.6(2.3～5.7)	0.012**
HBV RNA＋DNA/HBV DNA比	0.7(－1.6～2.8)	－0.2(－1.0～0.9)	0.030**
Sequential therapyの併用(＋：－)	16：6	11：3	1.000*
治療期間(weeks)	31(19～221)	30(20～304)	0.758**

* Chi-square test or Fisher's test, ** Mann-Whitney U test

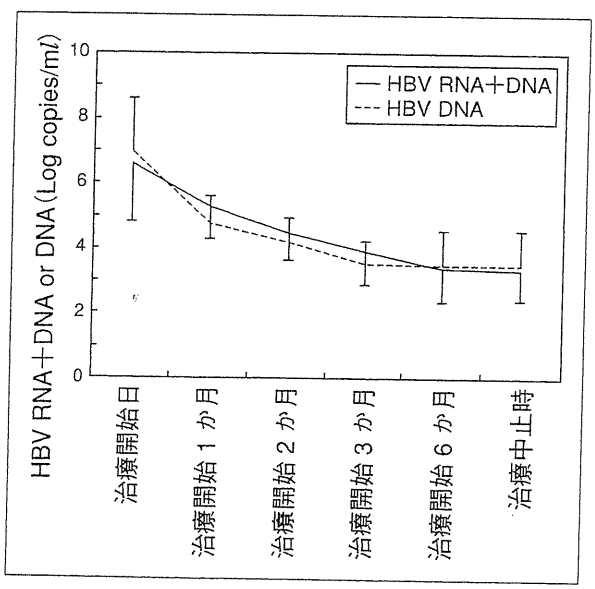

図4　HBV非上昇群におけるHBV DNAおよびDNA＋RNAの変化の相関
HBV非上昇群では，治療開始早期よりHBV DNA量とHBV DNA＋RNA量との間に乖離は認められず，治療中止時には，両者の値はほぼ程度であった．

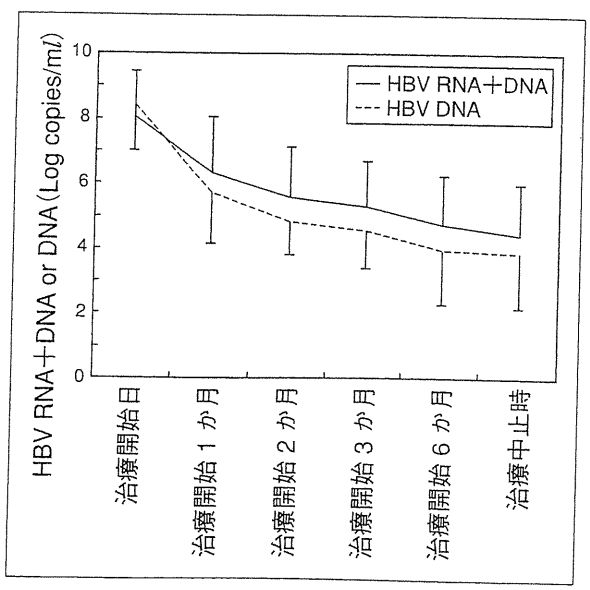

図5　HBV再上昇群におけるHBV DNAおよびHBV DNA＋RNAの変化の相関
HBV再上昇群では，治療開始早期よりHBV DNA量とHBV DNA＋RNA量との間に乖離を認め，乖離は治療中止まで持続した．

臨床経過には，治療中止時のHBV DNA＋RNA量とHBV RNA＋DNA/HBV DNA比(HBV DNA量とHBV DNA＋RNA量の乖離を反映)が強く関与していると考え，両因子を用いて，HBV DNAの累積再上昇率を検討した．その結果，中止時のHBV DNA＋RNA量が4.0Log copies/ml以下で，かつHBV RNA＋DNA/HBV DNA比が0.4以下であった群(N=16)では，その他の症例(N=20)よりもDNA累積再上昇率は有意に低く，中止後24週での再上昇率は31.2%だった(図6，*P*=0.006)．

ALT再上昇に関与する因子の検討

核酸アナログ療法中止後に，ALTが再上昇する因子について検討するため，ALTが24週以内に再上昇した15症例(ALT再上昇群)と再上昇しなかっ

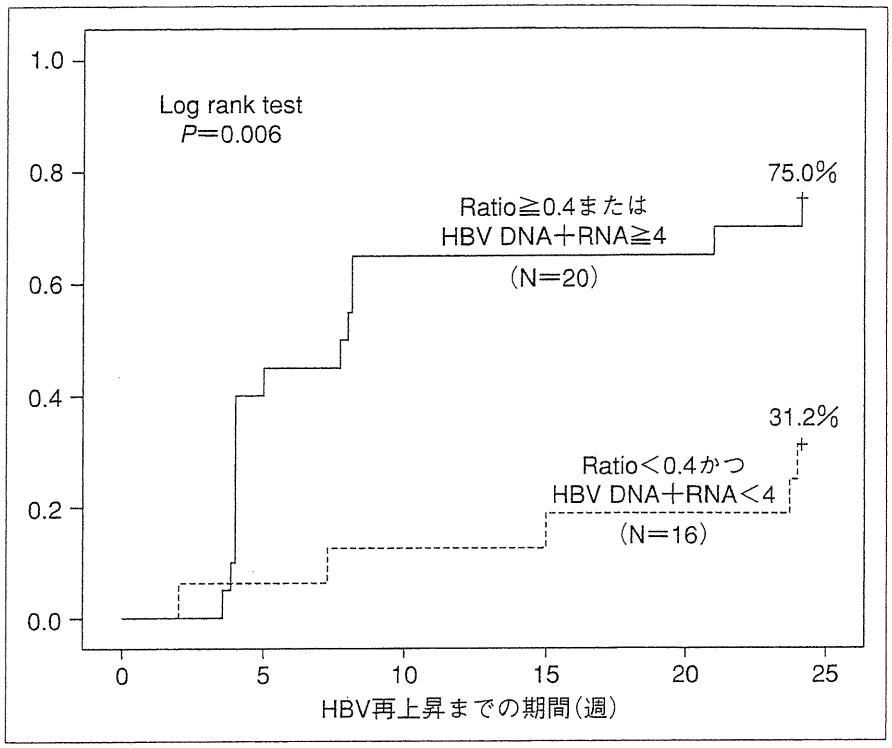

図6　核酸アナログ中止時のHBV RNA+DNA/DNA比およびHBV DNA+RNA値を用いた累積再燃率の比較

治療中止時のHBV RNA+DNA/DNA比が0.4未満かつHBV DNA+RNA値が4.0Log copies/m*l*未満の症例では，その他の症例に比べ，有意にHBV DNAの累積再上昇率は低く，24週後の累積再上昇率は有意に低かった(Log rank test)．

表3　核酸アナログ中止後24週以内のALT再上昇に関与する因子の検討

	再上昇あり(N=15)	再上昇なし(N=21)	*P* value
性別(男：女)	8：7	15：6	0.310*
HBV genotype(B：C：ND)	0：14：1	1：19：1	1.000*
年齢(歳)	40(25～59)	46(22～66)	0.446**
治療中止時			
血小板数(×10⁴/μ*l*)	15.9(8.9～28.7)	13.7(7.1～20.6)	0.298**
T. Bil(mg/d*l*)	0.8(0.3～1.3)	0.6(0.4～1.1)	0.312**
AST(IU/*l*)	24(16～42)	26(19～49)	0.559**
ALT(IU/*l*)	31(12～54)	23(11～67)	0.804**
HBe抗原(+：−)	8：7	3：18	0.020*
HBs抗原量(IU/m*l*)	3,521(481～11,607)	1,054(0.5～20,488)	0.161**
HBコア関連抗原量(LogU/m*l*)	5.4(3.6～8.2)	4.4(3.0～6.6)	0.099**
HBV DNA(Log copies/m*l*)	4.0(2.1～9.2)	3.3(1.8～6.1)	0.440**
HBV DNA+RNA(Log copies/m*l*)	4.4(3.1～8.7)	3.5(2.3～5.7)	0.017**
HBV RNA+DNA/HBV DNA比	0.8(−1.0～2.8)	−0.1(−1.6～1.4)	0.100**
Sequential therapyの併用(+：−)	11：4	16：5	1.000*
治療期間(weeks)	29(24～221)	31(19～304)	0.568**

* Chi-square test or Fisher's test, ** Mann-Whitney U test

た21症例(ALT非上昇軍)に群別し，治療前の臨床背景を検討した(表3)．その結果，DNA再上昇を検討した際に抽出された治療中止時のHBV DNA+RNA量やHBV RNA+DNA/HBV DNA比は，ALT再上昇との相関は認められなかった．その一方で，治療中止時のHBe抗原の有無とHBV DNA+RNA量への相関が認められた．そこで，治療中止時のHBe抗原の有無とHBV DNA+RNA量を

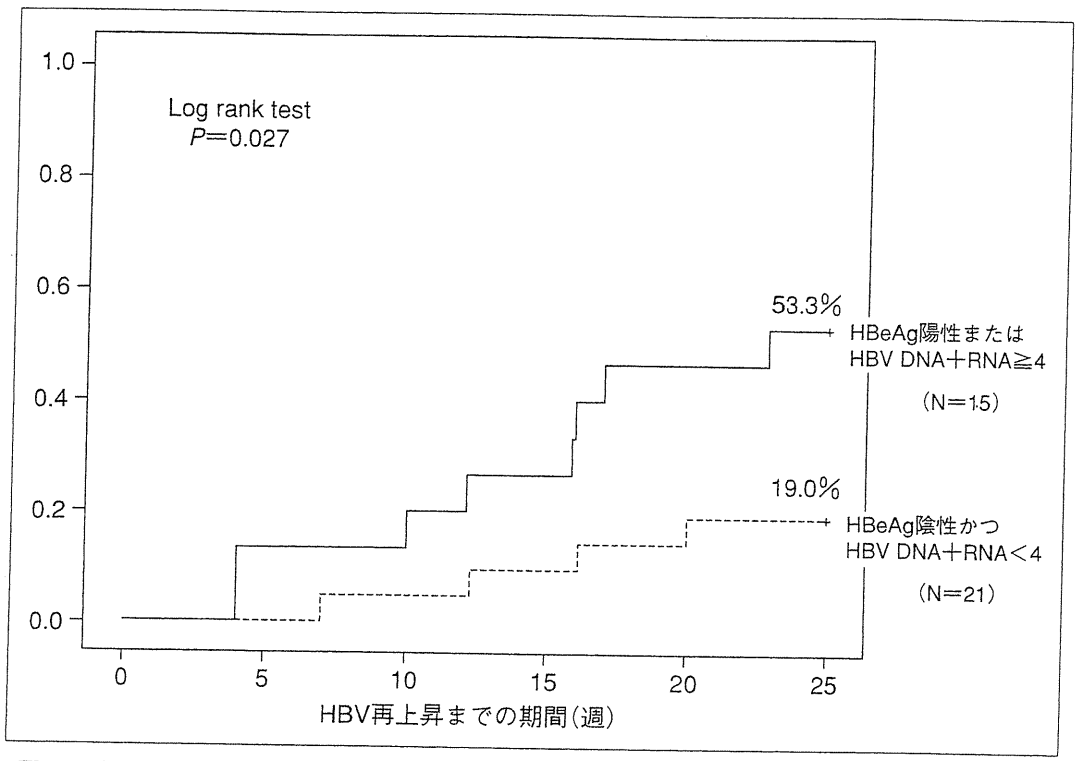

図7　核酸アナログ中止時のHBV RNA+DNA/DNA比およびHBV DNA+RNA値を用いた累積再燃率の比較

治療中止時のHBV DNA+RNA値が4.0 Log copies/m*l*未満かつHBe抗原陰性の症例では，その他の症例に比べ，有意にALTの累積再上昇率は低く，24週後の累積再上昇率は有意に低かった(Log rank test)．

用いて，HBV DNAの累積再上昇率を検討したところ，中止時にHBe抗原陰性かつHBV DNA+RNA量が4 Logcopies/m*l*未満であった群(N=21)では，その他の症例(N=15)よりもALT累積再上昇率は有意に低く，中止後24週での再上昇率は19.0%と低率であった(図7，$P=0.027$)．

考　察

核酸アナログ療法では，治療早期より血清HBV DNAは減少し，ほとんどの症例で，検出できないレベルまでウイルス量は低下する[13)~16)]．しかしながら，本治療法はウイルスの増殖を抑制し，肝炎の沈静を図ることが目的であり，ウイルスを完全に排除することはきわめて困難であり，治療を中止すると，きわめて高い確率でHBV DNAが再上昇し，肝炎を再燃する(図2)．本研究では，核酸アナログ治療の中止をより安全に行うためのマーカーを探索することを目的として検討を行った．治療中止後のHBV DNA再上昇に関与する因子としては，治療前のALT値，HBV DNA量および治療中止時のHBV RNA+DNA量，HBV RNA +DNA/DNA比が抽出され，ALT再上昇に関与する因子としては，治療前のHBeAgの有無と治療中止時のHBV RNA+DNA/DNA比が抽出された．結果的には，HBV DNA, ALTいずれの再上昇に関しても，ウイルス側の要因としてHBV RNA+DNA/DNA比が抽出されたことになる．HBV RNAは，細胞内で複製されたpregenome RNAが核酸アナログ治療により逆転写反応が行われず，そのままHBs蛋白に被われ，細胞外に放出されたものと考えられ，HBV DNA+RNA量とHBV DNA量の間に乖離が認められるのは，感染HBVの複製能力が高いことを意味しているものと考えられる．つまり，感染HBVの複製能力が高い場合，核酸アナログ療法中には，コア蛋白に被われたpregenome RNAのままの粒子が細胞内に蓄積し，一部は逆転写反応が行われないまま，細胞内に放出されるために，HBV DNA+RNA量とHBV DNA量の間に乖離が生じる．一方，感染HBVの複製能力が低い場合には，コア蛋白に

被われたpregenome RNAのままの粒子の細胞内への蓄積はわずかであり，ほとんどのウイルス粒子は逆転写反応が行われたのちに，細胞内に放出されるために，HBV DNA＋RNA量とHBV DNA量がほぼ同等となるものと推定される．そのため，感染HBVの複製能力が高い場合には，中止後早期にHBV DNAが増加し，肝炎再燃に至ったものと考えられる．しかしながら，本研究は対象症例が36例と少数であったことから，HBV RNA＋DNA/DNA比をHBV DNAやALTの再上昇に関与する独立因子として抽出するには至っておらず，症例を追加した上での検討が重要と考えられる．また，HBV DNAは，臨床の現場で測定可能であるが，HBV RNAの定量は臨床現場での測定は困難であり，また，測定方法も煩雑であるから，今後の臨床応用に向けたさらなる検討が必要と考えられる．

おわりに

現在，B型慢性肝疾患に対する治療の標準化に関するガイドラインでは，特に35歳未満の若年症例を対象として，drug freeを目指したIFN単独療法やsequential therapyが推奨されており，2011年度のガイドラインでは，sequential therapyの開始時期などについても提示されている．本研究では，安全な核酸アナログ療法の中止を目指す上で，HBV DNA＋RNA量が有用なマーカーとなりうる可能性を示したが，決して十分条件とは言えない．現在，各種HBV関連マーカーを中心とした中止基準の作成が試みられているが，安全かつ確実な核酸アナログ療法の中止を判断できるマーカーは見つかっておらず，今後の検討課題である．

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HBx protein is indispensable for development of viraemia in human hepatocyte chimeric mice

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The non-structural X protein, HBx, of hepatitis B virus (HBV) is assumed to play an important role in HBV replication. Woodchuck hepatitis virus X protein is indispensable for virus replication, but the duck hepatitis B virus X protein is not. In this study, we investigated whether the HBx protein is indispensable for HBV replication *in vivo* using human hepatocyte chimeric mice. HBx-deficient (HBx-def) HBV was generated in HepG2 cells by transfection with an overlength HBV genome. Human hepatocyte chimeric mice were infected with HBx-def HBV with or without hepatic HBx expression by hydrodynamic injection of HBx expression plasmids. Serum virus levels and HBV sequences were determined with mice sera. The generated HBx-def HBV peaked in the sucrose density gradient at points equivalent to the generated HBV wild type and the virus in a patient's serum. HBx-def HBV-injected mice developed measurable viraemia only in continuously HBx-expressed liver. HBV DNA in the mouse serum increased up to 9 $\log_{10}$ copies ml^{-1} and the viraemia persisted for more than 2 months. Strikingly, all revertant viruses had nucleotide substitutions that enabled the virus to produce the HBx protein. It was concluded that the HBx protein is indispensable for HBV replication and could be a target for antiviral therapy.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). HBV is a member of the family *Hepadnaviridae*, which consists of hepatotropic, small DNA viruses that infect their respective animal hosts (Ando *et al.*, 1999; Ganem & Schneider, 2001; Raney & McLachlan, 1991). HBV particles contain a 3.2 kb partially double-stranded circular DNA genome encoding four open reading frames (ORFs). The preS/S, pre-core/core, polymerase/reverse transcriptase and non-structural X protein (HBx) mRNAs are transcribed from each of the four ORFs (Seeger & Mason, 2000; Tang *et al.*, 2001). Although previous works have demonstrated that HBx protein is necessary for maximal HBV replication in cultured cells (Bouchard *et al.*, 2001; Keasler *et al.*, 2007; Leupin *et al.*, 2005; Tang *et al.*, 2005) and in mouse hepatocytes (Keasler *et al.*, 2007), the precise function of HBx in the virus life cycle remains poorly defined in human hepatocytes under physiological conditions because there is no natural infection–replication system available. Accordingly, all previous work has been done using hepatocarcinoma cell lines with transfection or mouse hepatocytes with hydrodynamic injection. Analysis of HBx under physiological conditions will provide more accurate information for the function of the HBx protein.

The nucleotide and amino acid sequences of the X genes are well-conserved among all mammalian hepadnaviruses. Expression of HBx protein in hepatocytes has been reported

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the HBV genome cloned into plasmid pTRE-HB-wt is AB206817.

both in humans (Su *et al.*, 1998) and in woodchucks (Dandri *et al.*, 1996; Jacob *et al.*, 1997). Previous reports have shown that the X protein of the woodchuck hepatitis virus (WHV) is important for the virus life cycle (Chen *et al.*, 1993; Sitterlin *et al.*, 2000a; Zhang *et al.*, 2001; Zoulim *et al.*, 1994). In contrast, in non-oncogenic avian hepatitis viruses, such as duck hepatitis B virus (DHBV), the X protein (DHBx) is not necessary for virus replication *in vivo* (Meier *et al.*, 2003). The HBx and WHV X proteins (WHx) localize both in the cytoplasm and in the nucleus (Dandri *et al.*, 1998; Doria *et al.*, 1995; Sitterlin *et al.*, 2000b; Wang *et al.*, 1991), and both of them have similar multi-phasic activities for transcription, DNA repair, cell growth and apoptotic cell death in tissue-culture cells (Arbuthnot *et al.*, 2000; Murakami, 2001). HBx and WHx have also been shown to stimulate virus replication in cell lines by activating viral transcription (Colgrove *et al.*, 1989; Melegari *et al.*, 2005; Zhang *et al.*, 2001) or by enhancing the reverse transcription activity of the viral polymerase (Bouchard *et al.*, 2001; Klein *et al.*, 1999). Although it has been shown that the WHx protein is indispensable for virus replication *in vivo* (Zoulim *et al.*, 1994), which of the above functions is indispensable remains unknown. As HBV infects only humans and chimpanzees, it has been difficult to perform intensive studies *in vivo*.

Recently, Mercer *et al.* (2001) reported that transplanted human hepatocytes in SCID mice homozygous for the Alb-uPA transgene resulted in replacement of the mouse liver. They also reported that the highly replaced mice are susceptible to hepatitis C virus (Mercer *et al.*, 2001). Tateno *et al.* (2004) also created human hepatocyte chimeric mice with an improved replacement rate. Using this chimeric mouse model and the cell-culture-created HBV, we showed previously that hepatitis B e antigen (HBeAg) is dispensable for virus infection and replication (Tsuge *et al.*, 2005).

In this study, we tested whether the cell-culture-generated HBx-defective (HBx-def) HBV infects and replicates in the chimeric mice. As HBx-def HBV did not develop measurable viraemia, we expressed the HBx protein in the chimeric mouse liver by hydrodynamically injecting HBx-expression plasmid. It was noted that this *trans*-complementation of HBx helped the replication of HBx-def virus in the chimeric mice, and revertant viruses showed nucleotide substitutions that reversed the introduced stop codon [CAA to TAA created by a C-to-T point mutation at nt 1395 (aa 7) in the HBx gene; Fig. 1a] and restored expression. The HBx protein is thus indispensable for infection and proliferation of HBV. The protein thus might be a target for therapy development against HBV.

RESULTS

Production of HBV particles and antigens in cell culture and effect of HBx ablation

We initially examined nucleotide sequences of the cell-line-produced HBV by direct sequencing of the PCR products using cell-culture supernatants. As expected, HBV DNA was released from HepG2 cells transfected with the HBx-def plasmid with an introduced stop codon mutation by calcium phosphate precipitation (data not shown). We then analysed hepatitis B surface antigen (HBsAg), HBeAg and HBV DNA in the supernatants 3 days after transfection. While HBV DNA titres were not significantly different between the wild-type (WT)- and HBx-def HBV-transfected cultures, the HBsAg and the HBeAg levels were significantly lower in HBx-def HBV- than in WT-transfected cultures (Fig. 1b).

To examine the particle formation in the transfection experiments, we analysed the density of generated HBV by sucrose density gradient sedimentation analysis. The density of the cell-culture-produced HBx-def HBV was compared with those of WT HBV and HBV obtained from human serum. As shown in Fig. 1(c), each of the three preparations of HBV sedimented at sucrose density 1.18 g ml^{-1}, suggesting that cell-culture-produced HBV particles were similar to those obtained from human serum.

Infectivity of HBx-def HBV particles

To analyse the infectivity of HBx-def HBV, we inoculated cell-culture-produced recombinant HBV (WT HBV or HBx-def HBV) into chimeric mice. All seven mice injected with cell-culture-generated WT HBV developed measurable viraemia 2–7 weeks after inoculation. The virus titre reached 6–10 log_{10} copies ml^{-1} and the viraemia persisted for more than 4 months (Fig. 2a). In contrast, we did not observe any measurable viraemia in HBx-def HBV-injected mice within a period of 16 weeks after inoculation (Fig. 2b). Only five of 16 HBx-def HBV-inoculated mice became occasionally positive for HBV DNA by nested PCR assay. We then examined the mouse livers 14 weeks after inoculation by immunohistochemical staining with anti-HB core (HBc) antibody. As shown in Fig. 2(c), human hepatocytes of WT-injected mice were positive for HBV core antigen (HBcAg). In contrast, the staining was negative in mouse liver injected with HBx-def HBV.

Effect of *trans*-complementation of entire and partial HBx protein on replication of HBx-def HBV

We then investigated the effect of *trans*-complementation of the HBx protein both *in vitro* and *in vivo*. Since the C-terminal two-thirds (aa 51–154) domain of HBx has been reported to contain a transactivation domain (Tang *et al.*, 2005), we constructed three plasmids (full length and residues 1–50 and 51–154), as shown in Fig. 3(a). To analyse the effect of co-transfection of these three plasmids on intracellular replication of HBV, the cells transfected using *Trans*IT-LT1 reagent were harvested 24 h after transfection and analysed by Southern blotting. As shown in Fig. 3(b), *trans*-complementation of HBx enhanced the