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G.知的所有権の取得状況

1．特許取得

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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：これまでわれわれは，患者血清用いたHCV感染マウスを用いて，肝炎ウイルスの分子生物学的検討および抗ウイルス薬の効果判定抗ウイルス薬の効果判定を行ってきた．本研究において，HCV 全長クローンを用いて，リバースジェネティクス系を構築し，genotype 1a，1b，2a，2b型の感染マウスの構築に成功した．また 1b 型クローンに薬剤耐性変異を挿入することにより，変異ウイルスの薬剤耐性能および複製能を検証する系の作製，さらにはCoreやISDR領域にアミン酸変異を挿入することにより，変異ウイルスのIFN感受性や複製能を解析する系の作製にも成功した．またHCV感染マウスを用いて，新規治療法の開発を行った．主なものとして，1）ソヤサポゲノールB 誘導体がIFN-αのIFN誘導遺伝子発現作用を促進し，抗HCV効果を増強させることを見いだした．2）HIVのentry阻害剤である Phosphorothioate oligonucleotide (PS-ON)を前投与することにより，HCV感染が抑制され，PS-ONは in vivo におてい感染阻害剤として有用であることを示した．3）プロテアーゼ阻害剤やポリメラーゼ阻害剤などの異なるHCV蛋白を標的とする薬剤を併用することにより，IFN製剤を使用せずともHCVの排除が可能であることを見いだした．

A. 研究目的

ヒト肝細胞キメラマウスを用いて，患者血清の投与あるいはHCVクローンを用いたリバースエネティクスによる感染モデルを構築し，HCV の分子生物学的検討，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，あるいは新規治療法の開発に有効利用する．

B. 研究方法

1b型変異型HCVクローンを用いた検討

1）クローニングした1b型HCV全長(KT9)にはコア領域のaa 70および91にR70QおよびL91Mのアミノ酸変異を認めた．一方，NS5AのISDR領域には変異を認めなかった．KT9クローン(Core-Mutant)あるいはコア領域のaa 70およびaa 91のアミノ酸変異を野生型のもどしたクローン（Core-Wild）を作製した．全長cDNAを挿入したplasmidより，in vitro transcritption法によりRNAを合成し，30 μgのRNAをヒト肝細胞キメラマウスの肝臓内に直接注入し，感染成立率，血中HCV RNA量，さらに1000 IU/gのIFN-αを2週間連日投与し，HCV RNAの減少量を比較検討した．

2）KT9クローンのNS3領域にプロテアーゼ阻害剤耐性であるA156S変異を挿入したクローン(KT9-NS3-A156S)も同様に作成し，マウス肝臓内に投与した．

抗ウイルス剤の効果判定

1）HCV陽性ヒト血清投与前日，投与日，投与1，3，5日後にhuman immunodeficiency virus (HIV)のentry阻害剤であるPhosphorothioate oligonucleotide (PS-ON)を10 mg/kg，腹腔内投与した．ヒト血清投与2週間後にマウス血中HCV RNAを測定し，感染成立の有無を確認した．

2）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4週間，単独あるいは併用投与し，マウス肝臓内ISGs発現量および血中HCV RNA低下量を測定した．

3）HCV感染マウスに28日間，Protease inhibitorであるtelaprevir (200 mg/kg，1日2回，連日経口投与)あるいはRNA polymerase inhibitor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

1b型変異型HCVクローンを用いた検討

1) Core-Mutant および Core-Wild の投与による感染成立(real-time PCR にて血中HCV RNA が定量可能)率は 93%(26 頭/28 頭) vs 94%(16 頭/17 頭)であり同程度であった. 感染が成立したマウスでの血中 HCV RNA も同程度であった. Core-Mutant 感染マウスおよびCore-Wild 感染マウスでの IFN-α の 2 週間投与による血中 HCV RNA 低下量は, -1.9 log vs -1.8 log (ヒト肝細胞 A 移植マウス)および -0.8 log VS -0.9 log (ヒト肝細胞 B 移植マウス)であり, 同程度の IFN 感受性を示した.

2) KT9-NS3-A156S変異クローンを用いた感染マウスは, KT9クローンを投与したマウスに比べ, 血中HCV RNAは明らかに低値であり, protease inhibitor耐性クローンは複製効率が低下していることが示唆された. これらのマウスに400-600 mg/kg/日のtelaprevirを2-4週間連日経口投与したところ, KT9クローン感染マウスでは血中HCV RNAは著明に低下したが, NS3-A156S変異感染マウスでのHCV RNA低下量は軽度であり, A156S変異は明らかにtelaprevir耐性を示した. KT9クローン感染マウスでは, telaprevir投与中, V36A変異の出現により, telaprevirの効果が減弱するマウスが存在した.

抗ウイルス剤の効果判定

1) Control群では, マウスのHCV感染を7頭中7頭(100%)に認めたの対し, PS-ON投与群では7頭1頭(14%)のみであり(p=0.001), PS-ONがin vivoにおいて, HCVの感染阻害に有用であることが示された.

2) 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)をした群では, 投与していない群に比べ, 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.

3) 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領域のV36A変異あるいはNS5B領域のS282T変異)の出現により投与中, breakthroughを生じた. 両剤を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. 4週間の投与中, ウイルス量の再上昇を認めず, 投与終了後も陰性化が維持された. 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. 結論

1) HCV Coreの変異は感染の成立, ウイルスの複製, IFN感受性には影響しておらず, 臨床的に認められる治療効果の差は免疫を介する何らかの要因があると思われた. 2) 変異クローンを用いた, 変異ウイルスの複製能および薬剤耐性能を検討するシステムを構築した. 3) HCV感染マウスを用いて新規治療法の開発が可能であった. 今後, 開発した治療法の臨床応用が期待される.

E. 考察

種々の変異を挿入した変異クローンを用いて, 変異HCV感染マウスの作成が可能であり, これらのシステムを用いて, 変異ウイルスの生物学的特徴および抗ウイルス剤の治療効果の検討が可能である.

F.健康危機情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況

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Ⅱ．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

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Ⅲ. 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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