田中友加，尾上隆司，井手健太郎，田原裕之，番匠谷将孝，小林剛，大下彰彦，天野尋暢，田代裕尊，大段秀樹：既存抗MHC抗体陽性同種移植、異種移植、血液型不適合移植におけるプロテアソーム阻害剤（ボルテゾミブ）のB細胞に対する免疫抑制効果の検討．第46回日本移植学会総会，京都，2010．10．20-22

19）尾上隆司，井手健太郎，伊禮俊充，田原裕之，番匠谷将孝，田澤宏文，堀田龍一，小林剛，大下彰彦，天野尋暢，田代裕尊，大段秀樹：生体部分肝移植における過小グラフト症候群に対するPGE１門脈注入療法の有用性の検討．第46回日本移植学会総会，京都，2010．10．20-22

20）田澤宏文，伊禮俊充，五十嵐友香，田中友加，尾上隆司，井出健太郎，田原裕之，番匠谷将孝，小林剛，大下彰彦，天野尋暢，田代裕尊，大段秀樹：血液型不適合肝移植後の血液型抗原認識B細胞性免疫寛容の証明．第46回日本移植学会総会，京都，2010．10．20-22

21）山下正博，井手健太郎，尾上隆司，番匠谷将孝，田澤宏文，堀田龍一，寺岡義布史，平田文宏，橋本慎二，田代裕尊，大段秀樹：当院における高齢生体肝移植レシピエントの臨床的検討．第46回日本移植学会総会，京都，2010．10．20-22

22）井手健太郎，石田裕子，田中友加，尾上隆司，五十嵐友香，番匠谷将孝，田澤宏文，寺岡義布史，堀田龍一，山下正博，橋本慎二，平田文宏，森本博司，田代裕尊，大段秀樹：胆汁中Fractalkine測定による肝移植後グラフトバイオマーカーの開発．第46回日本移植学会総会，京都，2010．10．20-22

23）番匠谷将孝，尾上隆司，志々田将幸，五十嵐友香，田中友加，田原裕之，井手健太郎，梶谷桂子，田澤宏文，寺岡義布史，堀田龍一，山下正博，橋本慎二，平田文宏，田代裕尊，大段秀樹：肝類洞内皮細胞のアロ応答性T細胞制御能：in vivoモデルでの解明．第46回日本移植学会総会，京都，2010．10．20-22

24）寺岡義布史，田原裕之，井手健太郎，Basnet Nabin，田中友加，大段秀樹：CD４７-SIRPシグナルによるブタ－ヒト異種間T細胞応答．第46回日本移植学会総会，京都，2010．10．20-22

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
（分担）研究報告書（平成２２年度）
ヒト肝細胞キメラマウスを用いた治療抵抗性の肝炎に関する研究

ヒト肝細胞キメラマウスを用いた新規抗HCV薬の効果判定

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研究要旨：HCV を感染させたヒト肝細胞キメラマウスを用いて，種々の抗ウイルス剤の抗 HCV 効果を検討した. HCV 感染マウスにソヤサポゲノール B 誘導体を投与すると単独投与ではHCV 抑制効果を認めなかった．しかし IFN-α と併用することにより，IFN-α の抗 HCV 効果が増強された．HCV 感染マウスに NS3/4A プロテアーゼ阻害剤あるいはNS5B ポリメラーゼ阻害剤を単独投与すると，両薬剤とも抗HCV 効果を認めるものの, 耐性ウイルス出現による breakthrough を発症した. しかし両剤を併用投与することにより, breakthrough の予防，抗ウイルス効果の増強効果を認め，HCV の排除も可能であり，異なる HCV 蛋白を標的とする薬剤を組み合わせることにより，IFN 製剤を使用せずとも HCV の排除が可能であることが示された．HCV 感染マウスを用いて種々の抗ウイルス剤の効果判定および新規治療法の開発が可能であった.

A. 研究目的

ヒト肝細胞キメラマウスを用いて，新規候補となるHCV 感染阻害剤や抗HCV 剤のスクリーニング，あるいは種々の薬剤を組み合わせることにより，より有効な抗 HCV 治療法を開発する.

B. 研究方法

1) HCV感染マウスにインターフェロン-αあるいはソヤサポゲノール B 誘導体を投与し，マウス血中HCV RNA低下量, 肝臓内HCVコア抗原量および肝臓内IFN誘導遺伝子発現量を測定した.

2) HCV感染マウスに28日間, NS3/4Aプロテアーゼ阻害剤であるtelaprevir (200 mg/kg，1日2回，連日経口投与：)あるいはNS5Bポリメラーゼ阻害剤であるMK-0609 (3 mg/kg，1日2回，連日経口投与：)単独および両者を併用投与し，マウス血中HCV RNA量の測定した.

C. 結果

1) HCV感染マウスにIFN-α(1500 IU/g/日，連日腹腔内投与)あるいはソヤサポゲノール B 誘導体(1.5 mg/g feed 連日経口投与)を4週間，単独あるいは併用投与した．ソヤサポゲノール B 誘導体単独ではHCV RNAは低下しなかったが，IFN-α と併用投与したところ，4.7 log低下し，IFN-α 単独の1.9 log低下に比べ，有意に強い抗HCV効果を認めた(p=0.035)．また投与4週後の肝臓内コア抗原量はIFN-α/ソヤサポゲノール B 誘導体併用投与群で最も低かった. IFN-α を単回投与する3日前よりソヤサポゲノール B 誘導体(4.5 mg/g feed 連日経口投与)投与群では，非投与群に比べ，肝臓内のIFN誘導胃遺伝子(OAS-1, PKR, USP18)の発現量が有意に増加していた.

2) HCV感染マウスへのtelaprevirあるいはMK-0609の単独投与では，いずれも著明な抗ウイルス効果を認めたが，耐性ウイルス(NS3 ; V36A変異/NS5B ; S282T変異)により投与中，breakthroughを生じた．両剤を4週間併用投与することにより，耐性ウイルスの出現は予防され，より強い抗ウイルス効果を認め，マウス血中HCV RNAは投与1週後より陰性化した．観察した投与終了18週後も血中HCV RNAは陰性であり，肝臓内HCV RNAもPCRにて検出されず，おそらく完全排除されたものと思われた.

D. 考察

ソヤサポゲノール B 誘導体は，IFN による IFN 誘導遺伝子発現量の増加作用を促進させ，抗 HCV 効果を増強させるものと思われた．種々の HCV 蛋白を標的とした薬剤を組み合わせることにより，IFN 製剤を使用せずとも HCV の排除が可能となることが示唆された．

E. 結論

HC感染マウスを用いて種々の抗ウイルス剤の効果判定および新規治療法の開発が可能であった．

F.健康危機情報

特になし

G. 研究発表

1．論文発表

(1) Tsuge M, Hiraga N, Akiyama R, Tanaka S, Matsushita M, Mitsui F, Abe H, Kitamura S, Hatakeyama T, Kimura T, Miki D, Mori N, Imamura M, Takahashi S, Hayes CN, Chayama K. HBx protein is indispensable for development of viraemia in human hepatocyte chimeric mice. J Gen Virol. 2010;91:1854-64.

(2) Kamiya N, Iwao E, Hiraga N, Tsuge M, Imamura M, Takahashi S, Miyoshi S, Tateno C, Yoshizato K, Chayama K. Practical Evaluation of a Mouse with Chimeric Human Liver Model for Hepatitis C Virus Infection Using an NS3-4A Protease Inhibitor. J Gen Virol 2010;91:1668-7

(3) Mishima K, Sakamoto N, Sekine-Osajima Y, Nakagawa M, Itsui Y, Azuma S, Kakinuma S, Kiyohashi K, Kitazume A, Tsuchiya K, Imamura M, Hiraga N, Chayama K, Wakita T, Watanabe M. Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants. Virology 2010;405:361-9.

(4) Suda G, Sakamoto N, Itsui Y, Nakagawa M, Tasaka-Fujita M, Funaoka Y, Watanabe T, Nitta S, Kiyohashi K, Azuma S, Kakinuma S, Tsuchiya K, Imamura M, Hiraga N, Chayama K, Watanabe M. IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones. Virology 2010;407:80-90

(5) Ohara E, Hiraga N, Imamura M, Iwao E, Kamiya N, Yamada I, Kono T, Onishi M, Hirata D, Mitsui F, Kawaoka T, Tsuge M, Takahashi S, Abe H, Hayes CN, Ochi H, Tateno C, Yoshizato K, Tanaka S, Chayama K. Elimination of Hepatitis C Virus by Short Term NS3-4A and NS5B Inhibitor Combination Therapy in Human Hepatocyte Chimeric Mice. J Hepatol, in press

(6) Abe H, Imamura M, Hiraga N, Tsuge M, Mitsui F, Kawaoka T, Takahashi S, Ochi H, Maekawa T, Hayes CN, Tateno C, Yoshizato K, Murakami S, Yamashita N, Matsuhira T, Asai K, Chayama K. ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both in vitro and in vivo. J Hepatol, in press

(7) Chayama K, Hayes CN, Hiraga N, Abe H, Tsuge M, Imamura M. Animal model for study of human hepatitis viruses. J Gastroenterol Hepatol.26:13-18,2011.

2．学会発表

・今村道雄，平賀信彦，茶山一彰．HCV 感染マウスを用いた NS3/4A protease 阻害剤および NS5B polymerase 阻害剤併用療法の有用性の検討．第 14 回日本肝臓学会大会　横浜　平成 22 年 10 月 14 日．

・Imamura M, Hiraga N, Tsuge M, Takahashi S, Abe H, Hayes CN, Ochi H and Chayama K. The influences of viral mutations on HCV infectivity, replication ability and the susceptibility to interferon in human hepatocyte chimeric mice. The 7th APASL Single Topic Conference, Chiba, December 17-18, 2010

H. 知的財産権の出願・登録状況

特になし

Ⅲ. 研究成果の刊行に関する一覧表

別紙５：研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Abe H, Imamura M, Hiraga N, Tsuge M, Mitsui F, Kawaoka T, Takahashi S, Ochi H, Maekawa T, Hayes CN, Tateno C, Yoshizato K, Murakami S, Yamashita N, Matsuhira T, Asai K, Chayama K.	. ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both in vitro and in vivo.	J Hepatol			In press
Ohara E, Hiraga N, Imamura M, Iwao E, Kamiya N, Yamada I, Kono T, Onishi M, Hirata D, Mitsui F, Kawaoka T, Tsuge M, Takahashi S, Abe H, Hayes CN, Ochi H, Tateno C, Yoshizato K, Tanaka S, Chayama K.	Elimination of Hepatitis C Virus by Short Term NS3-4A and NS5B Inhibitor Combination Therapy in Human Hepatocyte Chimeric Mice.	J Hepatol			In press
Chayama K, Hayes CN, Hiraga N, Abe H, Tsuge M, Imamura M.	Animal model for study of human hepatitis viruses.	J Gastroenterol Hepatol	26(1)	13-18	2011
Suda G, Sakamoto N, Itsui Y, Nakagawa M, Tasaka-Fujita M, Funaoka Y, Watanabe T, Nitta S, Kiyohashi K, Azuma S, Kakinuma S, Tsuchiya K, Imamura M, Hiraga N, Chayama K, Watanabe M.	IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones.	Virology	407(1)	80-90	2010
Mishima K, Sakamoto N, Sekine-Osajima Y, Nakagawa M, Itsui Y, Azuma S, Kakinuma S, Kiyohashi K, Kitazume A, Tsuchiya K, Imamura M, Hiraga N, Chayama K, Wakita T, Watanabe M.	Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants.	Virology	405(2)	361-9	2010
Kamiya N, Iwao E, Hiraga N, Tsuge M, Imamura M, Takahashi S, Miyoshi S, Tateno C, Yoshizato K, Chayama K.	Practical Evaluation of a Mouse with Chimeric Human Liver Model for Hepatitis C Virus Infection Using an NS3-4A Protease Inhibitor.	J Gen Virol	91(Pt7)	1668-77	2010
Tsuge M, Hiraga N, Akiyama R, Tanaka S, Matsushita M, Mitsui F, Abe H, Kitamura S, Hatakeyama T, Kimura	HBx protein is indispensable for development of viraemia in human hepatocyte chimeric mice.	J Gen Viro	;91(Pt 7)	1854-64	2010

T, Miki D, Mori N, Imamura M, Takahashi S, Hayes CN, Chayama K.					
Amano H, Hino H, Tateno C, Emoto K, Imaoka Y, Yamasaki C, Itamoto T, Tashiro H, Asahara T, Ohdan H, Yoshizato K.	Therapeutic Potential of Propagated Hepatocyte Transplantation in Liver Failure.	J Surg Res.			In press
Tateno C, Kataoka M, Utoh R, Tachibana A, Itamoto T, Asahara T, Miya F, Tsunoda T, Yoshizato K.	Growth Hormone-Dependent Pathogenesis of Human Hepatic Steatosis in a Novel Mouse Model Bearing a Human Hepatocyte-Repopulated Liver.	Endocrinology			In press
Nishi H, Inagi R, Kawada N, Yoshizato K, Mimura I, Fujita T, Nangaku M.	Cytoglobin, a novel member of the globin family, protects kidney fibroblasts against oxidative stress under ischemic conditions.	Am J Pathol	Jan;178 (1)	128-39	2011
Yamasaki C, Kataoka M, Kato Y, Kakuni M, Usuda S, Ohzone Y, Matsuda S, Adachi Y, Ninomiya S, Itamoto T, Asahara T, Yoshizato K, Tateno C.	In vitro evaluation of cytochrome p450 and glucuronidation activities in hepatocytes isolated from liver-humanized mice.	Drug Metab Pharmacokinet.	Jan 14;25(6)	539-50	2011
Utoh R, Tateno C, Kataoka M, Tachibana A, Masumoto N, Yamasaki C, Shimada T, Itamoto T, Asahara T, Yoshizato K.	Hepatic hyperplasia associated with discordant xenogeneic parenchymal-nonparenchymal interactions in human hepatocyte-repopulated mice.	Am J Pathol.	177 (2)	654-665	2010
Ogawa T, Fujii H, Yoshizato K, Kawada N.	A human-type nonalcoholic steatohepatitis model with advanced fibrosis in rabbits.	Am J Pathol.	177 (1)	153-165.	2010
Kamiya N, Iwao E, Hiraga N, Tsuge M, Imamura M, Takahashi S, Miyoshi S, Tateno C, Yoshizato K, Chayama K.	Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3-4A protease inhibitor.	J Gen Virol.	91(Pt 7)	1668-1677	2010
Ogawa T, Iizuka M, Sekiya Y, Yoshizato K, Ikeda K, Kawada N	Suppression of type I collagen production by microRNA-29b in cultured human stellate cells.	Biochem Biophys Res Commun	391(1)	316-21	2010
Kubo S, Kataoka M, Tateno C, Yoshizato K, Kawasaki Y, Kimura T, Faure-Kumarl E, Palmer DJ, Ng P, Okamura H, Kasahara N	In Vivo Stable Transduction of Humanized Liver Tissue in Chimeric Mice via High-capacity Adenovirus-Lentivirus Hybrid Vector.	Hum Gene Ther.	21	40-50	2010
Sunagozaka H, Honda M, Yamashita T, Nishino R, Takatori H, Arai K, Yamashita T, Sakai Y, Kaneko S.	Identification of a secretory protein c19orf10 activated in hepatocellular carcinoma.	Int J Cancer.	-	-	(in press)

Misu H, Takamura T, Takayama H, Hayashi H, Matsuzawa-Nagata N, Kurita S, Ishikura K, Ando H, Takeshita Y, Ota T, Sakurai M, Yamashita T, Mizukoshi E, Yamashita T, Honda M, Miyamoto K, Kubota T, Kubota N, Kadowaki T, Kim HJ, Lee IK, Minokoshi Y, Saito Y, Takahashi K, Yamada Y, Takakura N, Kaneko S.	A liver-derived secretory protein, selenoprotein P, causes insulin resistance.	Cell Metab.	12(5)	483-95	2010
Honda M, Nakamura M, Tateno M, Sakai A, Shimakami T, Shirasaki T, Yamashita T, Arai K, Yamashita T, Sakai Y, Kaneko S.	Differential interferon signaling in liver lobule and portal area cells under treatment for chronic hepatitis C.	J Hepatol	53(5)	817-26	2010
Honda M, Sakai Y, Yamashita T, Yamashita T, Sakai A, Mizukoshi E, Nakamoto Y, Tatsumi I, Miyazaki Y, Tanno H, Kaneko S.	Differential gene expression profiling in blood from patients with digestive system cancers.	Biochem Biophys Res Commun.	400(1)	7-15	2010
Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, Yamashita T, Nakamura M, Shirasaki T, Horimoto K, Tanaka Y, Tokunaga K, Mizokami M, Kaneko S.	Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C.	Gastroenterology	139(2)	499-509	2010
Shirasaki T, Honda M, Mizuno H, Shimakami T, Okada H, Sakai Y, Murakami S, Wakita T, Kaneko S.	La protein required for internal ribosome entry site-directed translation is a potential therapeutic target for hepatitis C virus replication.	J Infect Dis.	202(1)	75-85	2010
Yamashita T, Honda M, Nio K, Nakamoto Y, Yamashita T, Takamura H, Tani T, Zen Y, Kaneko S.	Oncostatin m renders epithelial cell adhesion molecule-positive liver cancer stem cells sensitive to 5-Fluorouracil by inducing hepatocytic differentiation.	Cancer Res.	70(11)	4687-97	2010
Kushima Y, Wakita T, Hijikata M.	A disulfide-bonded dimer of the core protein of hepatitis C virus is important for virus-like particle production.	J. Virol.	84 (18)	9118-9127	2010
Zang L, Nishikawa M, Machida K, Ando M, Takahashi Y, Watanabe Y, Takakura Y.	Inhibition of nuclear delivery of plasmid DNA and transcription by interferon γ: hurdles to be overcome for sustained gene therapy.	Gene Ther	In press		2011

Miyakawa N, Nishikawa M, Takahashi Y, Ando M, Misaka M, Watanabe Y, Takakura Y	Prolonged circulation half-life of interferon γ activity by gene delivery of interferon γ-serum albumin fusion protein in mice	J Pharm Sci	In press		2011
Takiguchi N, Takahashi Y, Nishikawa M, Matsui Y, Fukuhara Y, Oushiki D, Kiyose K, Hanaoka K, Nagano T, Takakura Y.	Positive correlation between the generation of ractive oxygen species and activation/reactivation of transgene expression after hydrodynamic injection into mice	Pharm Res	In press		2011
Takahashi Y, Vikman E, Nishikawa M, Ando M, Watanabe Y, Takakura Y.	Persistent interferon transgene expression by RNA interference-mediated silencing of interferon receptors	J Gene Med	12(9)	739-746	2010
Hattori K, Nishikawa M, Watcharanurak K, Ikoma A, Kabashima K, Toyota H, Takahashi Y, Takahashi R, Watanabe Y, Takakura Y.	Sustained exogenous expression of therapeutic levels of IFN-gamma ameliorates atopic dermatitis in NC/Nga mice via Th1 polarization	J Immunol	184(5)	2729-2735	2010
Yamaoka A,Guan X, Takemoto S, Nishikawa M, Takakura Y.	Development of a novel Hsp70-based DNA vaccine as a multifunctional antigen delivery system	J Control Release	142(3)	411-415	2010
Kadokura M, Maekawa S, Ryota Sueki R, Miura M, Komase K, Shindo H, Amemiya F, Uetake T, Inoue T, Sakamoto M, Nakagawa M, Sakamoto N, Watanabe M, Enomoto N.	Analysis of the response to pegylated-interferon plus ribavirin therapy in chronicHCV-1b infection using comprehensive information of viral and host factors.	Hepatol Int.	In press		2011
Enomoto N, Maekawa S.	HCV genetic elements determining the early response to peginterferon and ribavirin therapy.	Intervirology.	53(1)	66-9	2010
Itakura J, Kurosaki M, Itakura Y, Maekawa S, Asahina Y, Izumi N, Enomoto N.	Reproducibility and usability of chronic virus infection model using agent-based simulation; comparing with a mathematical model	Biosystems.	99(1)	70-8	2010
Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, Sugiyama M, Matsuura K, Sugauchi F, Asahina Y, Nakagawa M, Watanabe M, Sakamoto M, Maekawa S, Sakai A, Kaneko S, Ito K, Masaki N, Tokunaga K, Izumi N, Mizokami M.	Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors.	J Hepatol.	Sep 19		2010

Moriishi K, Shoji I, Mori Y, Suzuki R, Suzuki T, Kataoka C, and Matsuura Y.	Involvement of PA28g in the propagation of hepatitis C virus.	Hepatology	52	411- 420	2010
Tani H, Shiokawa M, Kaname Y, Kambara H, Mori Y, Abe T, Moriishi K, and Matsuura Y.	Involvement of ceramide in the propagation of Japanese encephalitis virus.	J.Virol.	84	2798-2807	2010
Masaki T, Suzuki R, Saeed M, Mori KI, Matsuda M, Aizaki H, Ishii K, Maki N, Miyamura T, Matsuura Y, Wakita T, and Suzuki T.	Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription.	J.Virol.	84	5824- 5835	2010
Fukuhara T, Taketomi A, Motomura T, Okano S, Ninomiya A, Abe T, Uchiyama H, Soejima Y, Shirabe K, Matsuura Y, and Maehara Y.	Variants in IL28B in liver recipients and donors correlate with response to peginterferon and ribavirin therapy for recurrent hepatitis C.	Gastroenterology	39	1577- 1585	2010
Ito M, Masumi A, Mochida K, Kukihara H, Moriishi K, Matsuura Y, Yamaguchi K, and Mizuochi T.	Peripheral B Cells May Serve as a Reservoir for Persistent Hepatitis C Virus Infection.	J. Innate Immun.	2	2539- 2553	2010
Tripathi LP, Kataoka C, Taguwa S, Moriishi K, Mori Y, Matsuura Y, and Mizuguchi K.	Network based analysis of hepatitis C virus Core and NS4B protein interactions.	Mol. Biosyst.	6	1577- 1585	2010
Kawaoka T, Hiraga N, Takahashi S, Takaki S, Mitsui F, Tsuge M, Nagaoki Y, Kimura Y, Hashimoto Y, Katamura Y, Hiramatsu A, Waki K, Imamura M, Kawakami Y, Aikata H, HTashiro H, Ohdan H, Chayama K.	Prolongation of interferon therapy for recurrent hepatitis C after living donor liver transplantation: analysis of predictive factors of sustained virological response, including aminoacid sequence of the core and NS5A regions of hepatitis C virus.	Scand J Gastroenterol	45(12)	1488-1496	2010
Ushitora Y, Tashiro H, Takahashi S, Amano H, Oshita A, Kobayashi T, Chayama K, Ohdan H.	Splenectomy in Chronic Hepatic Disorders: Portal Vein Thrombosis and Improvement of Liver Function.	Dig Surg	28(1)	9-14	2011
Doskali M, Tanaka Y, Ohira M, Ishiyama K, Tashiro H, Chayama K, Ohdan H.	Possibility of Adoptive Immunotherapy With Peripheral Blood-derived CD3-CD56+ and CD3+CD56+ Cells for Inducing Antihepatocellular Carcinoma and Antihepatitis C Virus Activity	J Immunother	34(2)	129-138	2011

Ⅳ. 研究成果の刊行物・別刷

Research Article

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ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both *in vitro* and *in vivo*

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Background & Aims: ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B, attenuates liver disease in several animal models of acute and chronic liver injury. ME3738 is thought to inhibit replication of hepatitis C virus (HCV) by enhancing interferon (IFN)-β production, as determined using the HCV full-length binary expression system. We examined the effect of ME3738 combined with IFN-α on HCV replication using the genotype 1b subgenomic replicon system and an *in vivo* mouse HCV model.

Methods: HCV replicon cells (ORN/3-5B/KE cells and Con1 cells) were incubated with ME3738 and/or IFN-α, and then intracellular IFN-stimulated genes (ISGs) and HCV RNA replication were analyzed by reverse-transcription-real time polymerase chain reaction and luciferase reporter assay. HCV-infected human hepatocyte chimeric mice were also treated with ME3738 and/or IFN-α for 4 weeks. Mouse serum HCV RNA titer, HCV core antigen, and ISGs expression in the liver were measured.

Results: ME3738 induced gene expression of oligoadenylate synthetase 1 and inhibited HCV replication in both HCV replicon cells. The drug enhanced the effect of IFN to significantly increase ISG expression levels, inhibit HCV replication in replicon cells, and reduce mouse serum HCV RNA and core antigen levels in mouse livers. The combination treatment was not hepatotoxic as evident histologically and did not reduce human serum albumin in mice.

Conclusions: ME3738 inhibited HCV replication, enhancing the effect of IFN-α to increase ISG expression both *in vitro* and *in vivo*, suggesting that the combination of ME3738 and IFN might be useful therapeutically for patients with chronic hepatitis C.

Keywords: Human hepatocyte chimeric mouse; Interferon-stimulated genes.
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Abbreviations: HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL, interleukin; ISG, interferon stimulated gene; MxA, myxovirus resistance protein A; OAS, oligoadenylate synthetase; PKR, double stranded RNA-dependent protein kinase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator; USP18, ubiquitin specific peptidase 18.

Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. To date, the most effective therapy for viral clearance is a 48- or 72-week combination therapy of pegylated interferon (IFN)-α and ribavirin. However, successful eradication of the virus is achieved in only about 50% of treated patients [4–6]. Moreover, therapy induces significant adverse effects, such as fever, fatigue, and anemia [4], resulting in poor tolerability. More effective and less toxic treatment is, therefore, desired.

ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B [7], attenuates liver disease in several animal models of acute and chronic liver injury induced by concanavalin A, ethanol, lithocholate, and bile duct ligation [8–12]. ME3738 induces interleukin (IL)-6 expression, and serum amyloid A and α1-acid glycoprotein act as downstream targets of the IL-6 signal to protect against concanavalin A-induced liver injury [8–10]. The drug also prevents the progression of hepatic fibrosis in rats with bile duct ligation through suppression of activation and collagen synthesis of hepatic stellate cells [12].

Recently, Hiasa et al. reported that ME3738 inhibited HCV replication by enhancing IFN-β production using the HCV full-length binary expression system that uses full-length genotype 1a HCV complementary DNA plasmid with a T7 promoter sequence and an adenoviral vector expressing T7 polymerase [13]. However, it is not clear if the production of IFN-β and subsequent expression of IFN-stimulated genes (ISGs) was induced by the transcribed HCV genomes through detection by innate

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immune system receptors, including RIG-I. In addition, it is also not clear whether ME3738 has anti-viral effects on genotype 1b HCV, which is the most common and most IFN-resistant genotype in Japan [14].

Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice engrafted with human hepatocytes [15,16]. We and other groups had reported that this mouse model is useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease *in vivo* [17–19].

In the present study, we investigated the effects of ME3738 on HCV replication both *in vitro* and *in vivo* using the genotype 1b HCV replicon and HCV-infected human hepatocyte chimeric mice. The results demonstrate that ME3738 itself had an inhibitory effect on HCV replication, and when combined with IFN, ME3738 enhanced the anti-HCV effect of IFN by up-regulation of ISGs, such as oligoadenylate synthetase (*OAS*) *1*, myxovirus resistance protein A (*MxA*), and *ISG15* in HCV replicon cells. We also showed that the combination therapy increased *OAS1*, RNA-dependent protein kinase (*PKR*) and ubiquitin specific peptidase 18 (*USP18*) expression levels, and reduced virus levels effectively without liver cell damage in human hepatocyte chimeric mice.

Material and methods

Cell culture

Cells supporting replication of the genotype 1b-derived subgenomic HCV replicon, ORN/3-5B/KE cells [20] (kindly provided by N. Kato, Okayama University, Japan) and Con-1 cells [21], were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, non-essential amino acids, glutamine, penicillin, and streptomycin (complete DMEM) in the presence of G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA). ORN/3-5B/KE and Con1 replicon cells (2×10^4) were seeded onto 12-well plates and incubated for 3 days with or without ME3738 (Meiji Seika Kaisha, Tokyo, Japan) [9], human IFN-α (Dainippon Sumitomo Pharma Co., Tokyo), or the combination of both drugs.

Quantitation of HCV RNA and ISG mRNAs

RNA extraction and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [19]. Briefly, RNA was extracted from mice serum, livers, or cellular lysate using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV RNA was performed using the Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers used for amplification were 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCAC CCTATCA-3′. Quantitation of ISGs (*OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*) was performed using real-time PCR Master Mix (TOYOBO) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each ISG expression level was expressed relative to the endogenous RNA levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Luciferase reporter assay

After 72 h of IFN and/or ME3738 treatment, ORN/3-5B/KE cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to the luciferase assay according to the manufacturer's protocol.

Western blotting

The cells were ruptured with 250 μl lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15,000*g*. Cell lysates were subjected to Western blotting using antibodies against NS3 (Novocastra Laboratories, UK) and β-actin (Sigma, Tokyo, Japana) as described previously [22].

WST assay

Cell viability was determined by employing tetrazolium salt, WST-8, using the WST-8 Cell Proliferation Assay Kit (Dojindo Laboratories., Kumamoto, Japan), according to the instructions provided by the manufacturer.

Human serum samples

Human serum samples containing high titers of genotype 1b HCV (2.2×10^6 copies/ml) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots were stored in liquid nitrogen until use.

Animal treatment

All animal protocols in this study were in accordance with the guidelines of the local committee for animal experiments and under approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. We transplanted human hepatocytes into uPA$^{+/+}$/SCID$^{+/+}$ mice as described previously [16]. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were fed a normal chow containing 0.15% (w/w) ME3738 for 4 weeks, with or without IFN-α. IFN-α-treatment was provided daily by intramuscular injection of diluted IFN solution. Serum samples were collected every week, and human serum albumin (HSA) concentration and HCV RNA were measured. Mouse serum concentrations of HSA, which correlate with the repopulation rates, were measured as described previously [16]. Serum ME3738 concentrations were measured by liquid chromatography/mass spectrometry/mass spectrometry. After the fourth week of treatment, mice were sacrificed, and livers were either fixed with 4% buffered-paraformaldehyde for histological examination or frozen immediately in liquid nitrogen to measure HCV core antigen. To investigate the expression of ISGs in mouse livers, mice were kept for 1 week with or without 0.45% (w/w) ME3738 and then given a single injection of 1500 IU/g IFN-α. Four hours after injection, mice were sacrificed and liver samples were collected.

Quantitation of HCV core antigen in the mouse liver

Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000*g* for 30 min. HCV core antigen levels in the supernatant of liver homogenates were measured using enzyme immunoassay as described previously [23].

Statistical analysis

All data are expressed as mean ± SD. Levels of HCV RNA and ISG mRNAs were compared using the Mann–Whitney *U*-test. A p value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 14.0 software (SPSS, Tokyo, Japan).

Results

Antiviral activity of ME3738 on HCV subgenomic replicon

The effect of ME3738 on HCV replication was analyzed *in vitro* using subgenomic HCV replicon cells possessing the luciferase reporter. ORN/3-5B/KE cells were treated with either IFN-α or ME3738 for 72 h. The luciferase reporter assay demonstrated that the HCV RNA replication level decreased depending on the

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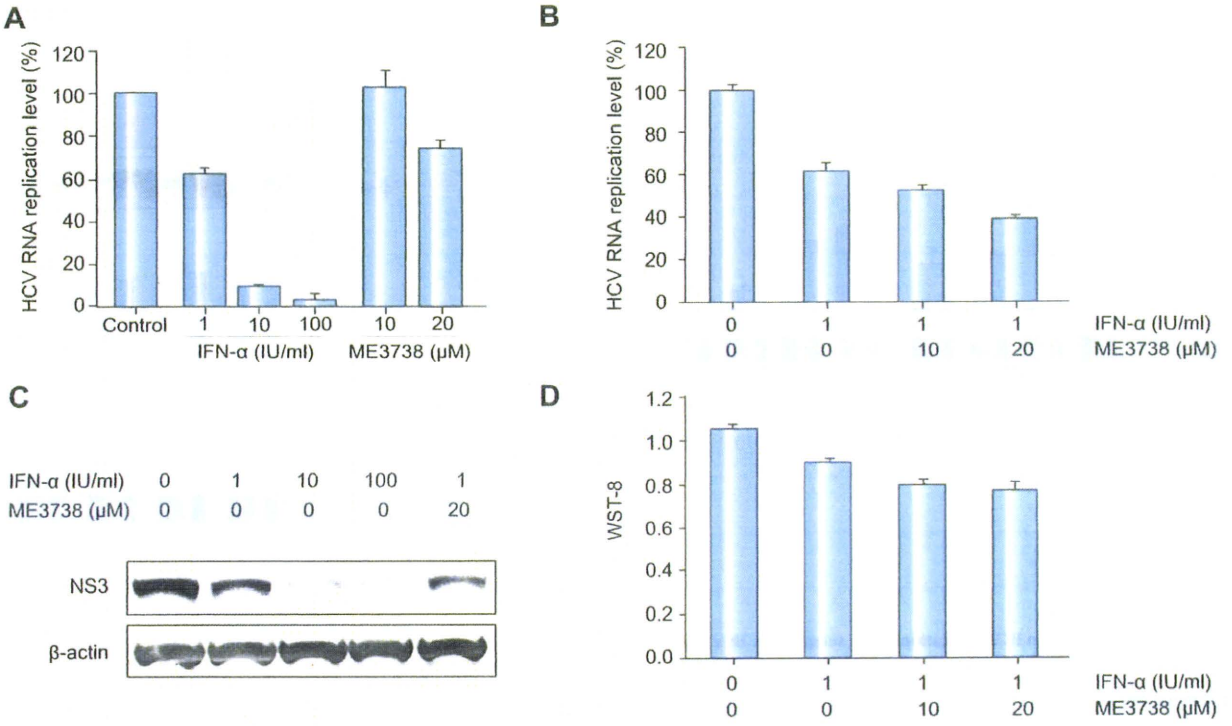

Fig. 1. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, ORN/3-5B/KE cells. ORN/3-5B/KE cells were treated for 72 h with the indicated concentration of interferon (IFN)-α alone, ME3738 alone, or IFN-α plus ME3738. (A and B) Intracellular HCV RNA replication levels were determined as luciferase activity and expressed relative to cellular viability. (C) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (D) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

IFN-treatment dose as reported previously (Fig. 1A) [20]. Treatment with 20 μM of ME3738 also reduced HCV RNA replication. Next, we investigated whether ME3738 enhances the effect of IFN-α. IFN-α (1 IU/ml) plus ME3738 inhibited the HCV RNA replication dose in a dependent manner with ME3738 (Fig. 1B). The level of cellular HCV NS3 protein was reduced depending on IFN-α-treatment and was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 1C). The viability of cells treated with IFN-α/ME3738 combination treatment was lower than that of the control treatment and almost the same as with IFN-α treatment alone (Fig. 1D).

The effect of ME3738 was also tested in a different replicon system, Con-1 cells. ME3738 reduced HCV RNA replication dose dependently in Con-1 cells (Fig. 2A). Similar to ORN/3-5B/KE cells, IFN-α (1 IU/ml) plus ME3738 inhibited HCV RNA replication dose in a dependent manner with ME3738 (Fig. 2A), and the level of cellular HCV NS3 protein was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 2B). The viability of cells treated with IFN-α/ME3738 combination treatment was lower but was not significant with IFN-α treatment alone (Fig. 2C). These results indicate that ME3738 itself has an inhibitory effect on HCV replication and enhances the effect of IFN-α.

Expression of ISGs in ME3738-treated replicon cells

We measured the levels of ISGs in drug-treated ORN/3-5B/KE cells and Con1 cells. IFN-α treatment significantly increased the expression levels of *OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*, which reached maximum levels at 24 h in ORN/3-5B/KE cells (Fig. 3A) and 8 h in Con1 cells (Fig. 3B). ME3738 treatment alone significantly increased the expression of *OAS1* in both cells. IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OA1S*, *MxA* and *ISG15* to levels higher than IFN-α alone in both cells. These results indicate that ME3738 enhances the effect of IFN-α to increase ISG expression, and this effect may contribute to the inhibition of HCV replication.

Effect of ME3738 on HCV replication in vivo

To further analyze the effects of ME3738, we used genotype 1b HCV-infected human hepatocyte chimeric mice [17,19]. Six weeks after HCV infection, when the mice developed stable viremia (10^6–10^7 copies/ml, data not shown), the animals were treated with ME3738 alone, IFN-α alone, or ME3738/IFN-α for 4 weeks (Fig. 4A). Mouse serum concentrations of ME3738 increased in ME3738- and ME3738 plus IFN-α-treated mice (Table 1). ME3738 alone did not reduce the levels of HCV RNA in mice, while IFN-α-treatment reduced the HCV RNA levels, as reported previously [17]. ME3738 plus IFN-α-treatment significantly reduced HCV to levels lower than that achieved by ME3738 or IFN-α alone. We also measured the HCV core protein level in the livers of treated mice. As shown by replicon experiments, core protein levels were reduced most effectively by the ME3738/IFN-α-combination therapy (Fig. 4B). Since the level of HSA did not decrease in these treatments, it was concluded that

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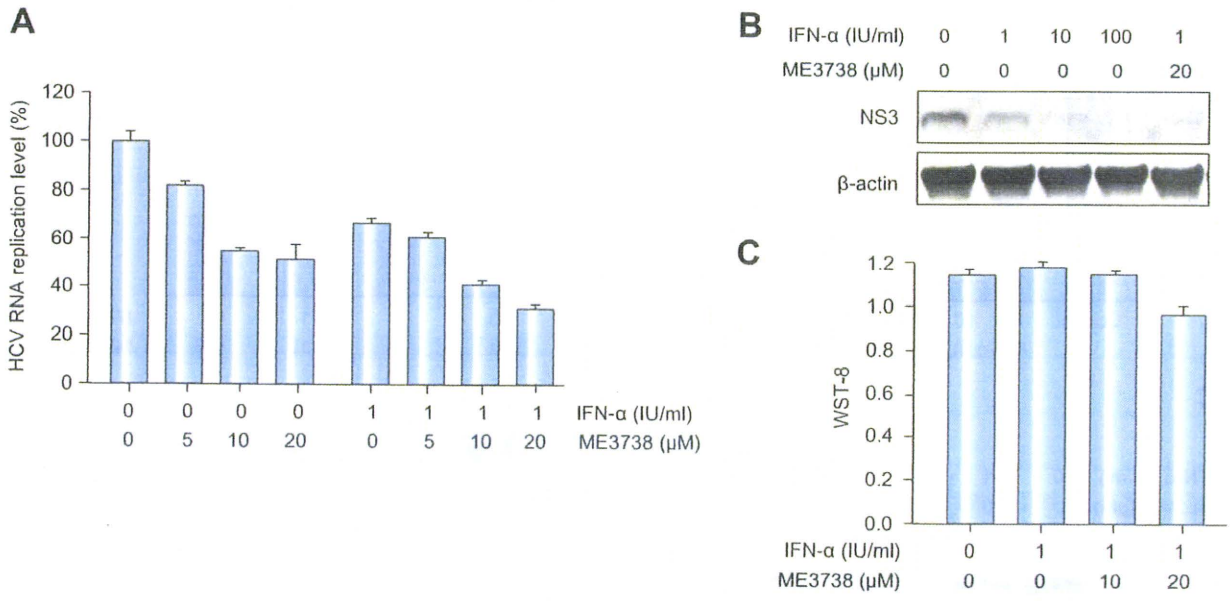

Fig. 2. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, Con1 cells. Con1 cells were treated for 72 h with the indicated concentration of ME3738 alone or IFN-α plus ME3738. (A) Intracellular HCV RNA replication levels were determined via real-time PCR. (B) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (C) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

the reduction of HCV in chimeric mice was not due to toxicity of the drugs (Fig. 4A). This was also supported by histopathological findings, including lack of cytotoxic changes in the livers of all four groups of mice (Fig. 4C). The effect of ME3738 to increase ISG expression was assessed in mouse liver following treatment with a high concentration of ME3738 for 1 week and a single injection of IFN-α. ME3738 alone showed no increase in the expression of ISGs in mouse livers (Fig. 5). IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OAS1*, *PKR* and *USP18* mRNA levels in mouse livers to levels higher than IFN-α alone. These results indicate that ME3738 inhibits HCV replication, enhancing the effect of IFN-α to increase ISG expression *in vivo*.

Discussion

Although the treatment outcome of chronic HCV infection has improved with the advent of pegylated IFN-α and ribavirin, the eradication rate of HCV is only about 50%. Many patients are unable to receive this therapy because of the harmful side effects or the financial costs. Development of effective, safe and inexpensive therapies should be encouraged.

ME3738 is reported to attenuate various liver pathologies in animals [8–12]. Furthermore, Hiasa et al. reported recently that ME3738 induces IFN-β mRNA expression and inhibits the replication of HCV [13]. We thus attempted in this study to evaluate the effect of ME3738, especially in combination with IFN-α, on HCV.

The results of the present study show that ME3738 induced the gene expression of *OAS* (Fig. 2) and inhibited HCV replication (Fig. 1A). Hiasa et al. reported that ME3738 enhanced the expression of *IFN-β* mRNA and that the enhanced production of IFN-β resulted in the increased expression of ISGs [13]. They showed also that the effect of ME3738 on HCV was abolished following the inhibition of IFN-β expression with siRNA or antibody. Our results are consistent with their findings. The extent of the increase in ISG expression was smaller in Hiasa et al. [13] than in our results. This is probably because they used the T7-genotype 1a-cDNA transient transfection-infection system to produce HCV in HepG2 or Huh7 cells [13,24,25] and assessed the effect of ME3738 by utilizing naturally produced IFN-β. The amount of IFN is likely to be very small in their system compared to that used in our study. We also tried to detect *IFN-β* mRNA in our replicon system but were unable to detect it in our replicon cells (Huh7 based ORN/3-5B/KE cells and Con1 cells). This is probably due to a defect of the innate immune system in producing IFN-β in those cells. This is consistent with their finding that ME3738 had an inferior effect in Huh7 cells than in HepG2 cells to produce ISG products [13].

As we showed in this study, ME3738 enhances the effect of IFN against HCV replication both *in vitro* (Figs. 1B and 2A) and *in vivo* (Figs. 4A and 4B). ME3738 enhanced the effect of IFN-α by increasing the expression levels of ISGs both *in vitro* (Fig. 3) and *in vivo* (Fig. 5). How ME3738 enhances the transcription of ISGs is unknown at this stage. ME3738 was reported initially to protect liver cells against injury through induction of IL-6 [8,9]. IL-6 is reported to provide protection to certain cells [26–28] by preventing apoptosis. In the present study, we tried to detect IL-6 protein in the serum and mRNA in the liver of ME3738-treated mice. However, the levels of both were too low to measure. Further studies should be conducted to elucidate the mechanism by which ME3738 enhances immunity against viral infections.

Our results showed that ME3738 did not reduce cell viability. We also showed that the drug is not hepatotoxic, as inferred by HSA level and liver histology. Since ME3738 is reported to

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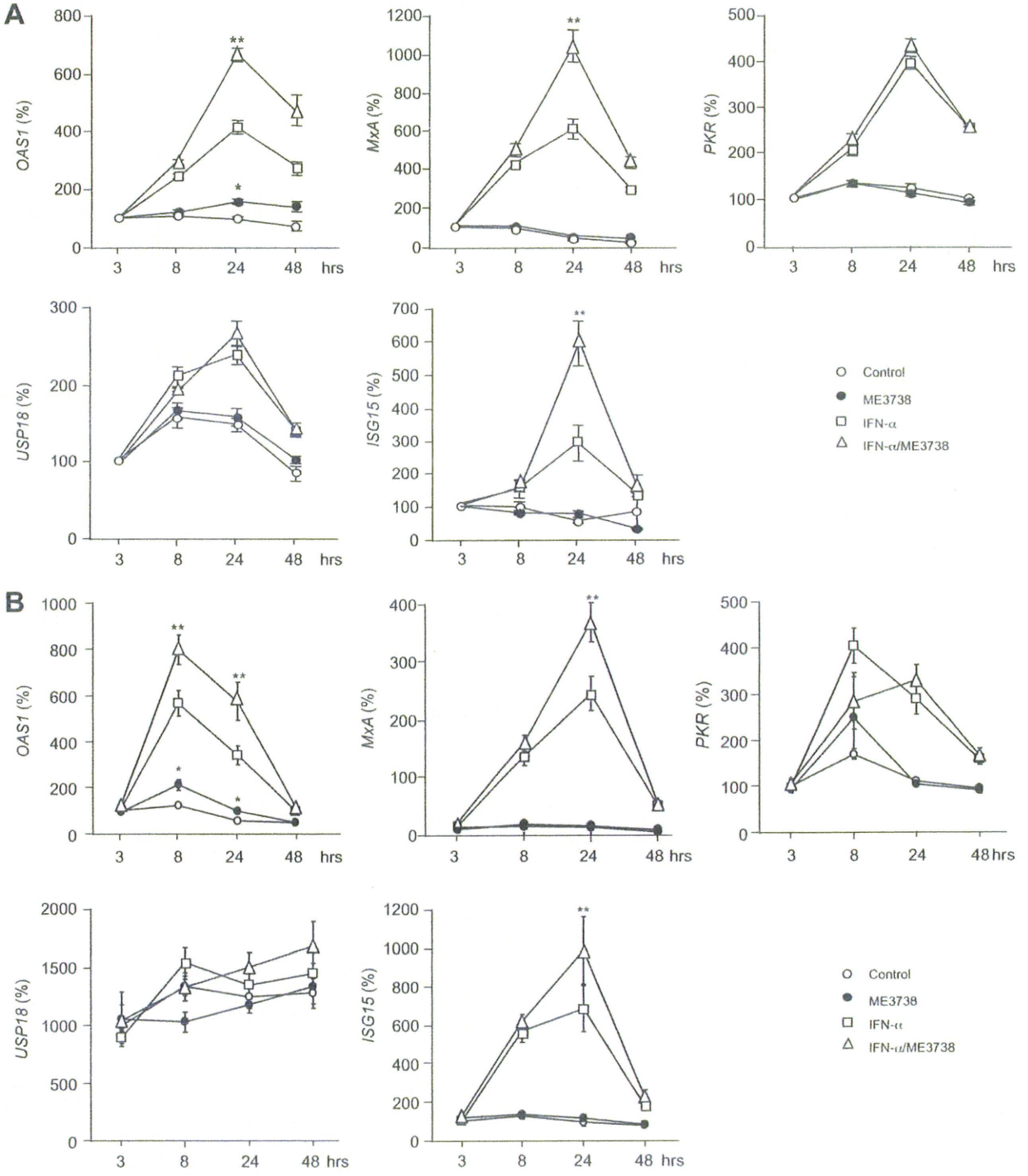

Fig. 3. Effects of ME3738 on the expression of interferon-stimulated genes. ORN/C-5B/KE cells (A) and Con1 cells (B) were treated with 20 μM of ME3738 and/or 1 IU/ml of interferon (IFN)-α for 48 h. Intracellular gene expression levels of oligoadenylate synthetase (*OAS*), myxovirus resistance protein A (*MxA*), double stranded RNA-activated protein kinase (*PKR*), *USP-18* and interferon-stimulated gene (*ISG*) *15* were measured at the indicated times. RNA levels were expressed relative to *GAPDH* mRNA. Data are shown as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α. ($^{*}p$ <0.05 compared with Control, $^{**}p$ <0.05 compared with IFN-α treatment).

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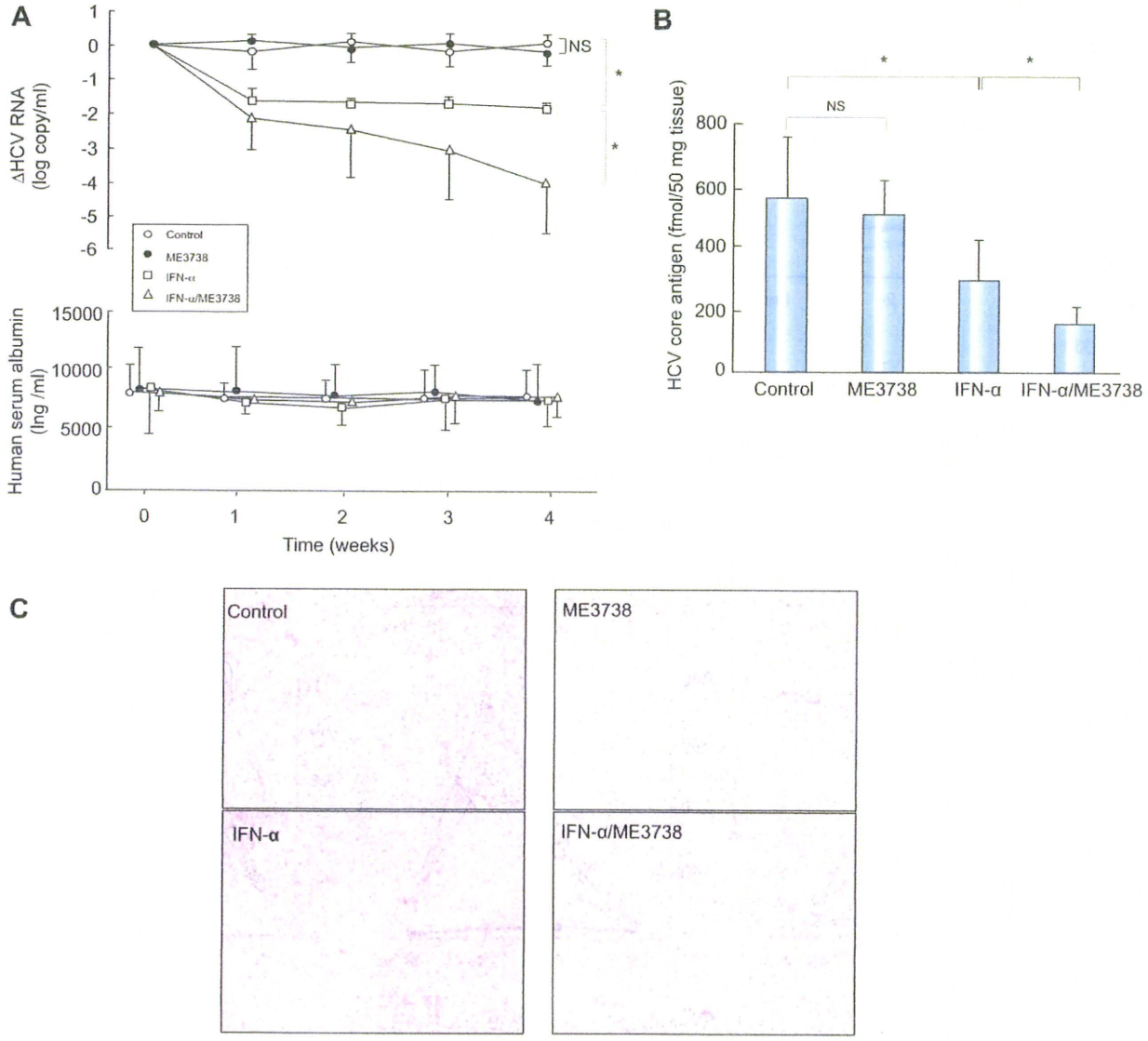

Fig. 4. ME3738 enhances the effect of IFN in mice with HCV infection. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were treated with ME3738 and/or interferon (IFN)-α for 4 weeks. (A) Mouse serum samples were obtained every week, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. HCV core antigen was measured in the mouse livers after 4 weeks of treatment (B). Data are mean ± SD of 6 mice. (*p <0.05; **p <0.01; NS, not significant). (C) Liver samples obtained from mice were stained with hematoxylin-eosin (Original magnification, 100×). Note the lack of specific changes in the mice of each group. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

Table 1. Concentrations of ME3738 in mouse serum samples.

	Control	ME3738	IFN-α	ME3738/ IFN-α
ME3738 (μM)	<0.01	4.02 ± 0.90	<0.01	2.44 ± 0.21

Concentrations of ME3738 in serum samples obtained from mice after 4 weeks of treatment were measured by liquid chromatography/mass spectrometry/mass spectrometry. Data are shown as mean ± SD of three mice. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

attenuate liver disease in several animal models of acute and chronic liver injury [8–12], the drug could be suitable for treatment of patients with chronic hepatitis C. In the current regimen of PEG-IFN and ribavirin combination therapy, IFN reduces the replication rate of the virus by inducing expression of ISGs in liver cells. Ribavirin enhances the effect of IFN synergistically through an unknown mechanism. ME3738 also enhances the effect of IFN similarly to ribavirin and may protect liver cells from apoptosis. Combination therapy using these three drugs might yield excellent anti-viral and anti-inflammatory effects. Alternatively, ME3738 could be used instead of ribavirin if the drug shows a superior effect in combination with IFN. Further animal and human studies should be conducted to develop an effective regimen for the treatment of patients with chronic hepatitis C.

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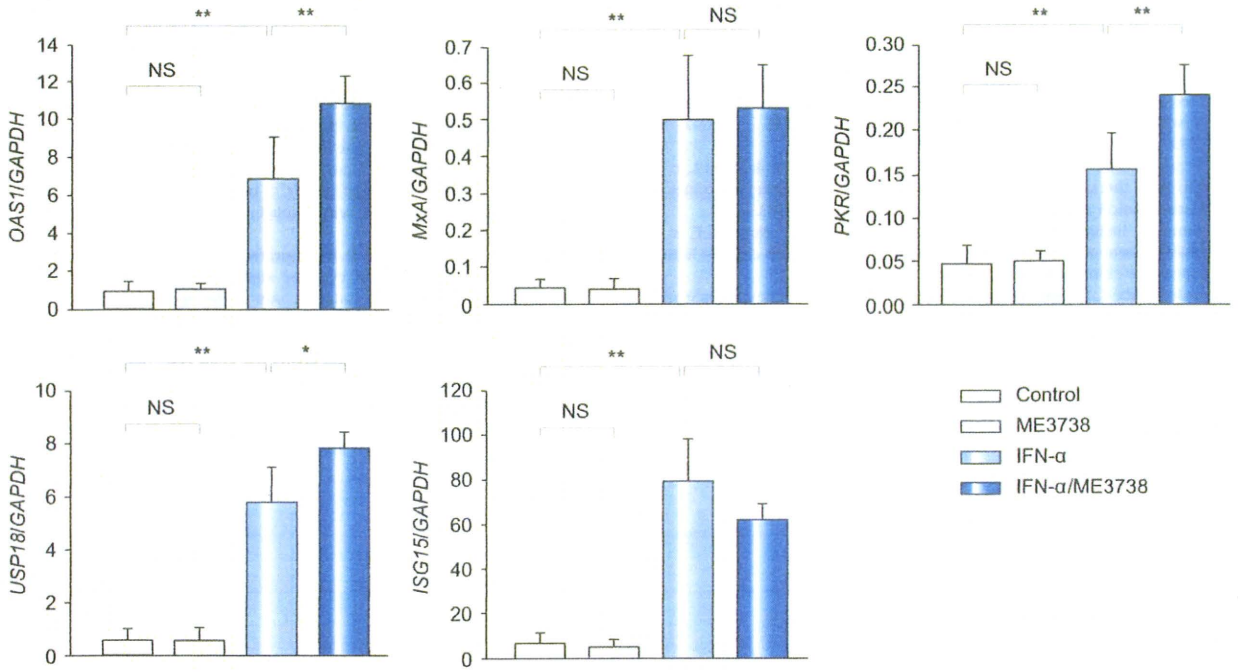

Fig. 5. Interferon-stimulated genes expression in mouse liver samples. Mice were treated with or without 0.45% (w/w) ME3738 for 1 week and then given a single injection of 1500 IU/g IFN-α. Four hours after IFN-α injection, interferon stimulated gene expression in mouse livers was measured. RNA levels are expressed relative to *GAPDH* mRNA. Data are presented as mean ± SD of six mice. Control: Mice treated with neither ME3738 nor IFN-α. (*p <0.05; **p <0.01; NS, not significant).

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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References

[1] WHO: Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J Viral Hepat 1999;6:35–47.

[2] Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12:671–675.

[3] Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 1998;28:1687–1695.

[4] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965.

[5] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.

[6] Hadziyannis SJ, Sette Jr H, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 2004;140:346–355.

[7] Sasaki K, Minowa N, Kuzuhara H, Nishiyama S. Preventive effects of soyasapogenol B derivatives on liver injury in a concanavalin A-induced hepatitis model. Bioorg Med Chem 2005;13:4900–4911.

[8] Klein C, Wüstefeld T, Heinrich PC, Streetz KL, Manns MP, Trautwein C. ME3738 protects from concanavalin A-induced liver failure via an IL-6-dependent mechanism. Eur J Immunol 2003;33:2251–2261.

[9] Kuzuhara H, Nakano Y, Yamashita N, Imai M, Kawamura Y, Kurosawa T, et al. Protective effects of alpha1-acid glycoprotein and serum amyloid A on concanavalin A-induced liver failure via interleukin-6 induction by ME3738. Eur J Pharmacol 2006;541:205–210.

[10] Fukumura A, Tsutsumi M, Tsuchishima M, Hayashi N, Fukura M, Yano H, et al. Effect of the inducer of interleukin-6 (ME3738) on rat liver treated with ethanol. Alcohol Clin Exp Res 2007;31:S49–S53.

[11] Nomoto M, Miyata M, Shimada M, Yoshinari K, Gonzalez FJ, Shibasaki S, et al. ME3738 protects against lithocholic acid-induced hepatotoxicity, which is associated with enhancement of biliary bile acid and cholesterol output. Eur J Pharmacol 2007;574:192–200.

[12] Maeda K, Koda M, Matono T, Sugihara T, Yamamoto S, Ueki M, et al. Preventive effects of ME3738 on hepatic fibrosis induced by bile duct ligation in rats. Hepatol Res 2008;38:727–735.

[13] Hiasa Y, Kuzuhara H, Tokumoto Y, Konishi I, Yamashita N, Matsuura B, et al. Hepatitis C virus replication is inhibited by 22beta-methoxyolean-12-ene-3beta, 24(4beta)-diol (ME3738) through enhancing interferon-beta. Hepatology 2008;48:59–69.

[14] Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, et al. Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. Hepatology 1994;19:1088–1094.

[15] Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927–933.

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[16] Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901–912.

[17] Hiraga N, Imamura M, Tsuge M, Noguchi C, Takahashi S, Iwao E, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. FEBS Lett 2007;581: 1983–1987.

[18] Kneteman NM, Weiner AJ, O'Connell J, Collett M, Gao T, Aukerman L, et al. Anti-HCV therapies in chimeric acid-Alb/uPA mice parallel outcomes in human clinical application. Hepatology 2006;43:1346–1353.

[19] Kimura T, Imamura M, Hiraga N, Hatakeyama T, Miki D, Noguchi C, et al. Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. J Gen Virol 2008;89:2108–2113.

[20] Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 2005;329:1350–1359.

[21] Lindenbach BD, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309: 623–626.

[22] Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, Takahashi S, et al. Dual effect of APOBEC3G on Hepatitis B virus. J Gen Virol 2007;88:432–440.

[23] Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, et al. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 1999;37:1802–1808.

[24] Bouvier-Alias M, Patel K, Dahari H, Beaucourt S, Larderie P, Blatt L, et al. Clinical utility of total HCV core antigen quantification: a new indirect marker of HCV replication. Hepatology 2002;36:211–218.

[25] Lanford RE, Guerra B, Lee H, Averett DR, Pfeiffer B, Chavez D, et al. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly(I)-poly(C), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. J Virol 2003;77: 1092–1104.

[26] Kolliputi N, Waxman AB. IL-6 cytoprotection in hyperoxic acute lung injury occurs via suppressor of cytokine signaling-1 induced apoptosis signal-regulating kinase-1 degradation. Am J Respir Cell Mol Biol 2009;40: 314–324.

[27] Xu G, Zhang Y, Zhang L, Ren G, Shi Y. The role of IL-6 in inhibition of lymphocyte apoptosis by mesenchymal stem cells. Biochem Biophys Res Commun 2007;361:745–750.

[28] Rollwagen FM, Madhavan S, Singh A, Li YY, Wolcott K, Maheshwari R. IL-6 protects enterocytes from hypoxia-induced apoptosis by induction of bcl-2 mRNA and reduction of fas mRNA. Biochem Biophys Res Commun 2006;347: 1094–1098.

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Research Article

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Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice

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Background & Aims: The current treatment regimen for chronic hepatitis C virus (HCV) infection is peg-interferon plus ribavirin combination therapy. The majority of developing therapeutic strategies also contain peg-interferon with or without ribavirin. However, interferon is expensive and sometimes intolerable for some patients because of severe side effects.

Methods: Using human hepatocyte chimeric mice, we examined whether a short term combination therapy with the HCV NS3-4A protease inhibitor telaprevir and the RNA polymerase inhibitor MK-0608 with or without interferon eradicates the HCV from infected mice. The effect of telaprevir and MK-0608 combination therapy was examined using subgenomic HCV replicon cells.

Results: Combination therapy with the two drugs enhanced inhibition of HCV replication compared with either drug alone. In *in vivo* experiments, early emergence of drug resistance was seen in mice treated with either telaprevir or MK-0608 alone. However, emergence was prevented by the combination of these drugs. Mice treated with a triple combination therapy of telaprevir, MK-0608, and interferon became negative for HCV RNA soon after commencement of the therapy, and HCV RNA was not detected in serum of these mice 12 weeks after cessation of the therapy. Furthermore, all mice treated with a high dose telaprevir and MK-0608 combination therapy for 4 weeks became negative for HCV RNA 1 week after the beginning of the therapy and remained negative after 18 weeks.

Conclusions: Eradication of HCV from mice with only 4 weeks of therapy without interferon points the way to future combination therapies for chronic hepatitis C patients.

Introduction

Chronic hepatitis C virus (HCV) infection is a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma [1,2]. The current standard treatment for patients chronically infected with HCV is the combination of peg-interferon (PEG-IFN) and ribavirin (RBV) [3–5]. However, this treatment results in a sustained viral response (SVR), defined as negative for HCV RNA 24 weeks after cessation of the therapy, in only about 50% of patients with genotype 1 HCV infection with high viral load [3–5]. In view of the lack of effectiveness of the current therapy, many molecules have been tested for development of novel anti-HCV therapies. Recently, a number of new selective inhibitors of HCV proteins, the so-called STAT-C (specifically targeted antiviral therapy for HCV) inhibitors, have been in development. The HCV NS3-4A protease inhibitor and the NS5B polymerase inhibitor, as well as an inhibitor of NS5A function, have been demonstrated to have potent anti-HCV effects and have proceeded to clinical trials [6].

Although the anti-viral effect of these drugs is quite potent, monotherapy using these drugs results in early emergence of drug-resistant strains [7,8]. Accordingly, these drugs are used in combination with PEG-IFN and RBV. However, because IFN-treatment is expensive and is frequently associated with serious adverse events, such as cytopenias, rash/itching, alopecia, and mental disorders [3–5,9], a new treatment strategy, especially one that does not use IFN, is needed for chronic hepatitis C patients.

Keywords: NS3-4A protease inhibitor; NS5B RNA polymerase inhibitor; Human hepatocyte chimeric mouse; Interferon.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; RBV, ribavirin; SVR, sustained virological response; STAT-C, specifically targeted antiviral therapy for HCV; uPA, urokinase-type plasminogen activator; SCID, severe combined immunodeficiency; RT-PCR, reverse transcript-polymerase chain reaction; HSA, human serum albumin.

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The immunodeficient urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes that can be infected with HCV [10]. We and other groups reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs such as IFN-alpha and the NS3-4A protease inhibitor [11–14]. In this study, we used the NS3-4A protease inhibitor telaprevir (VX950; MP424; Mitsubishi Tanabe Pharma Co., Osaka, Japan) [15] and the NS5B RNA polymerase inhibitor MK0608 (2′-*C*-methyl-7-deaza-adenosine) [16] and investigated the effect of a short term combination treatment with these drugs on HCV replication both *in vitro* and in *vivo*, and showed a successful elimination of viruses in HCV-infected chimeric mice without the use of IFN. Although the dose of the drugs used in this study might be intolerable in humans, elimination of the virus without IFN by only 4 weeks of therapy sheds light on approaches to developing combination therapies using multiple STAT-C agents without IFN.

Materials and methods

Cell culture

An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally, pHC-Vlbneo-delS [17]). The pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycine phosphotransferase, as described elsewhere [18,19]. Replicon RNA was synthesized *in vitro* by T7-RNA polymerase (Promega, Madison, WI) and transfected into Huh7 cells by electroporation. Huh7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37 °C under 5% CO_2. After culturing in the presence of G418 (Wako, Osaka, Japan), cell lines stably expressing the replicons were established (Huh7/Rep-Neo).

Luciferase assay

Replicon cell lines were treated with various concentrations of either telaprevir or MK-0608 for 72 hrs, and HCV RNA replication level was quantified by internal luciferase assay. Luciferase activities were quantified using a luminometer (Lumat LB9501; Promega) and the Bright-Glo Luciferase Assay System (Promega). The 50% inhibitory concentrations (IC_{50}) were defined as the drug concentrations producing a 50% reduction in the levels of luciferase activities relative to average levels in untreated cultures.

MTT assays

Cell viability was measured under the same experimental settings using a tetrazolium (MTT)-based viability assay (BioAssay, California, USA) according to the manufacturer's directions. The 50% cytotoxic concentrations (CC_{50}) were defined as the drug concentrations producing a 50% reduction in absorbance relative to the average level in untreated cultures.

Animal treatment

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [20]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA), correlated with the repopulation index [20], were measured as previously described [21]. Eight weeks after hepatocyte transplantation, mice were intravenously injected with 100 μl of HCV-positive human serum samples. Mice serum samples were obtained every one or 2 weeks after HCV infection, and HSA and HCV RNA levels were measured.

Treatment with anti-HCV drugs in HCV-infected mice

Telaprevir and MK-0608 were dissolved with a specific solvent. Eight weeks after HCV infection when the mice developed stable viremia (10^6 to 10^9 copies/ml), mice were administered either 200 mg/kg of telaprevir or 3–50 mg/kg of MK-0608 orally twice a day for 4 weeks. The specific solvent had no anti-HCV effect in this mouse model (data not shown). To analyze the effect of the combination treatment with telaprevir and MK-0608, these drugs were mixed and given together as a cocktail. Human IFN-alpha-treatment was provided daily by intramuscular injection of diluted IFN solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for 4 weeks.

Human serum sample

Human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis who had provided written informed consent to participate in the study. Serum samples were divided into small aliquots and stored in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

RNA extraction and amplification

RNA extraction, nested PCR and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [12,13]. Briefly, RNA was extracted from serum samples and extracted livers using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV cDNA was performed using Light Cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limit of real-time PCR is 10^3 copies/ml.

Sequence analysis

The nucleotide and amino acid sequences of the NS3 and NS5B region of HCV were determined by direct sequencing following PCR amplification of cDNA after reverse transcription of HCV RNA. The primers used to amplify the NS3 region were 5′-GTGCTCCAAGCTGGCATAAC-3′ and 5′-AGGACCGAGGAATCGAACAT-3′ as the first (outer) primer pair and 5′-CTAGAGTGCCGTACTTCGTG-3′ and 5′-ACTGATCCTGGAGGCGTAGC-3′ as the second (inner) primer pair. The primers used to amplify the NS5B region were 5′-TAAGCGAGGAGGCTGGTGAG-3′ and 5′-CCTATTGGCCTGGAGTGTTT-3′ as the first (outer) primer pair and 5′-GACTCAACGGTCACTGAGAG-3′ and 5′-CCTATTGGCCTGGAGTGTTT-3′ as the second (inner) primer pair. PCR was performed in a 25 μl solution, consisting of a reaction buffer (12.5 μl, 2× PCR buffer for FOD FX), 5 μl 2 mM dNTPs, 0.75 μl F primer (10 μM), 0.75 μl R primer (10 μM), 1 μl Temp DNA (10 pg–200 ng), 0.5 μl KOD FX, 4.5 μl D.W. RT-PCR reactions were carried out following the manufacturer's instructions (Biometra T-Personal; Montreal Biotech Inc., Kirkland, QC, Canada). Amplification conditions included an initial denaturation at 94 °C for 2 min, 35 cycles of amplification (denaturation at 94 °C for 2 min, annealing of primer at 56 °C (1st PCR) or 59 °C (2nd PCR) for 30 s; extension at 68 °C for 2 min 30 s (NS3, 1st PCR), 1 min 30 s (NS3, 2nd PCR), 2 min (NS5B, 1st PCR), or 1 min 10 s (NS5B, 2nd PCR)); and final extension at 68 °C for 5 min.

Results

Anti-viral activity of telaprevir and MK-0608 on HCV subgenomic replicon cells

The effect of telaprevir and MK-0608 on HCV replication was analyzed *in vitro* using HCV replicon cells. Huh7/Rep-Feo cells were treated with various concentrations of either telaprevir or MK-0608. Measured luciferase activity demonstrated that both drugs inhibited HCV replication in a dose-dependent manner (Fig. 1). The IC_{50} of telaprevir and MK-0608 was 0.53 and 0.51 μM, respectively, consistent with previous reports [7,16]. When

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