

特別講演 1

C型肝炎の自然免疫系遺伝子発現 プロファイルと抗ウイルス療法の治療効果

【司会】 小俣 政男

山梨県立中央病院

【演者】 朝比奈 靖浩

武蔵野赤十字病院 消化器科

特別講演 1

C型肝炎の自然免疫系遺伝子発現プロファイルと抗ウイルス療法の治療効果

朝比奈 靖浩*

はじめに

わが国において年間約3万4千人が肝臓癌で死亡しており、肝臓癌は死亡原因として重要な位置を占めている。肝細胞癌の75%はC型肝炎が原因であり、わが国における肝細胞癌の撲滅にはC型肝炎ウイルス(HCV)の駆除が極めて重要である。HCVの駆除にはインターフェロン(IFN)治療が最も基本的であるが、わが国のC型慢性肝炎の約70%を占めるHCV genotype 1bはIFNに抵抗性を示すことが知られ、特に高ウイルス量症例やIFN感受性決定領域(Interferon sensitivity determining region ; ISDR)にアミノ酸変異がない症例では、IFN単独24週の治療ではウイルス学的著効(sustained viral response ; SVR)が得られる率はこれまで10%以下であった。

近年、genotype 1b型かつ高ウイルス症例のいわゆる難治性C型慢性肝炎に対して、ペグインターフェロン(PEG-IFN)/リバビリン(RBV)併用療法が導入され、約50%の症例でSVRが得られるようになった。しかし、約15%は治療中HCV dynamicsの第2相以降のウイルス減少を認めないいわゆるnull responderで治療抵抗性である¹⁾。これらnull responderに対しては、治療抵抗性の機序を解明し、プロテアーゼ阻害薬などの新薬による治療も視野に入れた対策が必要である。しかし、これら難治例の治療抵抗性の機序は不明であり、またPEG-IFN/RBV併用

療法の治療前にnull responseを予測することは困難である。今回、筆者らはHCVの排除に重要と考えられている宿主の自然免疫機構に注目し、これらの遺伝子発現とPEG-IFN/RBV併用療法の治療効果との関連について検討したので概説する²⁾。

PEG-IFN/RBV併用療法の治療効果に関する要因

PEG-IFN/RBV併用療法は、承認以来すでに5年近くが経過し、実地臨床における治療成績の実態を多数例で解析することが可能になってきた。そこで、まず当院においてPEG-IFN α -2b/RBV併用療法を施行した561例のうちgenotype 1b型かつ高ウイルス量の473例を対象とし、難治要因に関わる因子を検討した。すでに最終治療効果を判定し得た症例について検討すると、予定投与量の80%以上を投与できた症例におけるSVR率(PP解析)は61%であったが、減量・中止例を含めたITT解析では37%となった。SVRに関する治療前因子を単変量解析で検討すると、年齢、性別のほか、過去のIFN治療効果、血小板数、ヘモグロビン値、血清クレアチニン値、血清 γ -GTP値、肝線維化が有意な因子として挙げられ、さらに体重、LDLコレステロール値、血糖値、肝脂肪化といった代謝に関連する因子に有意差があった。ウイルス学的には、ウイルス量は有意ではなかったが、ISDR変異とHCV

*武蔵野赤十字病院 消化器科(〒180-8610 東京都武蔵野市境南町1-26-1)

コア70番・91番変異が有意であった。これらについて非SVRに寄与する独立因子を多変量解析を用いて検討すると、①高齢、②女性、③架橋形成を伴う高度線維化、④ISDR野生型、⑤HCVコア変異型の5因子が難治要因として抽出された。

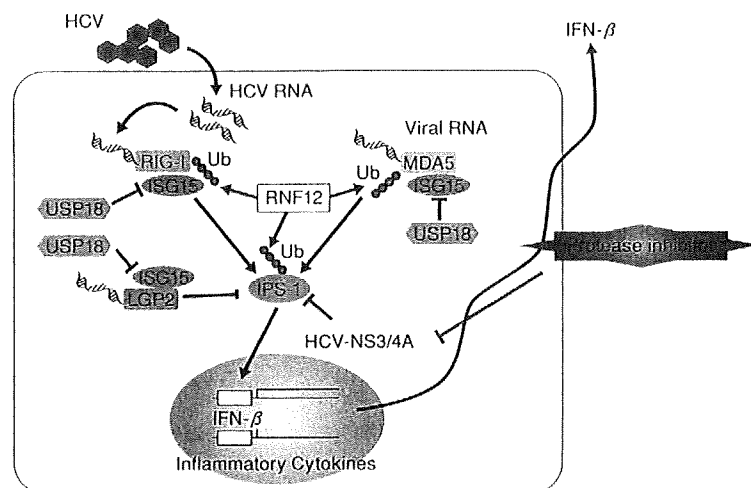
一方、予定投与量の80%以上が投与できているにも関わらず、治療中HCVが陰性化しない nonvirological response (NVR) の症例は、13%に認められた。NVRに関与する治療抵抗因子としては、①HCVコア変異、②架橋形成を伴う高度線維化、③血清γ-GTP高値、④血清クレアチニン低値が多変量解析により抽出された。したがって、これらの臨床的検討から治療抵抗性に関わる因子としては、ウイルス因子の他に何らかの宿主因子が関与している可能性が考えられた。しかし、その機序の詳細は未だ十分に明らかではなく、宿主因子とウイルス因子の相互の関わり合いについても不明である。

C型肝炎ウイルスと自然免疫

そこで、筆者らは、IFN不応性に関わる宿主側要因として宿主自然免疫系に注目した。すなわち、近年の *in vitro* の研究によるとHCVに対する生体防御には宿主自然免疫が重要とされるが³⁾、この自然免疫系にはIFN系が深く関わっていることから、IFN治療抵抗性には宿主自然免疫が何らかの影響を及ぼしていると考えた。

HCVが細胞に感染すると、まずHCV由来のRNAが細胞内のウイルスセンサーであるRIG-I (retinoic acid inducible gene I) によって探知され、そのシグナルがアダプター分子であるIPS-1 (IFN-β promoter stimulator-1 : 別名MAVS, Cardif, VISA) を介して核に伝達されIFN-βが産生される。この自然免疫の作動がHCV感染に際して生体側で起こる最初の防御機構であるとされる(図1)。

図1 HCV感染と宿主自然免疫



Asahina Y et al : *Gastroenterology* 134 : 1396-1405, 2008. 改変

RIG-IIは細胞質に存在するヘリカーゼであり、ウイルス由来の5'の三リン酸構造や3'のポリU配列を認識し、形質細胞様樹状細胞以外のほとんどの細胞においてウイルス感染センサーとして必須の役割を担っている⁴⁾。RIG-Iと構造上非常に類似したファミリー分子であるMDA5も同様の機能を有するウイルスセンサー分子であることが示されているが、ピコルナウイルスなどRIG-Iとは違ったウイルス種を認識しているとされる⁵⁾。RIG-IまたはMDA5がウイルス由来RNAを感知するとその三次構造が変化し、下流で機能するアダプター分子であるIPS-1と会合する。IPS-1はミトコンドリアにアンカリングしている蛋白であり、RIG-IやMDA5が探知したウイルス感染シグナルを核に伝えるのに必須である⁶⁾⁻⁹⁾。

これらRIG-I/IPS-1系は、いろいろな宿主分子により制御を受けている可能性が示されている。たとえば、RIG-Iと同様の分子構造をもつRIG-Iファミリー分子のLGP2はIPS-1との結合に必要なCARDドメインを欠くため、シグナルを核に伝達することができずRIG-I/IPS-1系の制御に関与すると報告されている⁵⁾。また、ユビキチン様蛋白であるISG15は、STAT1やJak1と結合しIFNシグナル伝達を修飾する一方、RIG-IやIPS-1などと結合することにより(ISGylation)その機能を修飾していると考えられている¹⁰⁾。そして、ISG15とその結合蛋白とを解離させる特異的プロテアーゼであるUSP18はISGylationに対して抑制的に働くことが報告されている¹¹⁾¹²⁾。さらに、最近このRIG-I/IPS-1系の特異的ユビキチンE3リガーゼであるRNF125が同定され、ubiquitin-proteasome pathwayによりRIG-I, MDA5およびIPS-1を分解し抑制的に調節していることが示されている¹³⁾。

一方、非常に興味深いことにHCVのNS3/4AセリンプロテアーゼはIPS-1をミトコンドリアとアン

カリングしている部分で切断することが示されており、HCVはRIG-I/IPS-1系を標的とすることで巧みに宿主の自然免疫系から逃れている可能性が示唆されている(図1)⁶⁾¹⁴⁾。したがって、これまでの*in vitro*の研究から、RIG-I/IPS-1系は宿主によるHCVの排除およびそれに対するHCVの抵抗性に重要な鍵を握っていることは間違いないと考えられる。しかし、RIG-I/IPS-1系の状態と実際の抗ウイルス療法の治療効果との関連は不明で、ヒトにおける臨床的意義はほとんど解明されていない。そこで筆者らは、臨床検体を用いてこれら自然免疫系分子の遺伝子発現プロファイルとPEG-IFN/RBV併用療法の治療効果との関連を検討した。

自然免疫系遺伝子の 肝内発現プロファイルと抗ウイルス効果

PEG-IFN/RBV併用療法を施行した1b高ウイルス量のC型慢性肝炎74例を対象として、治療直前に肝生検を施行し、総RNAを抽出後リアルタイムPCR法を用いて細胞内ウイルスセンサーであるRIG-I, MDA5や修飾・制御系分子であるLGP2, ISG15, USP18, RNF125およびアダプター分子であるIPS-1のmRNAの肝内発現を定量した(図2)²⁾。また、原発性胆汁性肝硬変などの非ウイルス性肝疾患を対照としてその肝内遺伝子発現プロファイルを比較した。

1. C型慢性肝炎と非ウイルス性肝疾患との比較 (図3)

まず、これら自然免疫系分子の肝内遺伝子発現プロファイルをC慢性肝炎患者と非ウイルス性肝疾患患者で比較した。その結果、C型慢性肝炎患者におけるRIG-I, MDA5, LGP2, ISG15, USP18, RNF125の肝内遺伝子発現は非ウイルス

性肝疾患患者に比し有意に高いことが分かり、これらの分子はHCV感染による内因性IFNにより肝内に発現誘導されていることが示唆され

た。それに対して、IPS-1は両者の中で肝内発現量に有意差はなく、比較的constitutiveに発現していると考えられた。

図2 治療プロトコール(上段)と研究方法(下段)

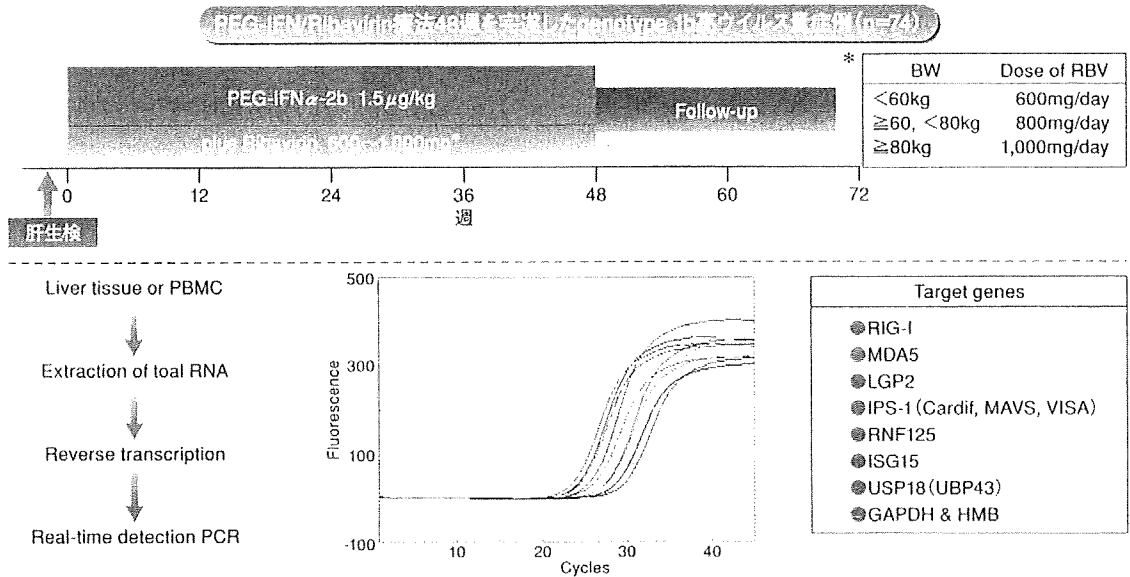
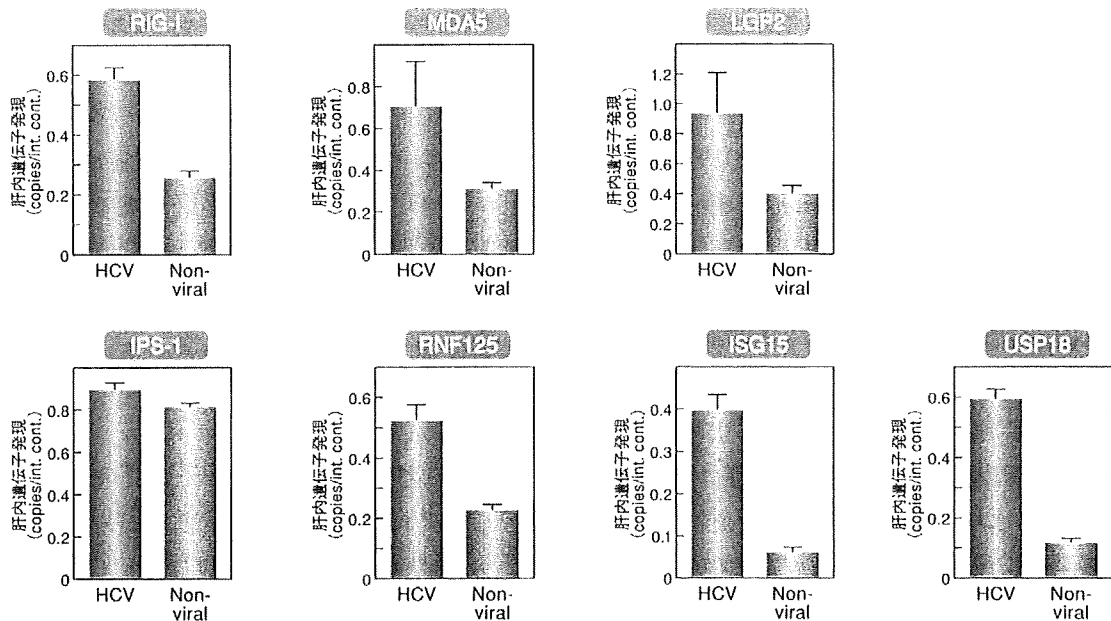


図3 C型慢性肝炎および非ウイルス性肝疾患における自然免疫系分子の肝内遺伝子発現の比較



Asahina Y et al: *Gastroenterology* 134: 1396-1405, 2008. 改変

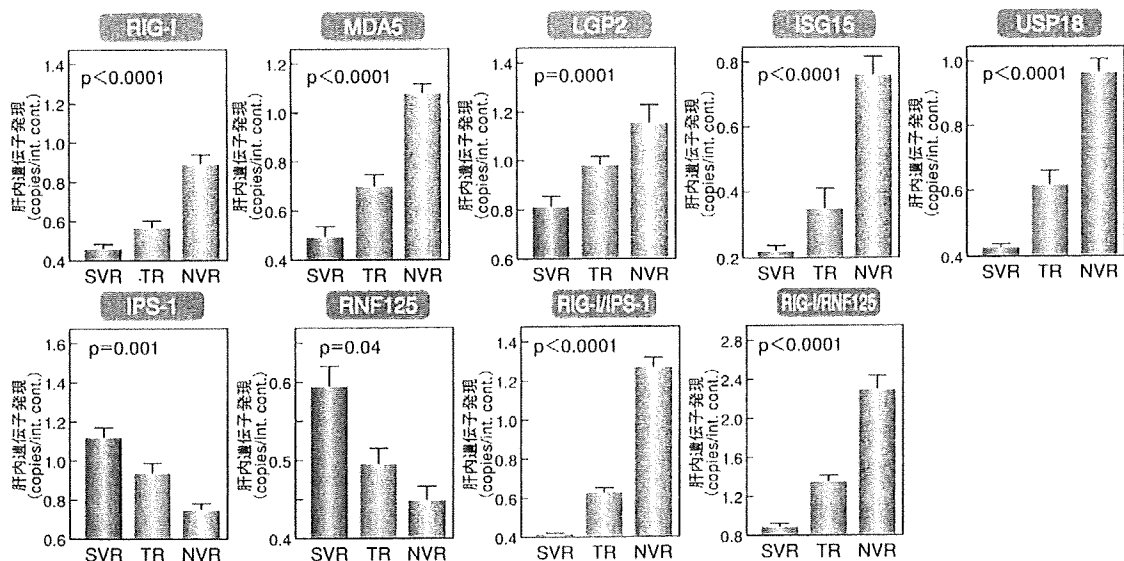
2. 治療前肝内遺伝子発現プロファイルとPEG-IFN/RBV併用療法の治療効果(図4)

次に、PEG-IFN/RBV併用療法を施行した症例を最終的なウイルス学的治療効果により、著効(SVR:n=30)、再燃(TR:n=24)および治療中HCVが全く消失しない無効(NVR:n=20)に分類し、これらの治療効果と治療前における肝内遺伝子発現との関連を解析した。その結果、RIG-I, MDA5, LGP-2, ISG15およびUSP18の肝内遺伝子発現は、治療中HCVが減衰しないNVR群でSVR群に比し有意に1.5~4倍高発現していた。それに対して、IPS-1とRNF125の治療前肝内遺伝子発現はNVR群で有意に低値で、RIG-I/IPS-1比またはRNF125比はNVRで有意に高かった(NVR:SVR=1.3:0.4, 2.3:0.8)。このNVR例におけるIPS-1の発現低下は、ウエスタン・ブロットにより蛋白レベルでも確認された(図5)。しかし、筆者らの検討では、HCVのプロテアーゼにより切断されたと考えられるIPS-1の切断体はウエスタン・ブロットでは検出されなかった。その

原因として、IPS-1の切断体は生体内での安定性が低いため、ウエスタン・ブロットのバンドとして検出されなかった可能性がある。

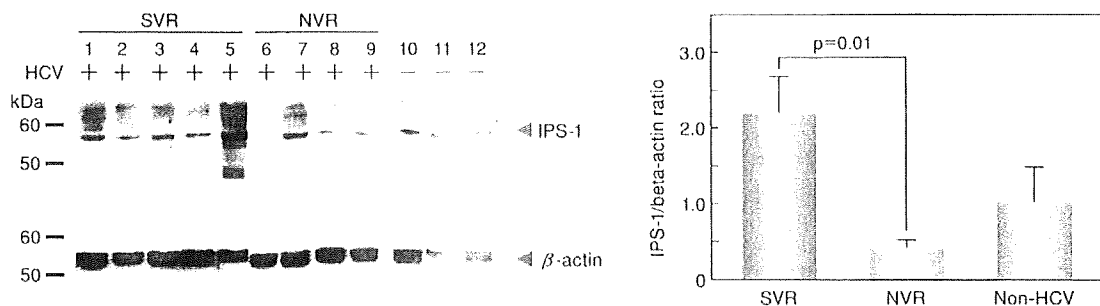
多変量解析でNVRに関与する因子を検討すると、ISG15またはUSP18発現およびRIG-I/IPS-1比と血小板数が独立因子として有意であった。ROC解析ではISG15, USP18発現およびRIG-I/IPS-1比のarea under the curveは0.9以上で、これらの遺伝子の治療前における肝内発現を定量することは、これまで困難とされてきたPEG-IFN/RBV併用療法の最終治療効果を治療前に予測するのに極めて有用と考えられた(図6)。一方、最近PEG-IFN/RBV併用療法のNVRに関与するHCV遺伝子変異としてコア70番および91番変異が報告されているが¹⁵⁾、筆者らの症例ではNVR例ではコア変異の有無に関係なくRIG-I/IPS-1比は高値であり、反対にSVR例では低値であった。多変量解析で検討すると、RIG-I/IPS-1比はこれらHCVコア遺伝子変異とは独立してNVRに関与している可能性が示唆された(表1)。

図4 PEG-IFN/RBV併用療法の最終ウイルス学的治療効果と自然免疫系分子の肝内遺伝子発現



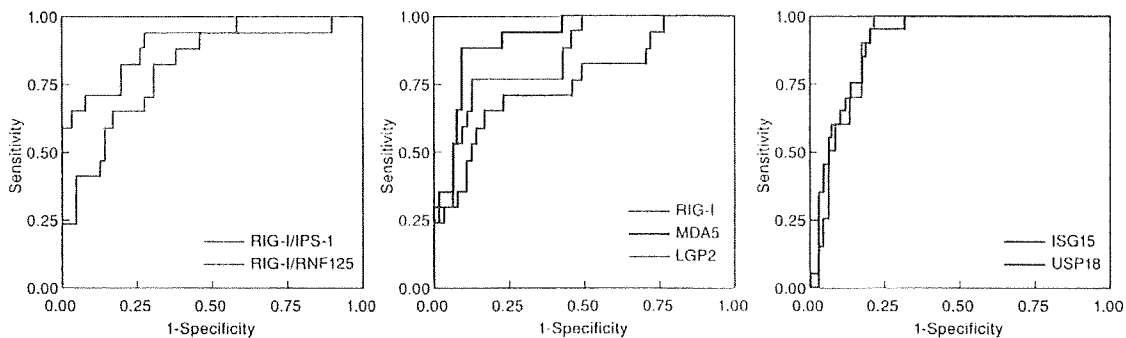
Asahina Y et al: *Gastroenterology* 134: 1396-1405, 2008. 改変

図5 ウエスタン・ブロットによる肝内IPS-1の蛋白発現と最終治療効果



Asahina Y et al : *Gastroenterology* 134 : 1396-1405, 2008. 改変

図6 NVR診断に関わるROC解析



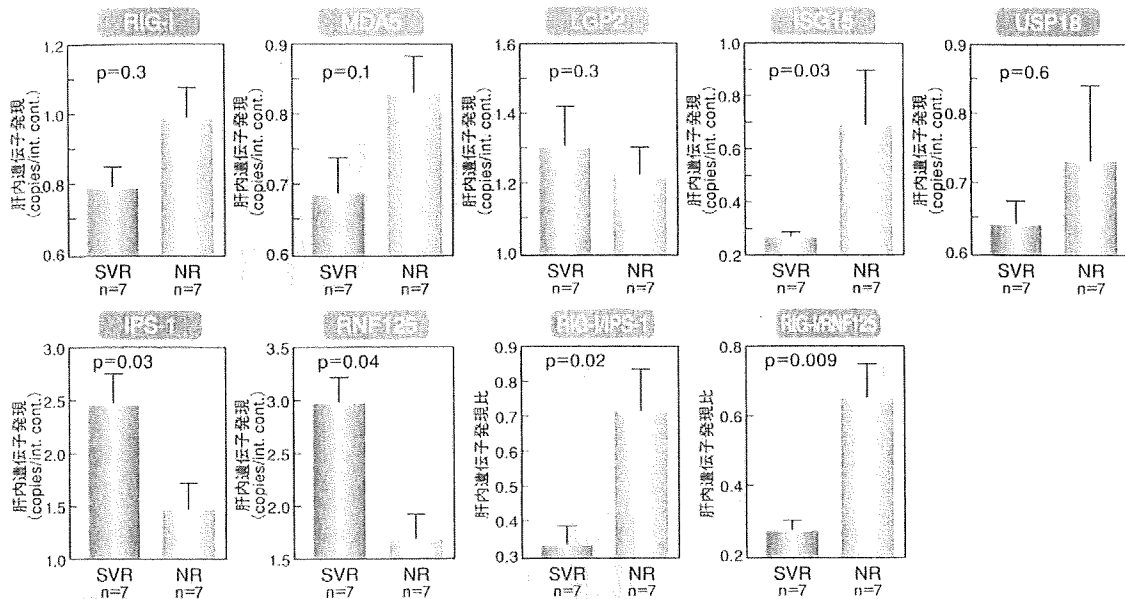
Variables	Az	95% CI	Cut-off	Sensitivity	Specificity	NPV	PPV
RIG-I	0.89	0.78-0.95	0.68	0.80	0.87	0.92	0.70
MDA5	0.92	0.86-0.98	0.84	0.82	0.89	0.93	0.74
LGP2	0.76	0.63-0.90	1.03	0.65	0.72	0.85	0.46
RIG-I/IPS-1	0.91	0.84-0.99	0.88	0.75	0.91	0.91	0.75
RIG-I/RNF125	0.81	0.69-0.93	1.05	0.82	0.62	0.91	0.43
ISG15	0.91	0.85-0.97	0.36	0.90	0.81	0.96	0.64
USP18	0.90	0.84-0.96	0.67	0.90	0.83	0.96	0.67

Asahina Y et al : *Gastroenterology* 134 : 1396-1405, 2008. 改変

表1 NVRに関する因子(多重ロジスティック解析)

Variables	Odds Ratio	95% CI	p value
RIG-I/ISP-1 ratio (by 0.2↑)	2.6	1.5- 4.5	0.0007
Core 71&91 double mut.	6.7	0.9-45.4	0.05
Platelet count (by 50×10 ³ /μL)	1.8	1.4- 9.6	0.01
Age (by 1 yr.)	1.0	0.9- 1.1	0.55
Gender (Female)	4.1	0.7-25.7	0.12

図7 Genotype 2におけるPEG-IFN/RBV併用療法の最終ウイルス学的治療効果と自然免疫系分子の肝内遺伝子発現



Asahina Y et al: *Gastroenterology* 134: 1396-1405, 2008. 改変

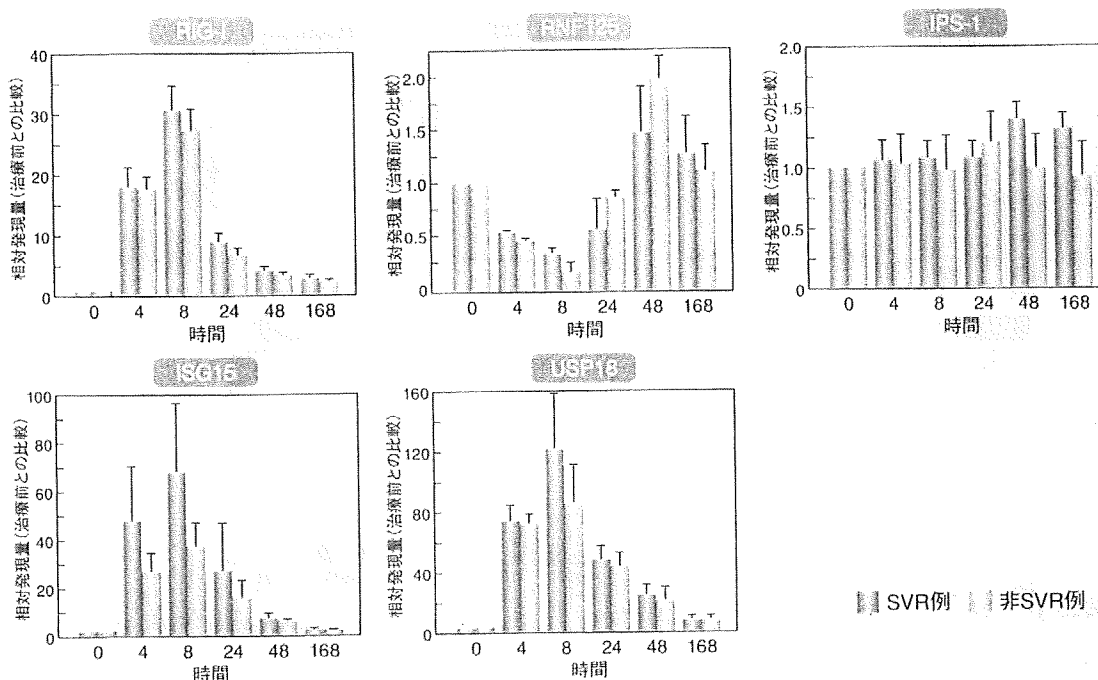
3. Genotype 2における自然免疫系遺伝子発現プロファイルと治療効果

Genotype 2はgenotype 1に比しIFN治療効果が高い。しかし、genotype 2におけるNVR例も極めて少ないが存在し、その機序については不明な点が多い。そこで、筆者らは、genotype 2においてもRIG-I/IPS-1系を中心とする自然免疫系遺伝子の発現状態とPEG-IFN/RBV併用療法の治療効果を検討した。それによると、genotype 2においても、NR例(n=7)ではSVRに比しISG15発現、RIG-I/IPS-1比およびRIG-I/RNF125比は高値であった(図7)。したがって、genotype 1bで認められた自然免疫系遺伝子発現と治療効果との関連は、genotypeにかかわらず認められる現象と考えられた。

自然免疫系遺伝子の経時的発現と治療効果

前述のように、RIG-Iなどの治療前における肝内遺伝子発現は、PEG-IFN/RBV併用療法不応例であるNVRで高発現していることがわかったが、PEG-IFN/RBV投与による反応性はどのようになっているであろうか。そこで、筆者らは、末梢血単核球中における、RIG-I, ISG15, USP18, RNF125およびIPS-1のPEG-IFN/RBV投与前後における経時的遺伝子発現動態を解析した²⁾(図8)。その結果、RIG-I, ISG15, USP18の発現量は、PEG-IFN/RBV投与の8時間後に治療前の30~120倍となり、治療により強く誘導されることがわかった。さらに、この遺伝子誘導は最終的にウイルスが駆除されたSVR例で高い傾向を認め、外因性IFNによる遺伝子の誘導能と治療効果との間に関連があることが示唆された。一方、抑制系遺伝子であるRNF125はRIG-Iの発現動態とは

図8 PEG-IFN/RBV併用療法治療中における自然免疫系遺伝子の発現動態と最終治療効果
[末梢血単核球における検討]



Asahina Y et al. *Gastroenterology* 134: 1396-1405, 2008. 改変

対照的で、PEG-IFN/RBV投与で抑制され投与後8時間で最低値となったが、その後発現が亢進しRIG-Iの発現が最も誘導された投与後48時間後にピークとなり、RIG-Iの発現が抑制されるにしたがって168時間後に治療前値に復した。このように、RNF125は実際の治療状態において、精巧にRIG-I系を制御していると考えられた。これらの分子の発現動態と異なって、IPS-1の発現動態はPEG-IFN/RBV投与により大きく影響は受けないことが観察され、治療中もconstitutiveに発現していることが示唆された。

宿主自然免疫系が治療効果へ関与するメカニズム

宿主自然免疫系が実際のPEG-IFN/RBV併用療法の治療効果にどのように関わるかのメカニズムの詳細については、未だ十分解明されていない。上述したように、筆者らの検討では、RIG-I, ISG15, USP18などのIFN誘導遺伝子はHCV感染による内因性IFNによりC型慢性肝炎患者において高発現しているが、PEG-IFN/RBV併用療法に不応性であるNVR例でより治療前の肝内発現が亢進していることがわかった。反対に、これらの遺伝子のIFN投与による反応性はSVR例でNVR例に比しより良好であることが観察された。このことから、NVR例では治療前に内因性IFNにより自然免疫系がすでにup regulationされているため、治療である外因性IFNに対する

反応性が現弱していることが示唆され、IFNに対する不応性のメカニズムを探る糸口になると考えられた。IFNに抵抗性を示す特定のHCVがこのような宿主の不応性を惹起するのか、宿主免疫自体に問題があるのかは不明であるが、これまでの筆者らの検討ではHCVコア遺伝子変異は自然免疫系遺伝子発現に関与していなかった。しかし、今後HCV全長遺伝子の解析などHCVと自然免疫との関連について詳細に検討する必要があると考えられた。

今回の筆者らの検討では、IPS-1はNVR例でRIG-Iと対照的にその発現がより低値であった。ウエスタン・ブロットにおいてこの低発現は蛋白レベルでも確認されたが、そのメカニズムは十分明らかではない。これまでの報告ではHCVは自身のNS3/4AセリンプロテアーゼによりIPS-1を分解し巧みにRIG-I/IPS-1系から逃避する可能性が示唆されているが¹⁶⁾、HCVが何らかの機序で転写レベルでもIPS-1を抑制している可能性は否定できない。この点については、HCVの治療抵抗性を解明し治療法を開発する上で極めて重要であり、今後のさらなる検討が期待される。

プロテアーゼ阻害薬治療中の自然免疫系遺伝子発現の経時的変化

現行のPEG-IFN/RBV併用療法に対して不応性であるNVR例に対しては、プロテアーゼ阻害薬などの新薬による治療が期待される。プロテアーゼ阻害薬は、HCVのNS3/4Aセリン・プロテアーゼを阻害することによりHCVの複製を強力に直接抑制することで主に抗ウイルス効果を発揮する。一方、NS3/4Aセリン・プロテアーゼがプロテアーゼ阻害薬により抑制されることにより、RIG-I/IPS-1系を中心とする宿主自然免疫能が改善することもプロテアーゼ阻害薬の効果として想定される。

筆者らは、テラプレビル単独で治療した症例において、治療中のRIG-I, IPS-1, ISG15, RNF125などの末梢血単核球中の経時的遺伝子発現について検討し、テラプレビル単独投与による強力な抗ウイルス効果に伴いRIG-Iなどの発現が低下する症例が多いことを認めており、さらに抗ウイルス効果による差について検討中である。

おわりに

これまで述べてきたように、RIG-I/IPS-1系の遺伝子発現は治療効果に深く関与しており、これらの遺伝子発現プロファイルを解析することはPEG-IFN/RBV併用療法の治療効果予測に有用と考えられた。すなわち、HCV感染により誘導される自然免疫は治療効果と密接に関連し、これらを解析することは治療効果予測や治療抵抗性の機序の解明、さらに自然免疫系を標的とした治療法の開発に有用である。さらに、最近宿主IL28B遺伝子のSNPsがPEG-IFN/RBV併用療法の治療効果に極めて強く関与することが報告された¹⁷⁾¹⁸⁾。自然免疫系の中心的役割を果たすI型IFNとIL28B遺伝子のコードするIFN- λ との関連などIFN抵抗性の機序に関わる今後の研究が期待される。

〈文献〉

- 1) Zeuzem S, Pawlotsky JM, Lukasiewicz E et al : DITTO-HCV Study Group. International, multi-center, randomized, controlled study comparing dynamically individualized versus standard treatment in patients with chronic hepatitis C. *J Hepatol* **43** : 250-257, 2005.
- 2) Asahina Y, Izumi N, Hirayama I et al : Potential relevance of cytoplasmic sensors and related regulators involving innate immunity in antiviral response. *Gastroenterology* **134** : 1396-1405, 2008.
- 3) Biron CA : Initial and innate responses to viral infections-pattern setting in immunity or disease. *Curr Opin Microbiol* **2** : 374-381, 1999.
- 4) Yoneyama M, Kikuchi M, Natsukawa T et al : The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* **5** : 730-737, 2004.
- 5) Yoneyama M, Kikuchi M, Matsumoto K et al : Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* **175** : 2851-2858, 2005.
- 6) Meylan E, Curran J, Hofmann K et al : Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437** : 1167-1172, 2005.
- 7) Kawai T, Takahashi K, Sato S et al : IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* **6** : 981-988, 2005.
- 8) Seth RB, Sun L, Ea CK et al : Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122** : 669-682, 2005.
- 9) Xu LG, Wang YY, Han KJ et al : VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* **19** : 727-740, 2005.
- 10) Zhao C, Denison C, Huijbregtse JM et al : Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. *Proc Natl Acad Sci USA* **102** : 10200-10205, 2005.
- 11) Schwer H, Liu LQ, Zhou L et al : Cloning and characterization of a novel human ubiquitin-specific protease, a homologue of murine UBP43 (Usp18). *Genomics* **65** : 44-52, 2000.
- 12) Malakhov MP, Malakhova OA, Kim KI et al : UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *J Biol Chem* **277** : 9976-9981, 2002.
- 13) Arimoto K, Takahashi H, Hishiki T et al : Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. *Proc Natl Acad Sci USA* **104** : 7500-7505, 2007.
- 14) Li XD, Sun L, Seth RB et al : Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci USA* **102** : 17717-17722, 2005.
- 15) Akuta N, Suzuki F, Kawamura Y et al : Prediction of response to pegylated interferon and ribavirin in hepatitis C by polymorphisms in the viral core protein and very early dynamics of viremia. *Intervirology* **50** : 361-368, 2007.
- 16) Loo YM, Owen DM, Li K et al : Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc Natl Acad Sci USA* **103** : 6001-6006, 2006.
- 17) Dongliang G, Fellay J, Thompson AJ et al : Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **2009**.
- 18) Tanaka Y, Nishida N, Sugiyama M et al : Genome-wide association of IL28B with response to pegylated interferon- α and ribavirin therapy for chronic hepatitis C. *Nat Genet* **2009**.

* * *

討 論

【小俣】 どうもありがとうございました。会場からご質問はございませんか。

【熊田(博)】 RIG-IとIPS-1の変化を年齢別に、50歳以下の症例と50歳以上の症例で分けて検討すればどうなりますか。

【朝比奈】 全体では年齢による差はみられず、年齢が高いとRIG-Iが高く発現するというようなことはありませんでした。ただ、個々の症例を見ると、年齢が高いLVR症例はRIG-Iの発現が高く、年齢が若い症例では低い。そのような症例が多いのですが、中には年齢が低いにもかかわらずRIG-Iが高く、LVRになる症例が含まれておりました。そのため、全体でみると50歳以上、50歳以下の間の差は消えてしまうというデータが得られました。

【熊田(博)】 年齢が若くて治らない症例は、RIG-Iが低いということですか。

【朝比奈】 年齢が若くて治らない症例にも、RIG-Iが上がっている症例はあります。若くてRIG-Iが高いと、やはりIFNが効いていないです。

【熊田(博)】 年齢が若くてIFNが効かない症例は、RIG-Iが高いのですね。

【朝比奈】 はい。

【林】 2点教えていただきたいことがございます。RIG-IとIPS-1の比をとっていますが、途中経過をみるとIPS-1はほとんど変わっていないため、実際にはRIG-Iの影響を受けているのだと思います。先生は最初に、ウイルスが消えないNVR症例は、基本的にRIG-Iが高いとおっしゃいました。これは、治療によってSVRが得られれば、RIG-I

がresponseするというお話だと思いますが、その関係はどのように理解すればよろしいでしょうか。私達もHCVに感染するとRIG-Iのinductionが起こると考えていますが、RIG-Iの高いNVR症例の治療経過をみると、SVRが得られたことでRIG-Iのresponseがみられます。

【朝比奈】 効かない症例では、おそらく治療前には、内因性IFNによりRIG-Iはup regulateされている状態にあるのだらうと思います。ただ、外因性IFNが入るとそれに対しては不応性を示すと考えられます。おそらく内因性IFNによって発現がup regulateされており、それ以上の反応が鈍くなっていると考えられます。

【林】 内因性IFN、外因性IFNの反応の差がわからないので解釈のしようがない気もしますが、そこに少し違和感を覚えました。

もう1点教えていただきたいことがございます。先生が最初におっしゃったように、ウイルスに感染してIPS-1の出現が障害される、自然免疫系の最初の部分が障害されるのでウイルスがうまく排除されないというのはその通りだと思います。その後IFN- β が出てきて、IFN後のシグナルについては現在のところ影響がございません。なぜ外因性IFNを投与してもウイルスの排除が起こらないのですか。

【朝比奈】 おそらく外因性IFNを投与したらJAK-STATは強力に動くのだと思いますが、実際にウイルスを排除するというになると、やはりRIG-I/IPS-1などの自然免疫系が深く影響を及ぼしているのではないかと考えています。

* * *

Absence of viral interference and different susceptibility to interferon between hepatitis B virus and hepatitis C virus in human hepatocyte chimeric mice[☆]

Nobuhiko Hiraga^{1,2}, Michio Imamura^{1,2}, Tsuyoshi Hatakeyama¹, Shosuke Kitamura¹, Fukiko Mitsui^{1,b}, Shinji Tanaka¹, Masataka Tsuge^{1,2}, Shoichi Takahashi^{1,2}, Hiromi Abe^{2,3}, Toshiro Maekawa^{2,3}, Hidenori Ochi^{2,3}, Chise Tateno^{2,4}, Katsutoshi Yoshizato^{2,4,5}, Takaji Wakita⁶, Kazuaki Chayama^{1,2,3,*}

¹Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

²Liver Research Project Center, Hiroshima University, Hiroshima, Japan

³Laboratory for Liver Diseases, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan

⁴PhoenixBio Co., Ltd., Higashihiroshima, Japan

⁵Osaka City University Graduate School of Medicine, Osaka, Japan

⁶Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Japan

Background/Aims: Both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate in the liver and show resistance against innate immunity and interferon (IFN) treatment. Whether there is interference between these two viruses is still controversial. We investigated the interference between these two viruses and the mode of resistance against IFN.

Methods: We performed infection experiments with either or both of the two hepatitis viruses in human hepatocyte chimeric mice. Huh7 cell lines with stable production of HBV were also established and transfected with HCV JFH1 clone. Mice and cell lines were treated with IFN. The viral levels in mice sera and culture supernatants and messenger RNA levels of IFN-stimulated genes were measured.

Results: No apparent interference between the two viruses was seen *in vivo*. Only a small (0.3 log) reduction in serum HBV and a rapid reduction in HCV were observed after IFN treatment, regardless of infection with the other virus. In *in vitro* studies, no interference between the two viruses was observed. The effect of IFN on each virus was not affected by the presence of the other virus. IFN-induced reductions of viruses in culture supernatants were similar to those in *in vivo* study.

Conclusions: No interference between the two hepatitis viruses exists in the liver in the absence of hepatitis. The mechanisms of IFN resistance of the two viruses target different areas of the IFN system.

© 2009 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Keywords: Superinfection; JFH-1; IFN-stimulated genes

Received 4 February 2009; received in revised form 14 July 2009; accepted 15 July 2009; available online 23 September 2009

Associate Editor: F. Zoulim

* C.T. and K.Y. are employees of PhoenixBio Co. Ltd., Higashihiroshima, Japan. The other authors who have taken part in this study declared that they do not have anything to disclose regarding funding from industry or conflict of interest with respect to this manuscript.

Corresponding author. Tel.: +81 82 2575190; fax: +81 82 2556220.

E-mail address: chayama@hiroshima-u.ac.jp (K. Chayama).

Abbreviations: GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HBcAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; OAS, 2',5'-oligoadenylate synthetase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

1. Introduction

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 million people are infected with HBV, and more than 170 million people are infected with HCV [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and lead to death due to liver failure and hepatocellular carcinoma [3]. To date, interferon (IFN) remains one of the most important drugs available for the treatment of both types of hepatitis viral infections. Although it is assumed that IFN suppresses viral replication through the effect of IFN-induced gene products such as mixovirus resistance protein A (MxA), RNA-dependent protein kinase (PKR), and 2',5'-oligoadenylate synthetase (OAS) [4], the precise mechanism of action of these proteins on both hepatitis viruses are unknown.

Coinfection with both viruses leads to a rapid and severe progression of chronic liver disease [5], with a higher risk of hepatocellular carcinoma [6]. Currently, there is a debate about whether or not there is interference between the two hepatitis viruses, with some favoring such interference [7] and others arguing against such a concept [8]. A number of mechanisms can cause interference between viruses. A major mechanism of interference is induction of IFN by one virus to prevent replication of the second virus; however, viruses develop their own strategies to resist the effect of IFN. In clinical practice, practitioners often perceive that reduction of HBV in serum by IFN therapy is poorer compared with HCV. HCV levels in sera of IFN-treated patients decrease relatively rapidly, and a proportion of patients eventually show complete eradication of the virus. Furthermore, the recent use of pegylated IFN (PEG-IFN) in combination with ribavirin has improved the eradication rate [9]. Eradication of HBV by IFN, however, is usually difficult, even when using IFN combined with ribavirin [10].

The mechanisms developed by viruses to resist host innate immunity, including IFN signaling, are well established in some viruses. Such mechanisms involve interruption of IFN signaling by interacting molecules that transduce the signal from the IFN receptor through the Janus kinase (Jak) signal transducer and activator of transcription (STAT) pathway [4]. Viral proteins of paramyxoviruses, for example, inhibit IFN signaling [11]. Several studies have also examined the mechanisms by which HCV resists the host immune system. These include degradation of Cardif adaptor protein by NS3A/4 protease [12]. Generally, expression of HCV protein is associated with inhibition of STAT1 function independent of STAT tyrosine phosphorylation [13]. Additionally, expression of the HCV core protein in cultured cells is associated with increased expression levels of the suppressor of cytokine signaling 3 (SOCS-3) [14]. The NS5A and E2 proteins are both inhibitors of PKR

[15]. These strong actions of HCV against innate immunity are consistent with the high chronicity rate of the virus. IFN, however, effectively reduces HCV replicon in Huh7 cells [16], suggesting that the virus has little potential to disturb the actions of IFN.

In contrast to HCV, the mechanisms of IFN resistance by HBV are poorly understood. To date, only a few studies have reported the molecular mechanisms of HBV resistance against the actions of IFN. The HBV-related resistance to IFN, for example, involves upregulation of protein phosphatase 2A (PP2A) as the primary event, which subsequently leads to inhibition of protein arginine methyltransferase 1 (PRMT1) and reduced STAT1 methylation [17]. In addition to these molecular mechanisms, microarray analyses of serial liver biopsies of experimentally infected chimpanzees showed striking differences in the early immune responses to HBV and HCV. HCV, for example, induced early changes in the expression of many intrahepatic genes, including genes involved in type 1 IFN response [18], whereas HBV did not induce any detectable changes in the expression of intrahepatic genes in the first weeks of infection [19].

HBV–HCV double infection is a good model to use for assessment of the mechanism of IFN resistance by these two viruses because one can test the effect of IFN on one virus in the presence of the other virus. Recently, Bellecave et al. [20] established a novel *in vitro* model system in Huh7 cells that allowed the analysis of both viruses in a replicating context and reported the absence of direct viral interference. To this end, we used human hepatocyte chimeric mice and cell culture systems in the present study. The results showed that the presence of HBV does not affect the actions of IFN on HCV and vice versa. These results suggest the lack of interference between the two viruses in liver cells and indicate that the reported interference between the two viruses might be via inflammation including death of infected cells by cytotoxic T cells, cytokines including IFN- α and IFN- β , and interleukins produced by hepatocytes and infiltrating T cells.

2. Materials and methods

2.1. Transfection of Huh7 cells with HBV DNA and HCV RNA

Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37 °C and under 5% CO₂. Cloning of HBV DNA and the plasmid construction were performed as described previously [21]. For production of stably transfected cell lines, Huh7 cells were seeded onto 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pTRE-HB-wt [21] was transfected by the calcium phosphate precipitation method. Twenty-four hours after transfection, the cells were split and cultured in Hygromycin B-DMEM selection medium (300 μ g/ml; Invitrogen Japan K.K., Osaka, Japan), while 50 colonies were isolated and cultured for identification of virus-producing cell lines. Clones positive

for both hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were selected and further analyzed for production of HBV particles. Finally, three cell lines that produced more than 10^5 copies per milliliter of HBV DNA in supernatant were selected and used for further experiments.

For transfection with HCV RNA, we used pJFH1, which contains the complementary DNA of full-length genotype 2a HCV clone JFH1 downstream of the T7 promoter [22]. *In vitro* synthesis of HCV RNA and electroporation into Huh7 cells were performed as described previously [22,23]. Briefly, cells were treated with trypsin, washed twice with ice-cold RNase-free phosphate-buffered saline, and resuspended in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) at a final concentration of 7.5×10^6 cells per milliliter. Then, 10 μ g of HCV RNA to be electroporated was mixed with 0.4 mL of cell suspension and subjected to an electric pulse (950 μ F and 260 V) using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish.

2.2. Generation of human hepatocyte chimeric mice

Generation of the urokinase-type plasminogen activator (uPA)^{+/+} and severe combined immunodeficiency (SCID)^{+/+} mice and transplantation of human hepatocytes were performed as described recently by our group [21,23,24]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and euthanasia were performed under ether anesthesia. The concentration of serum human serum albumin, which correlates with the repopulation index [24], was measured in mice as described previously [21]. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.

2.3. Human serum samples

Human serum samples containing high titers of either HBV DNA (5.3×10^6 copies per milliliter) or genotype 1b HCV (2.2×10^6 copies per milliliter) were obtained from patients with chronic hepatitis with a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use. Chimeric mice were injected intravenously with 50 μ L of either HBV- or HCV-positive human serum. Some mice were injected with HBV-positive human serum at 6 weeks after injection of HCV-positive human serum.

2.4. Analysis of HBV and HCV

HBsAg and HBeAg in culture supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Japan, Osaka, Japan). DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μ L H₂O [21,25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μ L RNase-free H₂O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20- μ L reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY, USA).

2.5. RNA extraction and measurement of mRNAs of interferon-induced genes by quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One nanogram of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random

Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, OAS, and PKR. Amplification and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). Results were normalized to the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6. Statistical analysis

Changes in HBV DNA and HCV RNA in mice sera were compared by Mann–Whitney test and unpaired *t* test. Differences in HBV DNA and HCV core antigen in mice sera and culture supernatants were analyzed by one-way analysis of variance followed by Scheffé's test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Infection of chimeric mouse with HBV and HCV and susceptibility to interferon

To investigate the interference between HBV and HCV and to examine the effect of IFN on both of these two viruses *in vivo*, we used six human chimeric mice. Each of six mice was inoculated intravenously with 50 μ L of serum samples obtained from either HBV- or HCV-positive patients. The median HBV DNA level in HBV-positive serum-inoculated mice was 1.4×10^8 copies per milliliter (range: 5.3×10^6 – 3.6×10^9 copies per milliliter) at 6 weeks after inoculation (Fig. 1A), similar to our recent observation [21]. Similarly, the median HCV RNA level in HCV-positive human serum-inoculated mice was 1.0×10^7 copies per milliliter (range: 1.2×10^6 – 0.8×10^7 copies per milliliter) at 4 weeks after inoculation (Fig. 1B), as reported recently by our group [23]. Six weeks after inoculation, three of six HBV- or HCV-infected mice were treated daily with 7000 IU/g per day of intramuscular IFN- α for 2 weeks. Treatment resulted in a decrease of only 0.3 log in mice serum HBV DNA level compared to that in mice without treatment (Fig. 1A). In contrast, the same therapy resulted in a rapid decrease in HCV RNA to undetectable levels, as confirmed by quantitative polymerase chain reaction (PCR; Fig. 1B).

To investigate the direct interference of the two viruses, we performed double-infection experiments. Ten chimeric mice were first inoculated intravenously with 50 μ L of HCV-positive human serum samples. Six weeks after HCV infection when the mice developed HCV viremia, 50 μ L of HBV-positive human serum samples were inoculated intravenously in 5 of 10 HCV-infected mice. All five mice became positive for both HBV and HCV at 2 weeks after HBV infection. No significant decrease in HCV RNA levels was observed in these superinfected mice before or after the development of HBV viremia (Fig. 2A). After HBV infection, there was no apparent decrease in HCV titer (Fig. 2B). Moreover, HBV DNA level in HBV–HCV-coinfected mice was comparable with that of only HBV-infected mice (Fig. 2B). These results sug-

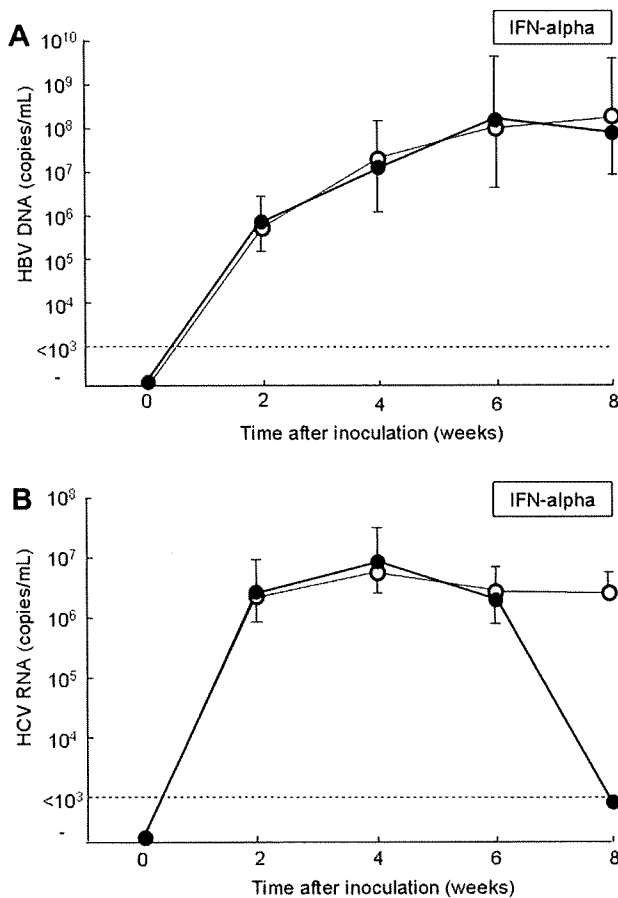


Fig. 1. Changes in serum virus titers in mice inoculated with hepatitis B virus (HBV) – positive or hepatitis C virus (HCV) – positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples. Six weeks after inoculation, three of six mice were treated daily with (closed circles) or without (open circles) 7000 IU/g per day of interferon-alpha intramuscularly for 2 weeks. Mice serum samples were extracted every 2 weeks after inoculation. Data are mean plus or minus standard deviation ($n = 3$). The horizontal dashed line represents the detection limit (10^3 copies per milliliter).

gest no interference between the two viruses in mice, which lack immunocytes known to cause hepatitis.

To further investigate if infection with either of the two hepatitis viruses alters the effect of IFN against the other virus, three HBV–HCV-coinfected mice were treated with IFN- α (Fig. 3A). Such treatment resulted in a rapid decrease in HCV RNA in all mice to undetectable levels as confirmed by quantitative PCR (Fig. 3B). In contrast, no significant decrease in HBV DNA titers was observed in these mice (Fig. 3B). These results are similar to the reduction of HCV RNA and HBV DNA in mice that were infected with either of these hepatitis viruses. These results indicate that HCV is more susceptible to IFN- α than HBV and that each virus does not alter the effect of IFN on the other virus. Because the effect of IFN on HCV was not disturbed by HBV, we assumed that HBV has no effect on the signal from IFN receptor to IFN-stimulated genes. It is possible,

however, that HBV and HCV replicated in different cells in these mice. Because it was impossible to detect HCV protein and RNA in HCV-infected mouse liver by histologic examination, we performed *in vitro* experiments.

3.2. Production of both HBV- and HCV-producing cells and the effect of interferon

To investigate the effect of IFN on HBV and HCV *in vitro*, we created cell lines that produce both HBV and HCV. First, we established stable HBV-producing Huh7 cell lines. Three cell lines (Clone-39, -42, and -53) that produced HBsAg, HBeAg, and HBV DNA into the supernatant were selected (Table 1). These cell lines continuously produced HBV for more than 3 months (data not shown). Next, JFH1 RNA was transfected into these HBV-producing cell lines to produce both HBV DNA and HCV proteins into the supernatant. HBV DNA levels in the supernatants of these cell lines decreased in Clone-39, increased in Clone-42, and did not change in Clone-53 after JFH1 transfection (Fig. 4A). In contrast, HCV core antigen levels in the supernatants were higher in two of the three cell lines (Clone-39 and -42) than in Huh7 cells, and the level was not different in the remaining cell line (Clone-53) (Fig. 4B). These results indicate that the production of each of the two viruses does not disturb the replication of the other virus.

3.3. Effects of interferon on HBV and HCV *in vitro*

The effects of IFN on virus production in both HBV- and HCV-producing cell lines was examined by adding different amounts of IFN- α (0, 50, and 500 IU/mL) into the culture. The mRNA levels of intracellular IFN-stimulated genes such as MxA, OAS, and PKR increased in a dose-dependent manner in all three cell lines as well as in parental Huh7 cells (Fig. 5A). Following the addition of IFN, no apparent reduction of HBV was noted in the supernatant of HBV–HCV-cotransfected cell lines (Fig. 5B). In contrast, the levels of HCV core antigen in the supernatant decreased in all three cell lines treated with IFN, and the decrease was dose-dependent (Fig. 5C).

4. Discussion

Although IFN treatment for chronic HCV infection has improved with the advent of PEG-IFN, the rate of viral eradication remains unsatisfactory [9]. The mechanism responsible for failure of IFN to eradicate the virus completely must be clarified. To study the mechanism of viral resistance against IFN, analysis of viral interference may give us some hints because one of the major mechanisms of interference is through the action of IFN.

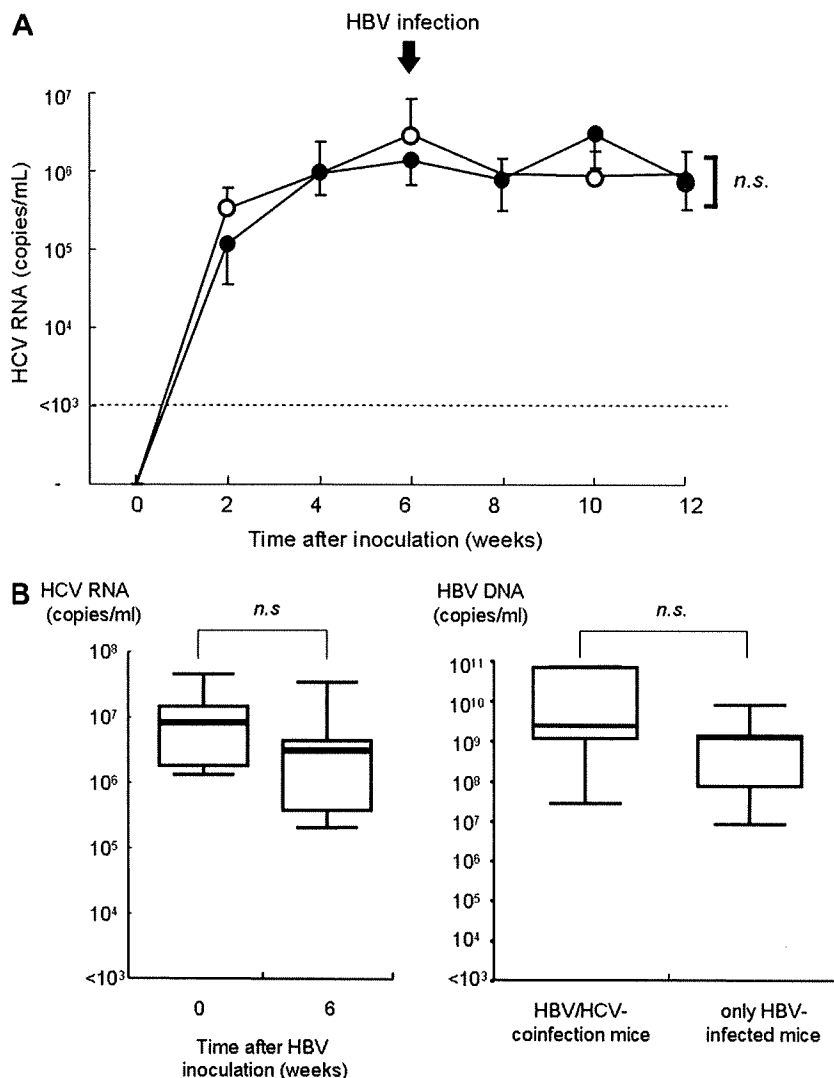


Fig. 2. Comparison of hepatitis C virus (HCV) and hepatitis B virus (HBV) titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining five mice (open circles) did not receive HBV inoculation. Data are mean plus or minus standard deviation ($n = 3$). (B) Serum HCV RNA titers in five mice infected with HCV before and at 6 weeks after HBV superinfection (left panel). Serum HBV DNA titers in five mice coinfecting with HBV and HCV were compared with those of five mice with HBV infection only (Fig. 1) at 12 weeks after HCV inoculation (right panel). In these box-and-whisker plots, lines within the boxes represent the median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Accumulation of mononuclear cells is usually seen in the livers of infected individuals, in association with the state of inflammation. It is thus difficult to examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte chimeric mouse model is ideal for investigating such interaction. We expected either reduction of HCV after inoculation of HBV in HCV-infected mice or failure to develop HBV viremia or low-level

HBV viremia in these mice due to viral interference; however, no reduction in HCV titers occurred in these mice, and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

Wieland et al. reported that HBV did not induce any genes during entry or expansion in HBV-infected chimpanzee livers and suggested that HBV was a stealthy virus early in the infection [19]. Because no reduction in HCV was noted during and after the development of high-level HBV viremia, we assume that HBV escapes innate immunity via an excellent mechanism without

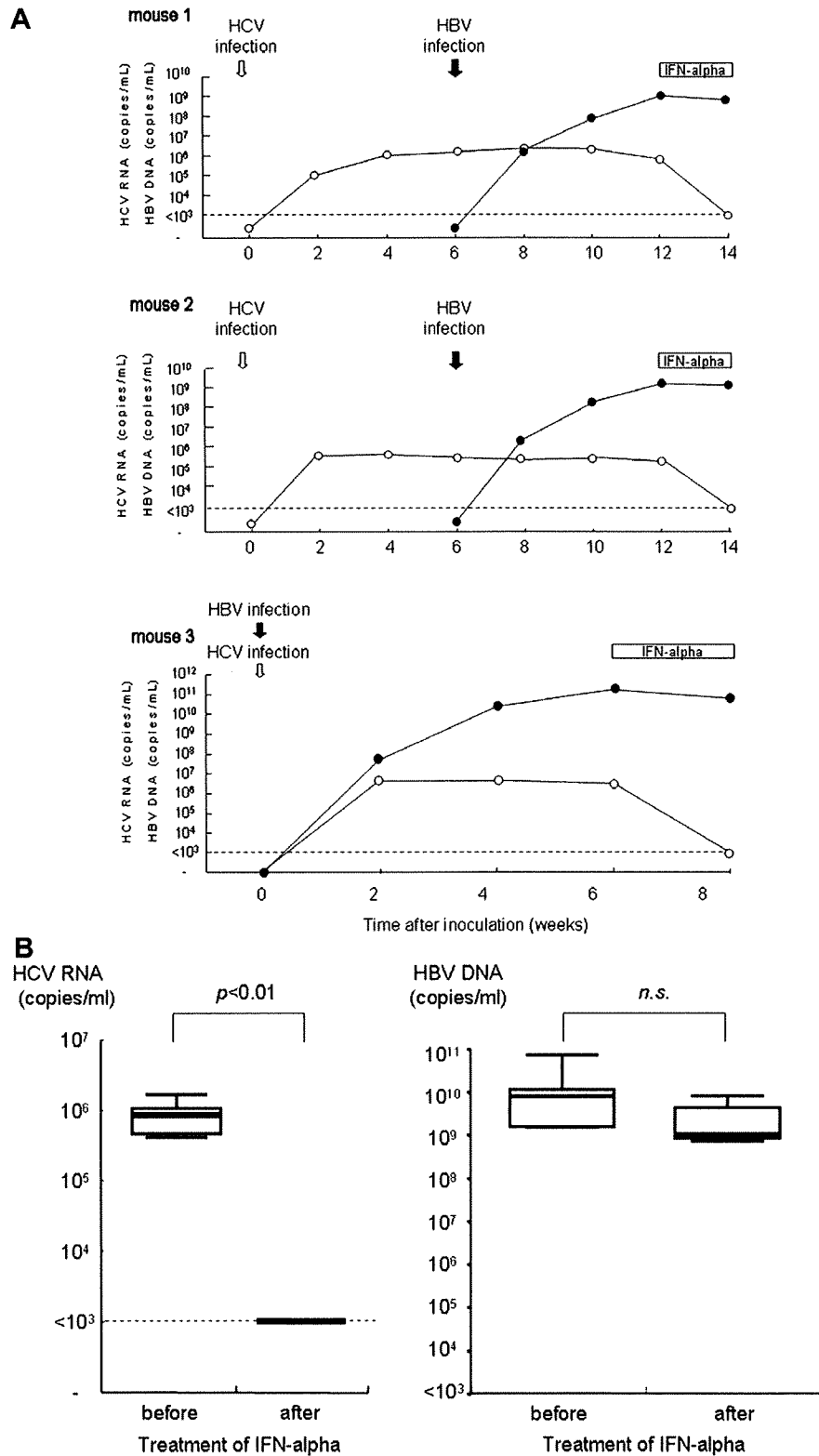


Fig. 3. Changes in serum hepatitis C virus (HCV) RNA and hepatitis B virus (HBV) DNA levels and effects of IFN on HBV–HCV-coinfected mice. Three mice (mouse 1, 2, and 3) were inoculated with both HBV- and HCV-positive human serum samples and treated daily with 7000 IU/g per day of interferon-alpha (IFN- α) intramuscularly for 2 weeks. Mice sera samples were obtained every 2 weeks after injection, and HCV RNA (open circles) and HBV DNA (close circles) were analyzed by quantitative polymerase chain reaction. (A) The horizontal dashed line represents the detectable limit (10³ copies per milliliter). (B) Serum HCV RNA and HBV DNA titers in mice before and after 2-week IFN- α treatment. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Table 1
Hepatitis B virus (HBV) markers in supernatants of stable HBV-transfected cell lines.

Clone	HBsAg (IU/L)	HBeAg (IU/L)	HBV DNA (log copies per milliliter)
39	0.46	4.57	5.2
42	8.16	1.34	5.3
53	0.08	9.29	5.4

Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

evoking the IFN production system in liver cells. Further study using double-infected mice treated with anti-HBV nucleotide analogs and anti-HCV protease inhibitors should be conducted to confirm the present findings.

With regard to the use of IFN as a treatment, we initially assumed that HBV infection would prevent the effect of IFN on HCV and possibly vice versa in double-infection mice. Unexpectedly, the reduction of HCV by IFN therapy was quite similar in mice infected with HCV only and in those coinfecting with HBV and HCV (Figs. 1 and 3). This finding indicated that HBV does not disturb the effect of IFN through signal transduction from the IFN receptor through the Jak-STAT pathway. It was, however, considered possible that HBV and HCV infect different liver cells in mice and replicated without being affected by each other. It has been reported that the same liver cell could be infected with both HBV and HCV [20,26], but it was difficult in the present study to confirm that these two viruses replicate in the same liver cell of mice because it is difficult to visualize HCV antigen and RNA in pathologic sections of the mouse liver. To address this issue, we transfected HCV to stable HBV-producing cell lines

(Fig. 4). We thought that both HCV and HBV were produced from successfully HCV RNA transfected cells because transfected cells were stable HBV-producing cells. Presence of the both hepatitis viruses in the same hepatocytes has also been shown by a recent report by Bellecave et al. [20]. We showed in our cell line experiments that only HBV-transfected cell lines produced HBV and that cells cotransfected with HBV and HCV did not show a clear effect of HCV replication on HBV production (Fig. 4A). Similarly, stable production of HBV did not alter the replication of HCV (Fig. 4B). These data are consistent with a recent report [20] that showed that HCV could infect cells producing HBV and suggest a lack of interference between the two viruses in liver cells.

Using HCV-transfected HBV-producing cell lines, we demonstrated that presence of HBV did not disturb the actions of IFN on HCV (Fig. 5C). HCV utilizes certain machinery to disrupt the innate immune system; however, once exposed a large concentration of IFN, the virus shows high sensitivity, as shown in the replicon system [16,27]. Thus, HCV seems to have a relatively weak ability to disturb the antiviral actions of IFN compared with HBV. In contrast, HBV showed strong resistance against IFN in cells with diminished HCV replication [28]. The fact that HBV does not disturb IFN signaling but resists the actions of IFN suggests that HBV counteracts the actions of IFN at IFN-induced antiviral product levels.

Although the culture environment is different from the replicon system, the JFH1 strain seems relatively resistant to IFN [29]. This suggests that the core and envelope proteins, which are absent in the replicon system, might play a role in IFN resistance; however, we could not show any effect for HCV infection on the actions of IFN on HBV replication. This finding sug-

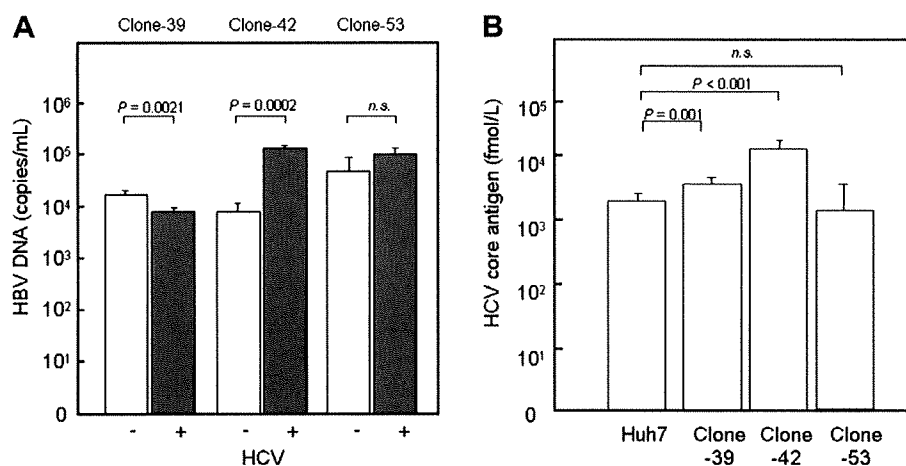


Fig. 4. Virus titers in supernatants of hepatitis B virus (HBV)-transfected or hepatitis C virus (HCV)-transfected cell lines. Huh7 cells were initially stably transfected with 1.4 genome-length HBV DNA. Three cell lines (Clone-39, -42, and -53) producing HBV DNA into the supernatant were selected. (A) HBV DNA levels in supernatants of HBV-producing cell lines 72 hours after transfection with JFH1 RNA (HCV positive) or control plasmid (HCV negative). (B) HCV core antigen levels in the supernatant of parental Huh7 cells and HBV-producing cell lines 72 h after transfection with JFH1 RNA. Data are mean plus or minus standard deviation ($n = 3$).

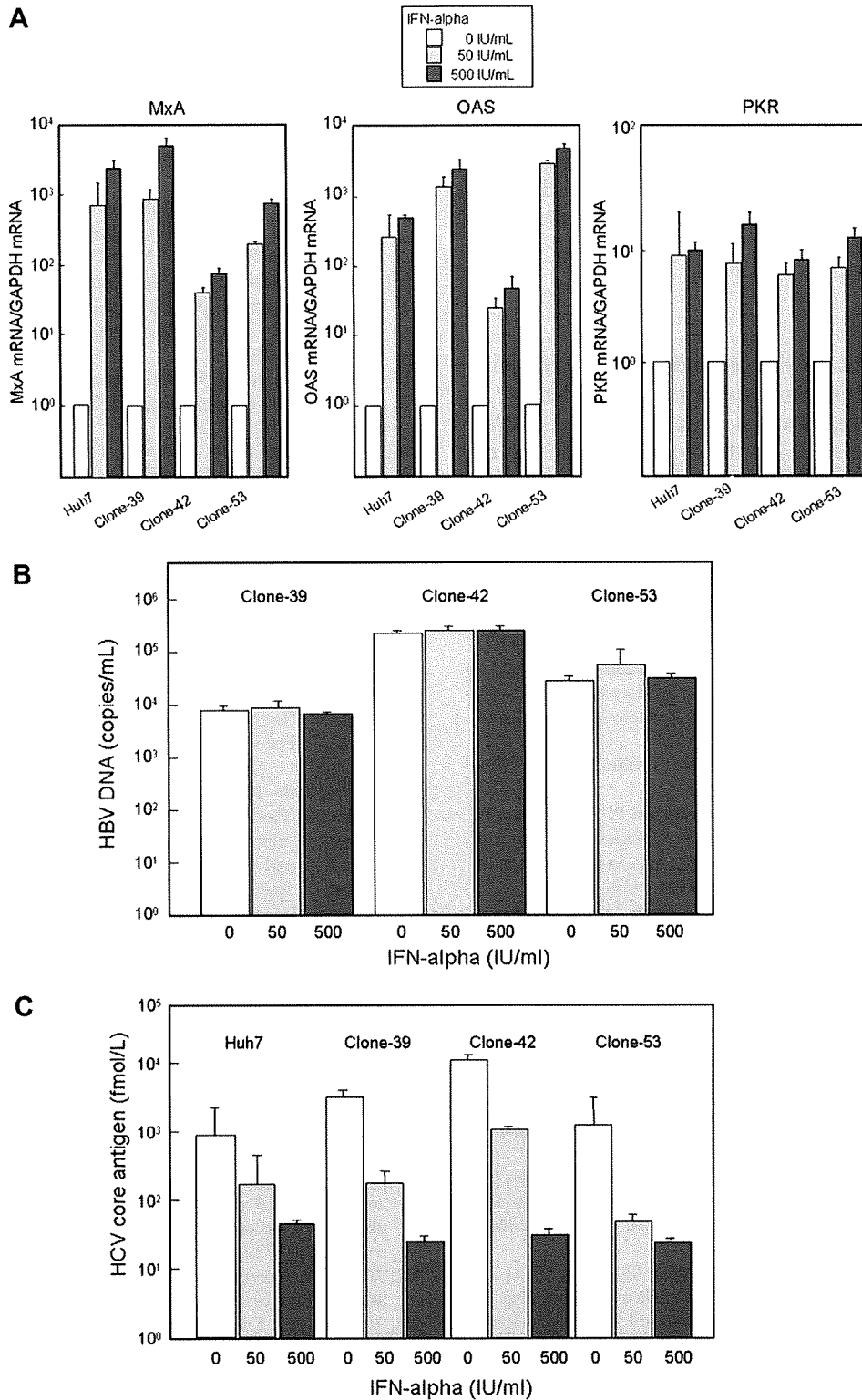


Fig. 5. Effects of interferon (IFN) treatment on hepatitis B virus (HBV) and hepatitis C virus (HCV) *in vitro*. Parental Huh7 cells and three HBV-transfected Huh7 cell lines (Clone-39, -42, and -53) were transfected with JFH1 RNA. Immediately after JFH1 transfection, the cell lines were treated with IFN- α (0, 50, and 500 IU/mL) for 72 h. (A) Intracellular gene expression levels of mixovirus resistance protein A (MxA), 2',5'-oligoadenylate synthetase (OAS), and RNA-dependent protein kinase (PKR) were measured. RNA levels were expressed relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) messenger RNA. (B) HBV DNA and (C) HCV core antigen in supernatants were measured. Data are mean plus or minus standard deviation ($n = 3$).