病理学

乏突起膠細胞の免疫組織化学的マーカー

Immunohistochemical markers of oligodendrocytes

複雑な突起網を形成する神経系の研究において，細胞の識別に役立つ特異抗体は欠かすことができない．乏突起膠細胞は髄鞘形成を特徴とする中枢神経系グリア細胞の一種で，髄鞘には特異的に発現する分子が豊富に存在することから，同細胞を認識する特異抗体の開発も容易に達成できるに違いない，と多くの研究者は当初楽観的に考えていたであろう．しかし，そうした分子を標的とした抗体は培養環境においては使用可能であっても，組織切片ではほとんど役に立たなかった．なぜならば同細胞の細胞体と髄鞘の連続性は電顕を駆使しても証明がかなり困難であり，そのような事情が横たわっていては，髄鞘を認識する抗体でいくら脳組織を免疫染色してみても細胞の同定に役立つはずもない．そのため，乏突起膠細胞の免疫組織化学的マーカーの開発は21世紀にもち越された宿題となった．

一方，乏突起膠腫は乏突起膠細胞あるいはその前駆細胞からの発生が想定される脳腫瘍で，成人の大脳白質に好発し，類円形核とperinuclear halo，網目状の血管，石灰化などを特徴とする．典型的な構築を形成する症例の場合，病理診断は比較的容易であるが，個々の腫瘍細胞は特異的な指標に乏しく，しかも同腫瘍は髄鞘を形成せず，髄鞘関連分子の発現もはっきりとしない．ただし，このような形態を示す脳腫瘍が化学療法によく反応することは事実であり，適切な病理診断に役立つマーカーがこれまでずっと渇望されつづけていた．

その問題解決の糸口は乏突起膠細胞の発生学的研究から見出された．同細胞の発生分化を制御する転写因子Olig1，Olig2が齧歯類やニワトリにおいてあいついで同定された[1]．一般に転写因子は核内で作用する．よってOligも核に発現しているならば，その抗体は乏突起膠細胞のよい免疫組織化学的マーカーなることが期待され，著者を含む国内外の複数のグループがそれぞれ独自に抗体を開発し，この仮説が実証された[2]．乏突起膠腫においてもほぼ100%に発現することが明らかにされ，乏突起膠細胞由来であることを示唆する結果を得たが，星状膠腫においてもかなり陽性率が高いことがわかり，グリオーマの鑑別にはかならずしも役に立たないという課題は残された[2]．最近の研究でOlig2は，発生期においてニューロンとグリアの系譜を分ける重要な分子であることが指摘され[3]，このことがグリオーマ全般において幅広くOlig2が発現していることと関係があるかもしれない．

また，微小管関連蛋白であるTPPP/p25はαsynucleinが蓄積するParkinson病，びまん性Lewy小体病，多系統萎縮症などにおいて，αsynucleinとほぼオーバーラップして発現する[4]．一方で，正常脳では乏突起膠細胞の細胞体にTPPP/p25が選択的に発現しており，マーカー分子としての有用性が示唆されている[5]．

Olig2，TPPP/p25のいずれもが完全な特異抗体とはいえないが，ともにホルマリン固定パラフィン包埋組織において使用可能であり，特性を理解して使用すれば十分な実用性を発揮する．乏突起膠細胞の核ならびに細胞体を選択的に認識する抗体が出揃い，今後は乏突起膠細胞の病理学的理解がいっそう深まることが期待される．

1) 竹林浩秀，池中一裕：オリゴデンドロサイトと運動ニューロンの発生を制御するOlig2遺伝子．細胞工学，**22**：406-411，2003.
2) Yokoo, H. et al.：Anti-human Olig2 antibody as a useful immunohistochemical marker of normal oligodendrocytes and gliomas. *Am. J. Pathol.*, **164**：1717-1725, 2004.
3) Marshall, C. A. et al.：Olig2 directs astrocyte and oligodendrocyte formation in postnatal subventricular zone cells. *J. Neurosci.*, **25**：7289-7298, 2005.
4) Kovacs, G. G. et al.：Natively unfolded tubulin polymerization promoting protein TPPP/p25 is a common marker of α-synucleinopathies. *Neurobiol. Dis.*, **17**：155-162, 2004.
5) Nishie, M. et al.：Oligodendrocytes within astrocytes ("emperipolesis") in the cerebral white matter in hepatic and hypoglycemic encephalopathy. *Neuropathology*, **26**：62-65, 2006.

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薬理学・毒性学

キメラマウス

Chimeric mice with human hepatocytes

なぜ，キメラマウスか

科学は2つの方法論を用いて自然現象を理解しようとしている．ひとつは現象に関与している物質の同定と分離である．他のひとつはその物質が現象を成立させるために演じている役割(機能)の決定である．たとえば，10個の物質が成立させている現象があるとすると，われわれは"同定"と"機能"作業をそれぞれ独立して10回行う．しかし，10回の作業では真に理解した気持ちにならないであろう．それはこの10個の物質だけ

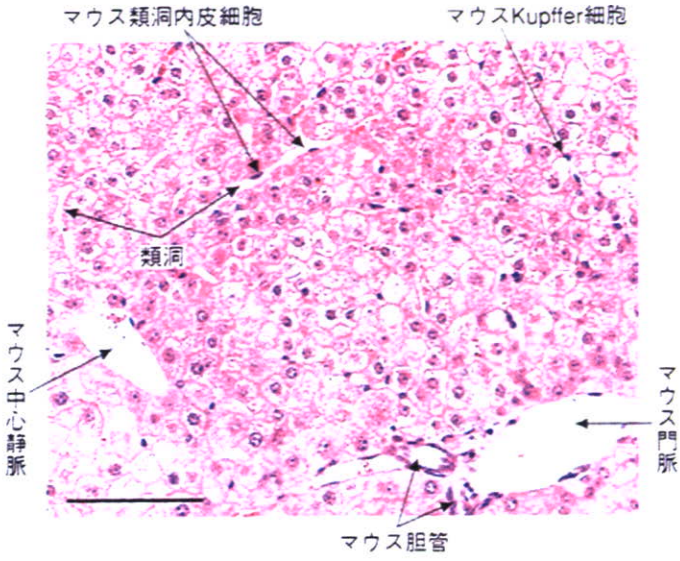

図 1　ヒト肝細胞キメラマウス肝組織

ヒト肝細胞を uPA/SCID マウスへ移植し，移植後 82 日目に肝を採取した．マウス肝切片のヘマトキシリン・エオジン染色像を示す．視野のすべての領域にはマウス肝細胞はみられず，ヒト肝細胞で置換されている．ヒト肝細胞の間には類洞とよばれる毛細血管が存在し，マウス類洞内皮細胞がみられる．その他の血管や胆管構造はマウス由来の細胞で構成されている．Bar＝100 μ.

を，そしてこれをすべて使って現象を再現できるか検証していないからである．再現するには，再現系の温度や各物質の濃度などの物理パラメータを確定しなければならない．再現に成功したとき，われわれはその現象を真に理解したと思える．このような考えで，著者は個々の物質の“同定”作業とその“機能”の解明作業を“分析”作業とよび，現象の最終的理解のために必須な作業を“再構成”作業とよんでいる．科学は分析と再構成を使い分けながら自然現象の理解を進めている．

著者らは肝の再生医療の観点から再生現象に関心をもっている．肝再生現象を理解するためには多くの事項を理解する必要があるが，基本的なことは“肝細胞が生体内で示す旺盛な増殖能力の原因の理解”である．これを理解することができれば，体外に取り出したヒト肝細胞を人工環境中で必要な数だけ増殖させて肝疾患患者に移植して救命することができるであろう．この原因解明の作業はいまだ道半ばである．

“肝細胞の旺盛な増殖能力の理解”を得るための科学的方法はまず，①肝細胞が活発に増殖することを可能にしている物質の分析である．つぎに，②これら物質だけを利用して実際に肝細胞を人工的環境下(再構成系)で活発に増殖させることである．たとえば，著者らはこれまでに同定された肝細胞増殖促進物質を添加した培養液中で，ヒト肝細胞を数代にわたって培養し，増殖させることに成功している[1]．しかし，体内での増殖能力の再現にはほど遠い．肝細胞増殖現象に関する“分析”の到達点がいまだ低いのである．それどころか，人体を利用してヒト肝細胞の増殖能力を研究することができないので，分裂能力を“分析”する作業自体があまり進展していない．

さらに，肝細胞増殖能力を判定する *in vitro* の再構成系を開発することにはかなり困難があったので，*in vivo* の再構成系の開発を行っている．人体に代わるものとして実験動物(マウス)にヒト肝細胞を移植し，ホストの肝細胞をヒト肝細胞で置換させ，ヒト肝細胞をもつ“キメラマウス”を作製できれば，ヒト肝細胞の増殖能力を自然により近いと考えられる体内で，しかも動物実験として分析できるとの期待からである．

ヒト肝細胞キメラマウス

凍結ヒト肝細胞を，肝障害をもつ免疫不全マウスに移植した[2]．肝障害マウスであるウロキナーゼ型プラスミノゲンアクチベータ(uPA)を肝に発現させたトランスジェニックマウス(uPA マウス)と，免疫傷害マウスである SCID(severe combined immunodeficiency)マウスを交配して得られる uPA SCID マウスをホストにした．7.5×10^5個のヒト肝細胞を脾に注入すると，一部の細胞は肝に運ばれ，そこに生着し増殖を開始し，ヒト肝細胞のコロニーを形成した．その後，増殖を続け置換率が 50%を超えるようになると，ドナーの補体因子がマウス血中に分泌され，ホストの肺などに傷害を与えるようになる．そこでヒト補体因子阻害剤を注射すると，ホストは健康になりドナー肝細胞はさらに増殖を続けるようになる．

現在著者らは，移植後約 60 日で 70%以上の置換率をもつヒト肝細胞キメラマウスを 50%の生成率で作製できるようなった．最高置換率 96%を記録している．この場合，マウスの肝重量が約 1.5 g で，肝 1 g 当りの肝細胞数は約 1.6×10^8個であるので，キメラマウス 1 匹当り約 2.3×10^8個のヒト肝細胞がいることになる．ヒト肝細胞の生着率はおよそ 15%であると考えられるので，ヒト肝細胞は 60 日間でおよそ 2,000 倍に増殖し，したがって，およそ 11 回分裂したことになる．キメラマウスの肝の切片

を組織学的に調べると，ヒト肝細胞がマウスの胆管や血管細胞などと協力して自然の肝を構築してマウスの生命活動を可能にしていることがわかる(図1)．

おわりに

このマウスに種々の薬物を投与する研究がはじまっており，これまでの研究結果は，このマウスはヒト型の代謝反応を行うことを示している．医薬品開発のツールとして創薬開発に貢献できると期待している[3]．将来的には分析して得られた物質だけを用いて，人工的にヒト肝細胞の活発な増殖能力を再現させたいと思っている．

1) Yamasaki, C. et al.：Growth and differentiation of colony-forming human hepatocytes *in vitro*. *J. Hepatol.*, **44**：749-757, 2006.
2) Tateno, C. et al.：Near-completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.*, **165**：901-912, 2004.
3) Tsuge, M. et al.：Infection of human hepatocyte chimeric mouse which genetically engineered hepatitis B virus. *Hepatology*, **42**：1046-1054, 2005.

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腎臓内科学

腎病変における遺伝子治療はどこまで具現化したか

Clinical application of gene therapy in kidney disease

1990年アデノシンデアミナーゼ欠損症に対する遺伝子治療が開始されて以来，2006年1月の時点で1,145の遺伝子治療プロトコールが提出され，そのうち，2/3が癌の遺伝子治療であり，単一遺伝子病，血管病，感染症が10%弱を占める(http://www.wiley.co.uk/genmed/clinical/)．腎病変に関する遺伝子治療は腎癌に限られているが，本稿で概説する基礎研究がその他の腎疾患に臨床応用される日も遠くないと思われる．

腎癌に対する遺伝子治療

わが国で行われている臨床研究はGM-CSF遺伝子治療のみである．手術で採取した腎癌細胞に*ex vivo*でレトロウイルスベクターを用いてGM-CSF遺伝子を導入し，その細胞を腎癌に接種するものである．この臨床研究でGM-CSF遺伝子治療の安全性は確認されたものの，臨床で奏功した症例は報告されていない[1]．海外ではB-7.1遺伝子改変腫瘍細胞ワクチンとinterleukin-2治療を組み合わせる遺伝子治療などが行われており，従来の免疫療法を超える効果が期待されている．

遺伝子病に対する遺伝子治療

本来，遺伝子治療の対象となる疾患はAlport症候群のような単一因子の遺伝性疾患が適しており，正常な遺伝子を導入し，染色体上にある欠陥のある遺伝子と組換えを行うことが理想ではあるが，現在までの遺伝子治療はすべて補充療法であり，原因遺伝子の治療ではない．Tryggvasonらはアデノウイルスを用いた遺伝子導入法により，Ⅳ型コラーゲンα5鎖の遺伝子をブタの糸球体に導入し，発現したα5鎖が糸球体基底膜でα3，4鎖とトリプルヘリックスを形成することを報告しているが，新しく形成された基底膜では蛋白尿改善効果はみられなかったという[2]．最近では先天性ネフローゼ症候群の原因遺伝子が解明されてきており，遺伝子導入法の改善に伴い，現在有効な治療法のないAlport症候群など先天性疾患に対して遺伝子治療が劇的な治療法となる可能性がある．

多発性嚢胞腎もまた，遺伝子治療による根本的な治療が期待されるが，たとえばPKD1遺伝子は46個のエクソンを含む50 kb以上の遺伝子であり，正常遺伝子を導入するためには遺伝子の巨大さがネックとなる．むしろ嚢胞形成や細胞増殖の抑制を目的とした遺伝子治療が有効かもしれない．

Fabry病はα-galactosidase A(α-GalA)遺伝子異常に起因する代謝異常症であるが，腎にスフィンゴ糖脂質が進行性に沈着し，腎障害をきたす．遺伝子組換えヒトα-GalAを用いた酵素補充療法が開始されているが，遺伝子治療もまたよい適応となる可能性がある．アメリカでは患者の間葉系幹細胞にα-GalA遺伝子をレトロウイルスベクターにて導入し，その細胞を移植する臨床研究が行われており，腎障害に対する効果も期待される．

腎炎に対する遺伝子治療

遺伝子治療の臨床試験に関する指針の改正により，遺伝子治療の対象疾患は従来の致死的疾患から身体の機能を著しく損なう疾患へと拡大した．このように遺伝子治療の環境も整備され，遺伝子治療はもはや特別な治療法ではなく，疾患治療におけるひとつの選択肢として考えられる時代になってきた．著者らは非常に強力な遺伝子ノックダウンの手段として注目されているshort interference RNA(siRNA)をエレクトロポレーション法によりThy-1腎炎モデルラットに導入し，腎炎に対する治療効果があるかを検討した．遺伝子導入を行った腎炎糸球体ではターゲットとしてTGF-β発現が抑制され，細胞外基質産生が抑制されること

が確認された[3]．現時点での腎炎に対する遺伝子治療の臨床研究は行われていないが，今後大動物などを用いて安全性や効果が確認され，これまでの基礎研究が臨床応用されることが期待される．

移植腎に対する遺伝子治療

現在，閉塞性動脈硬化症に対して hepatocyte growth factor(HGF)遺伝子治療が行われているが，著者らはブタの腎に HGF 遺伝子をエレクトロポレーション法により導入し，移植腎に対する HGF 遺伝子治療の安全性と有効性に関する検討を行った．エレクトロポレーション法を用いて HGF 発現プラスミドベクターを導入後，移植腎の保護効果を検討したところ，コントロール群に比較して HGF 遺伝子導入群では間質の線維化が抑制されていた．また，腎以外の臓器への導入は認められず，遺伝子導入が局所的に行えることを確認している[4]．

1) Tani, K. et al.：Phase I study of autologous tumor vaccines transduced with the GM-CSF gene in four patients with stage Ⅳ renal cell cancer in Japan：clinical and immunological findings. *Mol. Ther.*, **10**：799-816, 2004.
2) Heikkila, P. et al.：Adenovirus-mediated transfer of type Ⅳ collagen α5 chain cDNA into swine kidney *in vivo*：deposition of the protein into the glomerular basement membrane. *Gene Ther.*, **8**：882-890, 2001.
3) Takabatake, Y. et al.：Exploring RNA interference as a therapeutic strategy for renal disease. *Gene Ther.*, **12**：965-973, 2005.
4) Isaka, Y. et al.：Electroporation-mediated HGF gene transfection protected the kidney against graft injury. *Gene Ther.*, **12**：815-820, 2005.

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形成外科学

脂肪由来幹細胞を利用した脂肪組織再生

Adipose tissue regeneration by adipose-derived stem cells

外傷や先天異常に伴う広範囲軟部組織欠損や，乳癌術後の乳房欠損に対する治療方法としては従来，マイクロサージャリーなどの技術を駆使した自家軟部組織移植が応用されてきた．しかし，本法は手術時間が比較的長いうえ，ドナーの犠牲も少なからず問題となることがある．このような疾患に対するあらたな治療法として再生医学的アプローチが注目されている．とりわけ多分化能を有する幹細胞を用いた手法により，低侵襲かつ手技的にも容易に行える可能性が示唆されている．

以下に幹細胞のドナーとしての脂肪組織の有用性と，脂肪組織再生の研究の現状および臨床展望について記述する．

脂肪由来幹細胞とは

脂肪，骨，軟骨などの間葉系組織に分化する間葉系幹細胞のドナーとしては骨髄間質が有名であり，すでに臨床応用されており，その有用性も報告されている．しかし近年，骨髄以外の組織にも同様の細胞集団が存在することが指摘されており，脂肪組織はそのなかでも，①採取が容易である，②大量に採取可能である，③ドナーの犠牲がほとんどない，などの点で非常に有用である[1]（図 1）．この脂肪由来幹細胞（adipose derived stem cells：ASCs）は *in vitro* において，脂肪，骨，軟骨，骨格筋，心筋，神経，血管，肝細胞など非常に多くの成熟細胞に分化することが証明されている[2,3]．この ASCs により三次元構造を有する大型の脂肪組織を再生することで，上述した疾患の治療に結びつくものと考えられる．

In vivo による脂肪再生

著者らはマウスを用いた実験で，あらかじめ *in vitro* で脂肪への分化誘導をかけた ASCs をフィブリン糊に混入させ皮下に注入移植すると，移植した ASCs が脂肪組織の形成を起こすことを確認している．また，ASCs を吸引した成熟脂肪に混在させて移植することで，ASCs の血管再生効果を利用し移植後の組織吸収を抑制できるとの報告もあり，cell assisted lipotransfer(CAL)とよばれている．前者は，いったん採取した ASCs を凍結保存し必要に応じて培養増殖させることでいつでも利用可能である利点を有し，後者は，FDA が推奨す

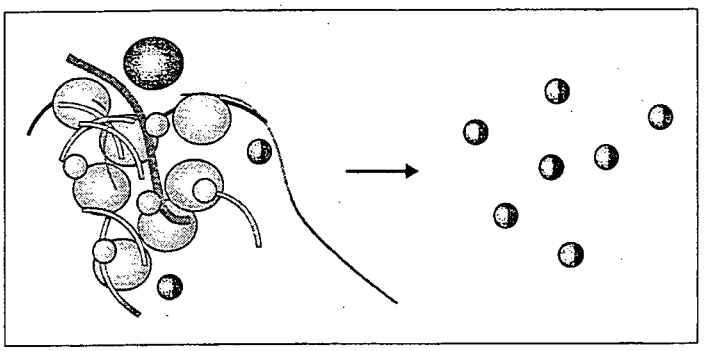

図 1　脂肪組織中の脂肪由来幹細胞
脂肪組織を酵素分解処理することで幹細胞を含んだ間質細胞を抽出できる．通常 500 ml の脂肪組織から約 10^8個の ASCs が抽出可能である．

ヒト肝細胞キメラマウス

Chimeric mice with human hepatocytes

立野知世[1)]・森川良雄[2)]・吉里勝利[1, 3)]

Key Words
ヒト肝細胞，キメラマウス，移植，ヒト化モデル動物

Abstract

私達はマウスの肝臓のほとんどをヒト肝細胞で置換させる技術を開発した。ヒト肝細胞は免疫不全でかつ肝障害を持つマウスの肝臓に生着し，増殖することができる。このヒトの肝細胞を持つキメラマウスはヒト肝臓における肝細胞の性質を保持していたことから，医薬候補品のヒトにおける薬物代謝，薬効，および毒性を予測するための新たなツールとして期待される。

■ はじめに

医薬品の開発には，マウス，ラット，イヌ，およびサルなどの多くの動物が使われているが，薬効や毒性には種差があることが知られており，動物実験結果がヒトの臨床試験結果を必ずしも反映していない。そこで，私達はマウスの肝臓のほとんどがヒト肝細胞で置換されたヒト肝細胞キメラマウス（以下，キメラマウス）を開発した。

■1. uPA/SCIDマウスの作製と性質

ウロキナーゼプラスミノーゲンアクチベータトランスジェニック（uPA）マウスとSCIDマウスを掛け合わせてuPA/SCIDマウスを作製した[1)]。uPA遺伝子にはアルブミンエンハンサープロモーターが接続されてあるため，肝細胞においてのみuPAが高発現し血液中に分泌される。uPA/SCIDマウスの肝細胞は，uPAの発現により萎縮しており増殖することができないが，肝臓におけるHGFの活性が高いことが知られている。また，UPA/SCIDマウスは重度免疫不全の性質を持つため，異種であるヒト肝細胞はこのマウスの肝臓に生着することができる。現在，このuPA/SCIDマウスにSCIDマウスを繰り返し戻し交配することにより，このマウスの背景遺伝子を均一化することに努めている。

■2. ヒト肝細胞キメラマウスの作製

生後8日目のマウスの尾からDNAを抽出し，遺伝子検査によりuPAとSCIDの遺伝子をホモ接合型で持つマウスを選択した。生後3週目のホモ接合型uPA/SCIDマウスの脇腹を約5 mm切開し脾臓を引き出し，27Gの注射針を用いて約1 x 10^6個のヒト肝細胞を移植した（図A）[1)]。通常，移植には米国から輸入した子供の凍結保存肝細胞を融解して用いている。ドナーによってマウス肝臓への生着や増殖のしやすさが異なるため，キメラマウス作製に適したドナー細胞を選択する必要がある。また，大人の肝細胞より子供の肝細胞の方が高置換率のキメラマウスを高頻度に得ることができることがわかってきた。通常ヒト肝細胞の凍結保存チューブから約1 x 10^7個の生存肝細胞を得ることができるため，凍結チューブ1本から約10匹のキメラマウスを作製することができる。

■3. ヒト肝細胞キメラマウスの性質

子供のドナー肝細胞をuPA/SCIDマウスに移植すると，マウス血中のヒトアルブミン濃度は対数的に増加し，移植後60日頃にはプラトーに達する（図B）。マウスの肝臓切片を作製し，ヒト特異的サイトケラチン8/18 (hCK8/18) 抗体で染色すると，ヒト肝細胞のみ染め分けることができる（図C）。肝臓切片あたりのhCK8/18陽性領域の面積の割合

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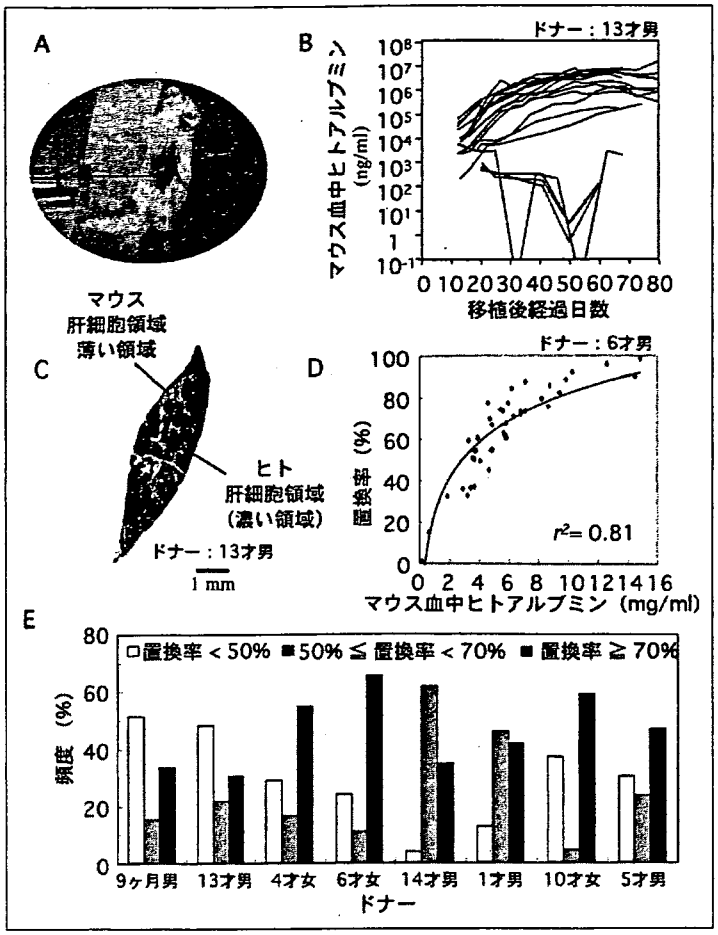

図　キメラマウスの作製と性状。A. uPA/SCIDマウスへのヒト肝細胞の移植。B. キメラマウス血中ヒトアルブミン濃度の推移。C. キメラマウス肝臓切片のhCK8/18染色像。D. キメラマウス血中ヒトアルブミン濃度と置換率の相関。E. 各ドナーで作製したキメラマウス置換率の割合。

を求め，キメラマウスの置換率とした。マウス血中ヒトアルブミン濃度と置換率には相関がある。ドナー肝細胞によって多少異なるが，マウス血中ヒトアルブミン濃度が6 mg/ml以上のキメラマウスの置換率はほぼ70%以上となる（図D）[1]。また，子供の肝細胞を移植した場合，移植したマウスのうち約半分のマウスが置換率70%以上となる（図E）。

現在では，キメラマウス作製技術は（株）フェニックスバイオに移転されている。（株）フェニックスバイオでは，現在置換率70%のキメラマウスを年間1000匹以上生産することができ，さらに増産を計画している。これらのマウスは医薬品開発のための受託試験に利用されている。

キメラマウスの肝臓における薬物代謝や解毒に関わる酵素であるシトクロームP450，第2相酵素，そしてトランスポーター遺伝子やタンパク質は，ヒトの肝臓と近いレベルで発現していることが示されている[1~3]。ヒトB型，C型肝炎ウィルスはヒトやチンパンジーの*in vivo*の肝臓にしか感染しないため，これまで，ウィルスの感染メカニズムや抗ウィルス剤を開発するための実験系が存在しなかった。キメラマウスは，ヒトB型，C型肝炎ウィルスを感染させることが可能であることも示されている[4]。さらに，キメラマウスは，肝臓をターゲットとした遺伝子治療ベクターの有効性や安全性を確かめる実験モデルとしても有用であることが示された[5]。

■ おわりに

これまで，*in vivo*のヒト肝臓における遺伝子およびタンパク質発現，酵素活性に対する医薬候補品などの影響を調べることは不可能であった。また，ヒトにおける医薬候補品の薬物代謝，薬効，および肝毒性を動物実験により予測してきたが，充分なものとはいえなかった。キメラマウスは，*in vivo*における医薬候補品などのヒトにおける薬物代謝，薬効，および肝毒性を予測することができる，画期的なモデル動物になりえると考えている。現在のところ，キメラマウスは医薬候補品のヒトにおける薬物動態を予測する系や，肝炎ウィルスに対する抗ウィルス薬のスクリーニング系として実際に利用されている。現在，キメラマウスが薬効や肝毒性を調べるためのツールとしても有効かどうか，データを積み重ねているところである。

さらに，キメラマウスを用いることによって，これまで調べることが困難であった，*in vivo*におけるヒト肝細胞の増殖，分化，老化などのメカニズムについて明らかにすることが可能となり，臨床における肝臓疾患治療への一助となることを期待している。

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Effect of Hepatitis C Virus (HCV) NS5B-Nucleolin Interaction on HCV Replication with HCV Subgenomic Replicon

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We previously reported that nucleolin, a representative nucleolar marker, interacts with nonstructural protein 5B (NS5B) of hepatitis C virus (HCV) through two independent regions of NS5B, amino acids 208 to 214 and 500 to 506. We also showed that truncated nucleolin that harbors the NS5B-binding region inhibited the RNA-dependent RNA polymerase activity of NS5B in vitro, suggesting that nucleolin may be involved in HCV replication. To address this question, we focused on NS5B amino acids 208 to 214. We constructed one alanine-substituted clustered mutant (CM) replicon, in which all the amino acids in this region were changed to alanine, as well as seven different point mutant (PM) replicons, each of which harbored an alanine substitution at one of the amino acids in the region. After transfection into Huh7 cells, the CM replicon and the PM replicon containing NS5B W208A could not replicate, whereas the remaining PM replicons were able to replicate. In vivo immunoprecipitation also showed that the W208 residue of NS5B was essential for its interaction with nucleolin, strongly suggesting that this interaction is essential for HCV replication. To gain further insight into the role of nucleolin in HCV replication, we utilized the small interfering RNA (siRNA) technique to investigate the knockdown effect of nucleolin on HCV replication. Cotransfection of replicon RNA and nucleolin siRNA into Huh7 cells moderately inhibited HCV replication, although suppression of nucleolin did not affect cell proliferation. Taken together, our findings strongly suggest that nucleolin is a host component that interacts with HCV NS5B and is indispensable for HCV replication.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis around the world (1, 7). Chronic infection with HCV results in liver cirrhosis and may lead to hepatocellular carcinoma (53, 54). HCV is an enveloped positive-strand RNA virus belonging to the genus *Hepacivirus* in the family *Flaviviridae*. The HCV RNA genome is ~9.6 kb in length and consists of a 5′ nontranslated region (NTR), a large open reading frame, and a 3′ NTR. The 5′ NTR contains an internal ribosome entry site, which mediates the translation of a single polyprotein of ~3,000 amino acid (aa) residues (61, 64). This polyprotein is cleaved by host and viral proteases into at least 10 different products (33). At the amino terminus of the polyprotein are the core protein, E1, and E2, followed by p7, a hydrophobic peptide with unknown function, and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The 3′ NTR consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region and is critical for HCV RNA replication and HCV infection (13, 29, 69, 71).

HCV is unique among positive-strand RNA viruses in that it causes persistent and chronic infections. In addition, the high mutation rate of the gene encoding the E2 protein allows it to escape host immune surveillance, which is strongly associated with chronic inflammation of the liver (19, 23, 66, 67). As a result, HCV replication has become a target for the treatment of chronically infected individuals. The RNA-dependent RNA polymerase (RdRp) NS5B is the central catalytic enzyme in HCV RNA replication. Several recombinant and catalytically active forms of NS5B have been expressed and purified from insect cells and *Escherichia coli*, and these proteins have provided insights into the biochemical and catalytic properties of NS5B (2, 12, 34, 68). Studies of HCV replication in vitro have to overcome several difficulties, since replication requires all or most NS proteins and/or host proteins and occurs at the membrane. An understanding of the biology of HCV replication has been facilitated by the development of subgenomic and full-length HCV replicons, which express HCV proteins and replicate their RNA when transfected into human hepatoma-cell-derived Huh7 cells and other cell lines (22, 24, 35).

Nucleolin is a major nucleolar phosphoprotein, and nucleolin-specific antibodies have been used to identify nucleoli (14, 59). Nucleolin has been shown to be an RNA chaperone and/or shuttling protein for various host and viral components in nucleoli, nucleoplasm, cytoplasm, and the plasma membrane (18, 37, 41). We previously reported that the transient expression of NS5B causes the redistribution of endogenous nucleolin from the nucleus to the cytoplasm and that nucleolin and NS5B interact, in vitro and in vivo, through two independent regions of NS5B, aa 208 to 214 and 500 to 506. We also showed that the C-terminal region of nucleolin inhibited NS5B RdRp activity through this interaction in vitro (20). Because full-length nucleolin was not available in that experimental condition (70), we could not determine the exact role of this interaction in vivo.

To further investigate the interaction between nucleolin and NS5B, we focused on NS5B aa 208 to 214. We prepared a

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series of mutant replicons in which each amino acid within this region was altered to alanine(s). Here, we report that the W208 residue is critical for transient HCV replication as well as for binding to nucleolin in vivo. HCV replication was considerably inhibited in cells in which endogenous nucleolin was transiently down-regulated by small interfering RNA (siRNA). Our results strongly suggest the involvement of nucleolin in HCV replication through its interaction with NS5B and that nucleolin acts as a positive modulator of HCV replication.

MATERIALS AND METHODS

Construction of plasmids. The plasmid pNNRZ2RU (28), which harbors a subgenomic replicon derived from MT-2C cells infected with HCV (a genotype 1b isolate, M1LE [GenBank accession no. AB080299]) and contains wild-type M1LE replicon (M1LE/wild) cDNA, was digested with MluI and BglII, and the obtained fragment was inserted into the MluI and BglII sites of the vector pGL3Basic (Promega) to create pGL3-*MluI-BglII*. The intermediate vector pGL3-*MluI-BglII*-S232I was constructed by introducing the point mutation S232I of NS5A into the MluI and SacI sites of pGL3-*MluI-BglII* by site-directed mutagenesis using primers carrying the necessary nucleotide changes. Subsequently, mutations were introduced into pGL3-*MluI-BglII*-S232I, which was digested with MluI and BglII. The resulting DNA fragments were subsequently ligated into the MluI and BglII sites of pNNRZ2RU. Plasmids containing the individual NS5B substitutions W208A, K209A, S210A, K211A, K212A, C213A, and P214A and the 7-amino-acid alanine substitution, cm211, were constructed by introducing each mutation into the Eco81I and NdeI sites of pGL3-*MluI-BglII*-S232I by site-directed mutagenesis using primers carrying the necessary nucleotide changes.

The vector pNKFLAG (49) was used to express amino-terminally FLAG-tagged proteins. The plasmid pNNRZ2RU was subcloned by PCR using the primers 5′-TATCGAGCTCGATGTCAATGTCCTACTCATGGACAGGT-3′ (NS5B For), which contains an artificial initiation codon downstream of the SacI site, and 5′-ATGGATGGATCCGCGGGGTCGGGCGCGAGACAGGCT-3′ (NS5Bt Rev), which contains a BamHI site. NS5Bt, containing full-length NS5B truncated by 21 aa at the C terminus, was subcloned into the SacI and BamHI sites of pNKFLAG to create pNKFLAGNS5Bt.

The plasmid pNKGST/Nucleolin (20) was used for the expression of glutathione-*S*-transferase (GST)-fused nucleolin proteins. FLAG-labeled plasmids containing the individual NS5B substitutions W208A, K209A, S210A, K211A, K212A, C213A, and P214A and the 7-amino-acid alanine substitution cm211 were constructed by introducing fragments of pGL3-*MluI-BglII*-S232I containing each mutation into the EcoRI and SmaI sites of pNKFLAGNS5Bt.

The sequences of all the constructs were confirmed using the dideoxy sequence method. The plasmids pLMH14 and pLMH14/GHD (40) were used as templates for replicon RNA LMH14 and LMH14/GHD, respectively.

Cell culture. We used two kinds of Huh7 cells, one derived from our own laboratory's original Huh7 cells, designated Huh7-DMB (56), and the other cured of MH14 gamma interferon, designated cured MH14 (40). Huh7-DMB cells were used for colony-forming assays, and cured MH14 cells were used for luciferase assays. Both types of Huh7 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin, and 100 μg of streptomycin.

In vitro transcription and purification of RNA. All plasmids harboring replicon RNA were linearized with XbaI and column purified (PCR purification kit; Promega). RNA was synthesized and purified as described previously (56).

RNA transfection and selection of G418-resistant cells. Subconfluent Huh7 cells were trypsinized, washed once with phosphate-buffered saline (PBS) that does not contain Ca and Mg [PBS(−)], and resuspended at 10^7 cells/ml in OPTI-MEM (Gibco-BRL, Invitrogen Life Technologies). One hundred nanograms of *neo* replicon RNA, with or without 1 μM of each siRNA, was added to 400 μl of each cell suspension in a cuvette with a gap width of 0.4 cm (Bio-Rad). The mixture was immediately transfected into Huh7 cells by electroporation with a GenePulser II system (Bio-Rad) set to 270 V and 975 μF. Following a 10-min incubation at room temperature, the cells were transferred into 10 ml of growth medium and seeded into a 10-cm-diameter cell culture dish. To select G418-resistant cells, the medium was replaced with fresh medium containing 1 mg/ml of G418 (GENETICIN; Gibco-BRL, Invitrogen Life Technologies) 24 h after transfection. After changing the medium twice per week for 4 weeks, the colonies were stained with Coomassie brilliant blue (0.6 g/liter in 50% methanol–10% acetic acid).

DNA transfection. Using the same electroporation protocol as described above, 500 ng of pCI-Neo (Promega), which encodes a neomycin resistance marker under the control of a cytomegalovirus (CMV) promoter/enhancer, with or without 1 μM of each siRNA, was transfected into Huh7 cells. G418-resistant cells were selected in medium containing 0.5 mg/ml G418. Four weeks after transfection, the colonies were stained with Coomassie brilliant blue.

Using DMRIE-C reagent (Invitrogen Life Technologies), 300 ng of pGL3 control (Promega), encoding luciferase under the control of a CMV promoter/enhancer, was cotransfected with or without 2 μM of each siRNA according to the manufacturer's instructions. Luciferase activity was assayed 48 and 72 h after transfection.

RNA transfection and luciferase assay. We used a luciferase assay to monitor luciferase replicon activity. Briefly, cured MH14 cells seeded onto 48-well plates were transfected with 250 ng of luciferase replicon RNA, with or without 2 μM of each siRNA, using DMRIE-C reagent according to the manufacturer's instructions. Cell proteins were extracted in a lysis buffer supplied in the Dual-Luciferase Reporter Assay system (Promega), and their luciferase activity was measured. Each assay was performed at least in triplicate, and means and standard deviations were determined.

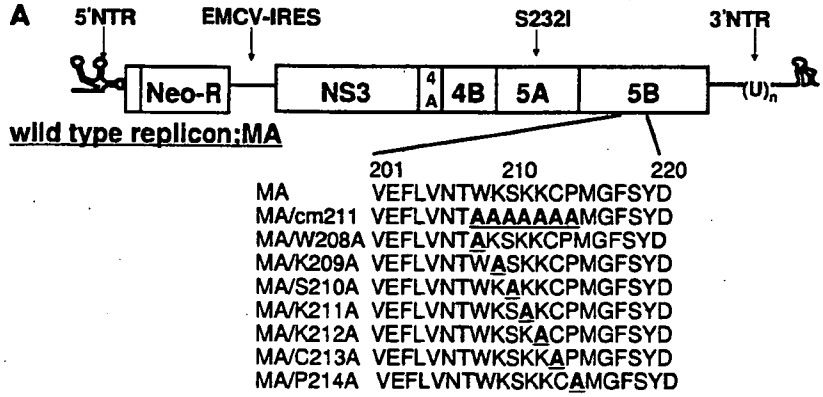
Preparation of cell extracts, coprecipitation with glutathione resin, and Western blot analysis. COS1 cells were transiently transfected using the calcium-phosphate method. The cells were harvested, washed with PBS(−), and sonicated in PBS lysis buffer [PBS(−) containing 150 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol] containing 10 μg each of aprotinin and leupeptin per ml. Total cell lysates were diluted 10-fold with PBS lysis buffer, mixed with 20 μl of glutathione-Sepharose 4B beads (glutathione resin) (Amersham Biosciences), and incubated for 3 h on a rotator in a cold room. After extensive washing with PBS(−) containing 1.0% Triton X-100, the bound proteins were eluted, fractionated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE), transferred onto nitrocellulose membranes, and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody (Sigma). The proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences). As a loading control, the nitrocellulose membranes used for Western blot analysis with anti-FLAG M2 monoclonal antibody were reprobed with anti-GST monoclonal antibody (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions (Amersham Biosciences).

siRNA. We purchased siRNA for luciferase GL3 duplex (si-Luc), siRNA for nonspecific control RNA duplex (si-Mix), siRNA for nucleolin (si-Nuc) (GGA AGACGGUGAAAUUGAU-deoxyribosylthymine [dT]dT), and siRNA for HCV (CCUCAAAGAAAAACCAAAC-dTdT) from B-Bridge International, Inc., and we purchased siRNA for GFP from QIAGEN.

Western blot analysis for endogenous nucleolin. Using the electroporation protocol described above, 1 μM of each siRNA was transfected into Huh7-DMB cells. After 48 h, the cells were harvested, washed with PBS(−), and sonicated in PBS lysis buffer. Total cell lysates were fractionated by SDS–10% PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis with rabbit polyclonal anti-nucleolin antibody (103C) (20), mouse monoclonal anti-nucleolin antibody (C23, sc-8031; Santa Cruz Biotechnology, Inc.), and mouse monoclonal anti-β-actin antibody (Sigma). The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

RESULTS

We previously reported that NS5B from HCV subtype 1b isolate JK-1 and nucleolin interact in vitro and in vivo and that two regions of NS5B, amino acids 208 to 214 and 500 to 506, are both indispensable for binding to nucleolin. We also reported that the C-terminal region of nucleolin inhibited the RdRp activity of NS5B in a dose-dependent manner (20). Although the effect of full-length nucleolin could not be determined, because we could not obtain recombinant full-length nucleolin, these results strongly suggested that nucleolin may be a component of the HCV replication complex and, through its interaction with NS5B, may modulate HCV replication. To further investigate this question, we determined the biological effect of the interaction between NS5B from HCV subtype 1b



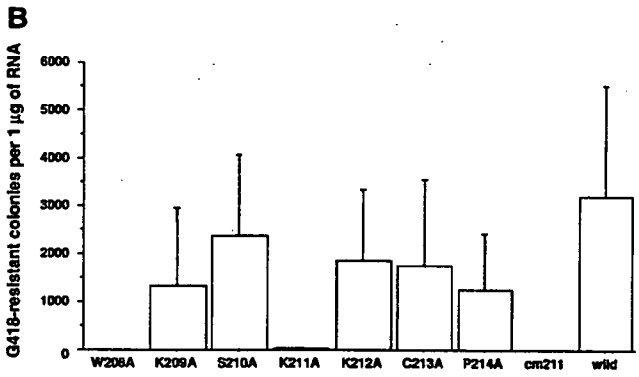


FIG. 1. (A) Schematic representation of the mutant replicons used in this study. MA is a replicon carrying the point mutation S232I in NS5A and was used as the wild-type replicon. The mutant replicons MA/cm211, MA/W208A, MA/209A, MA/S210A, MA/211A, MA/212A, MA/213A, and MA/214A carry NS5B mutations in MA, as shown. Numbering starts from the beginning of NS5B. EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site. (B) Effect of each mutation on HCV RNA replication. Huh7-DMB cells were electroporated with 1 μg of each replicon RNA, and G418-resistant cells were selected with 1 mg/ml G418 and stained 4 weeks later. This figure shows the mean number of G418-resistant cell colonies per 10-cm-diameter cell culture dish per 1 μg replicon RNA.

isolate M1LE and nucleolin on HCV replication using an HCV subgenomic replicon system.

Scanning of aa 208 to 214 in an HCV subgenomic replicon. First, we tested the importance of NS5B aa 208 to 214, a region essential for nucleolin binding, in HCV RNA replication. For this purpose, we prepared eight mutant replicons (Fig. 1A). The wild-type replicon was represented by MA, in which S232 of NS5A was altered to I, because this mutant replicon can efficiently replicate in Huh7 cells (36, 56). In the replicon MA/cm211, each of the amino acids at positions 208 to 214 of NS5B was changed to alanine, whereas in the replicons MA/W208A, K209A, S210A, K211A, K212A, C213A, and P214A, each individual amino acid residue was changed to alanine. All of these mutant replicons were transfected into Huh7-DMB cells, which were selected with G418, and the number of G418-resistant colonies was used as an indication of HCV RNA replication. In cells transfected with MA/cm211 and MA/W208A, we observed no G418-resistant colonies, whereas in cells transfected with the six other point mutant replicons, as well as in cells transfected with MA/K211, we detected G418-resistant colonies, but they were fewer than those detected with wild-type replicon MA (Fig. 1B). Our negative control, the mutant replicon M1LE/5B-VDD, in which the GDD motif of NS5B was mutated to VDD, yielded no G418-resistant colonies (data not shown). The results of this experiment indicated that the region of NS5B at aa 208 to 214, especially W208, is essential for HCV RNA replication.

Interaction between nucleolin and NS5B. Although we have shown that NS5B from isolate JK-1 binds to nucleolin, it was necessary to show this in isolate M1LE. Due to the poor recovery of soluble full-length NS5B, we utilized NS5Bt (68), a soluble form of NS5B in which the C-terminal 21 aa were truncated, to dissect the interaction between NS5B and nucleolin. Previously, we confirmed that these 21 deleted amino acids were not essential for this interaction (20). FLAG-NS5Bt and GST-nucleolin were transiently coexpressed in COS1 cells, after which the lysates were subjected to a GST pull-down assay and the bound proteins were immunologically detected with anti-FLAG M2 and anti-GST antibodies. We found that GST-nucleolin could bind FLAG-NS5Bt from the M1LE isolate, whereas GST could not, indicating that nucleolin interacts with NS5B in both JK-1 and M1LE isolates (Fig. 2). To determine the essential region/residues of NS5B required for its binding to nucleolin, we again focused on aa 208 to 214 using the alanine scanning method (3). We prepared FLAG-NS5Bt/cm211, in which aa 208 to 214 were all replaced by alanine residues, and showed that it could not bind to GST-nucleolin in an in vivo immunoprecipitation assay (Fig. 2), indicating that aa 208 to 214 of NS5B in both M1LE and JK-1 isolates constitute a critical region for the binding of nucleolin. To identify the exact residue(s) within aa 208 to 214 critical for binding to nucleolin, we prepared seven alanine-substituted point mutants in which each amino acid was replaced by alanine, and we tested the ability of each point mutant to bind to GST-nucleolin. Using an in vivo immunoprecipitation assay, we found that of the seven point mutants, only FLAG-NS5Bt/W208A could not bind to GST-nucleolin (Fig. 2), indicating that W208 of NS5B is essential for this binding and may be essential for HCV replication.

Suppression of endogenous nucleolin by siRNA. To identify the siRNA sequence that knocks down the expression of endogenous nucleolin, we used the prediction services of

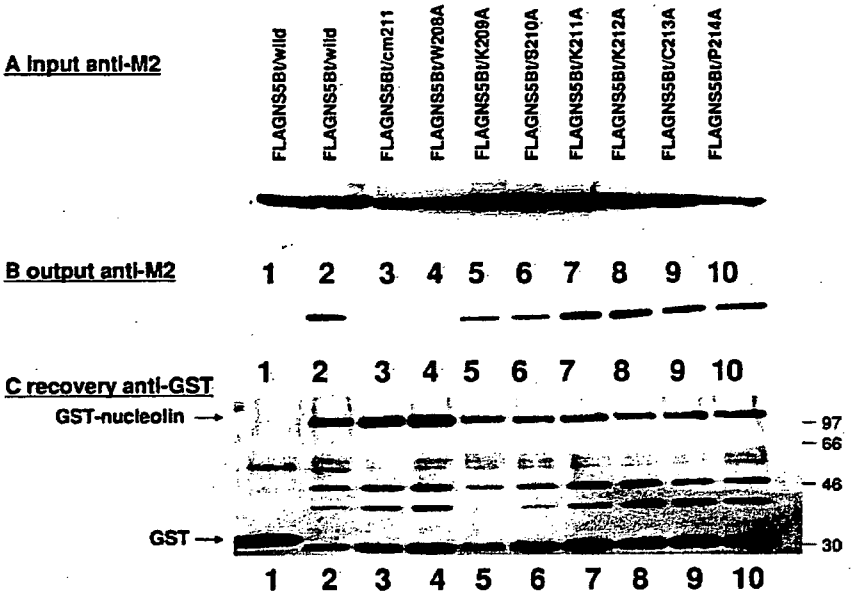


FIG. 2. Interaction between nucleolin and NS5B of HCV isolate M1LE and an essential residue for this interaction. COS1 cells were transiently cotransfected with mammalian expression vectors expressing FLAG-NS5Bt proteins (lanes: 1 and 2, wild type; 3, cm211; 4, W208A; 5, K209A; 6, S210A; 7, K211A; 8, K212A; 9, C213A; 10, P214A) and GST protein alone (lane 1) or GST-nucleolin protein (lanes 2 to 10). (A) Input of FLAG-NS5Bt proteins. Total lysates were fractionated by SDS–10% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. (B) Output of FLAG-NS5Bt proteins. Coprecipitants by glutathione resin were washed with PBS(−) containing 1.0% Triton X-100, fractionated by SDS–10% PAGE, and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. (C) Recovery of GST or GST-nucleolin proteins. The nitrocellulose membrane used for Western blot analysis of coprecipitants with anti-FLAG M2 antibody was reprobed with anti-GST antibody. Molecular masses (kilodaltons) are indicated to the right of the panel.

iGENE (Tsukuba, Japan). We selected one sequence, si-Nuc, and, as a control for siRNA transfection, we utilized siRNA for luciferase (si-Luc) (GL3 luciferase duplex). Forty-eight hours after electroporation of each siRNA, at a concentration of 1 μM, into Huh7-DMB, the lysates were analyzed by Western blotting analysis with two kinds of antibody to nucleolin. We found that both anti-nucleolin antibodies detected the expression of endogenous nucleolin. Although si-Nuc efficiently knocked down the expression of endogenous nucleolin, si-Luc did not (Fig. 3), showing the specificity of the former. In addition, real-time PCR showed that si-Nuc decreased nucleolin mRNA by about one-third compared with si-Luc (data not shown).

Effect of nucleolin suppression on HCV replication. To test the effect of nucleolin knockdown on HCV RNA replication, we transfected 1 μM of si-Nuc or si-Luc along with 100 ng of replicon MA RNA into Huh7-DMB cells and selected the cells with G418. As shown in Fig. 4, we found that cotransfection of si-Nuc reduced the number of G418-resistant colonies, whereas cotransfection of si-Luc did not (Fig. 4). As a control for the efficient transfection of siRNA, we used si-HCV, which targets the HCV internal ribosome entry site and can efficiently suppress HCV replication, as described previously (51). Using this siRNA, we observed no G418-resistant colonies, indicating that siRNA was efficiently transfected under these experimental conditions. To rule out the possibility that suppression of nucleolin may have a detrimental effect on cells and may inhibit HCV RNA replication, we transfected pCI-Neo, which encodes a neomycin resistance gene under the control of a CMV promoter/enhancer, into Huh7-DMB cells, with or without si-Nuc and si-Luc, and selected the cells with 0.5 mg/dl G418. We found that the suppression of nucleolin expression did not significantly reduce the number of G418-resistant colonies (data not shown). In addition, massive cell death was not observed after the transfection of any siRNA (data not shown). These results indicate that the transient suppression of nucleolin may not affect cell proliferation but that nucleolin may affect the HCV replication complex itself.

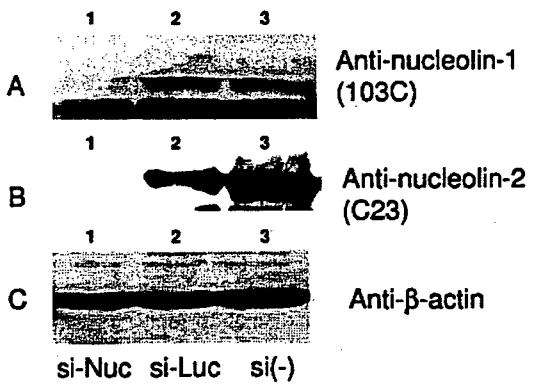


FIG. 3. Knockdown of endogenous nucleolin by siRNA. Huh7-DMB cells were electroporated with 1 μM si-Nuc and si-Luc. After 48 h, total cell lysates were fractionated by SDS–10% PAGE and subjected to Western blot analysis with the anti-nucleolin antibodies anti-nucleolin-1 (103C) in A and anti-nucleolin-2 (C23) in B and anti-β-actin antibody in C. Lanes: 1, cells transfected with si-Nuc; 2, cells transfected with si-Luc; 3, no siRNA [si(−)].

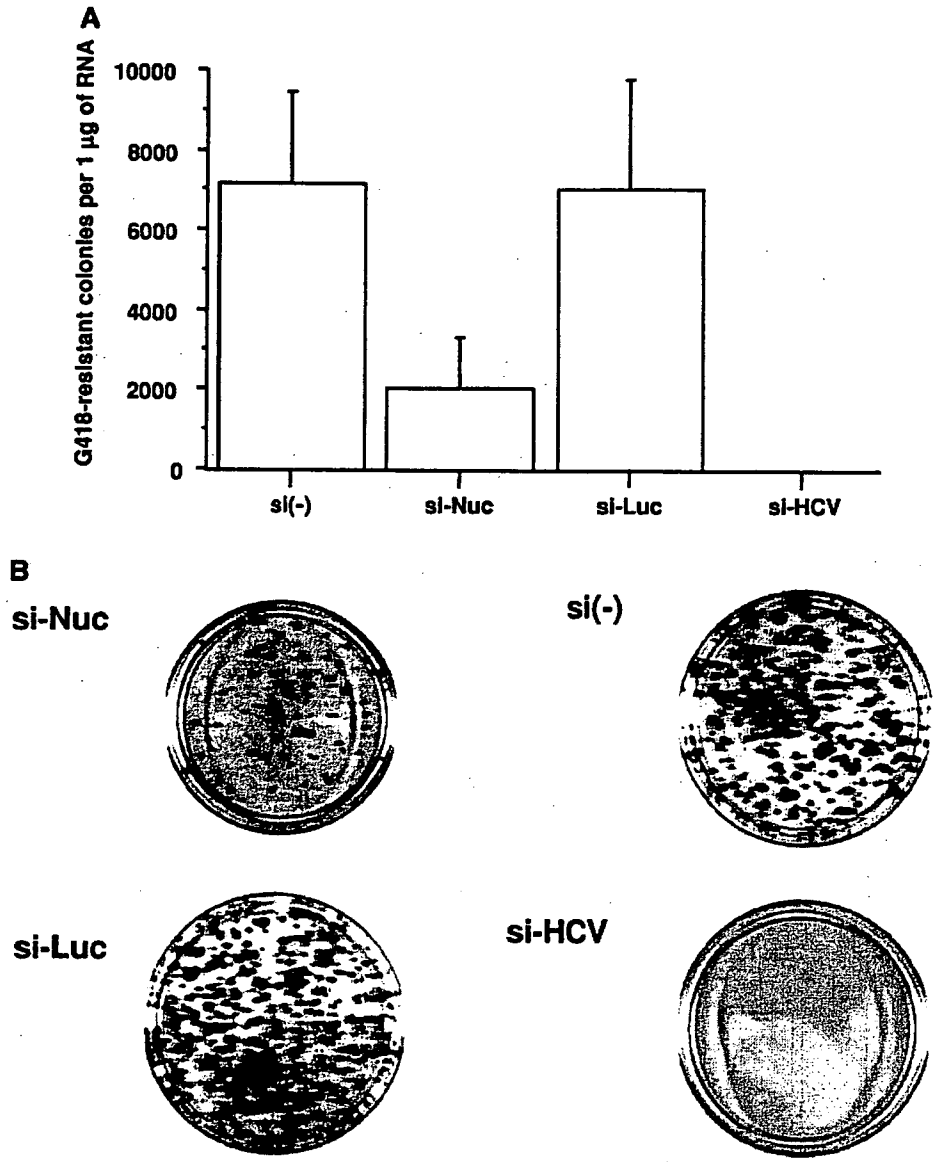


FIG. 4. Effect of suppression of endogenous nucleolin on HCV replication in the MA replicon. Huh7-DMB cells were electroporated with 1 μg of in vitro-transcribed MA RNA plus si-Nuc, si-Luc, si-HCV, or no siRNA [si(−)], and G418-resistant cells were selected with 1 mg/ml G418 and were stained 4 weeks later. (A) Mean number of G418-resistant colonies per 10-cm-diameter cell culture dish per 1 μg replicon RNA. Error bars indicate the standard deviations of the results from at least three independent experiments. (B) Visualization of G418-resistant colonies, as described in Materials and Methods.

Because the knockdown effect of siRNA does not continue for more than 3 weeks after transient transfection, the number of G418-resistant colonies may not be a good indicator of HCV RNA replication. We therefore performed a transient replication assay using a replicon in which the neomycin resistance gene was replaced by a luciferase gene, and luciferase activity was used as a marker of HCV RNA replication. Transfection of MH14 RNA, which was used as the wild-type replicon, into a subline of Huh7 cells resulted in highly efficient luciferase activity, whereas a polymerase-defective RNA replicon of MH14, MH4GHD, in which the catalytic GDD motif of NS5B polymerase was replaced by an inactive GHD motif, was used as a negative control (Fig. 5A). si-HCV and si-Luc suppressed the luciferase activity even at 24 h after transfection, but other siRNAs did not affect the luciferase activity, and luciferase activities in these siRNAs were similar to that of the control (no siRNA) at this point (Fig. 5B). We found that cotransfection of si-Nuc moderately suppressed both luciferase activity at 72 h after transfection and relative luciferase activity, whereas cotransfection of si-GFP and si-Mix did not (Fig. 5B and C). Cotransfection of si-HCV and si-Luc almost completely suppressed luciferase activity at 72 h after transfection. In a transient replication assay, the suppression of endogenous nucleolin also inhibited HCV replication.

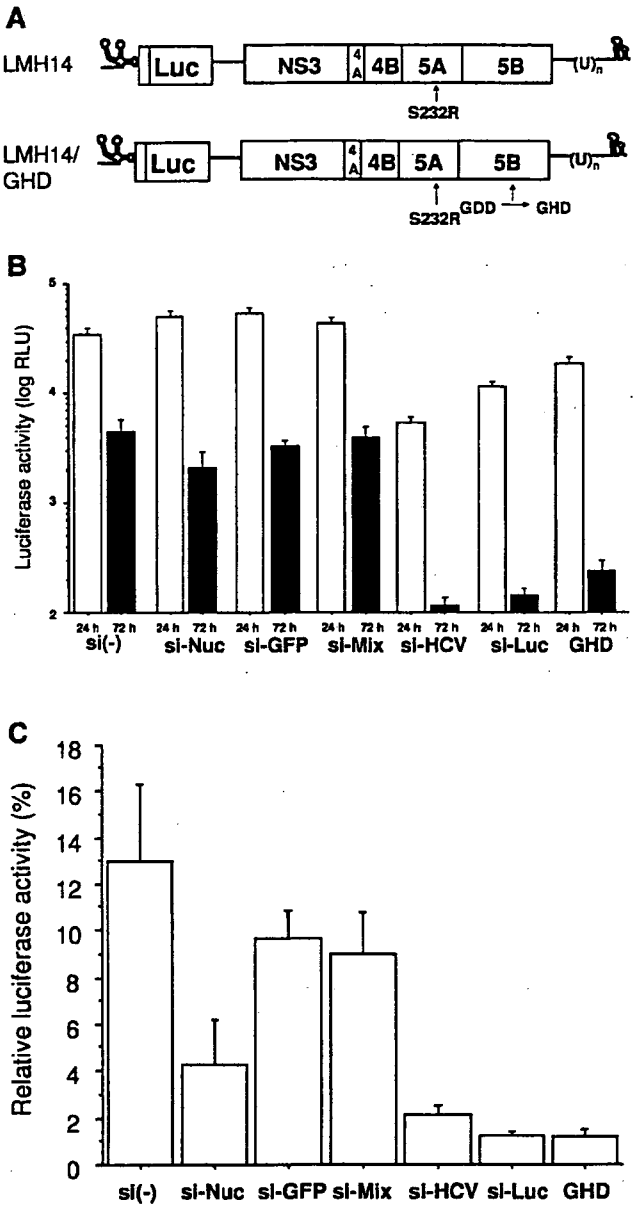


FIG. 5. Effect of suppression of endogenous nucleolin on HCV replication in the LMH14 replicon. (A) Schematic representation of the luciferase replicon. In the LMH14 replicon, the neomycin resistance gene was replaced by a luciferase gene, and S232 of NS5A was replaced by R. In the LMH14/GHD replicon, the NS5B GDD motif in LMH14 was changed to GHD and used as a negative control. (B) Cells were transfected with in vitro-transcribed LHH14 or LMH14/GHD RNA along with 2 μM of si-Mix, si-GFP, si-Nuc, si-Luc, si-HCV, or no siRNA [si(−)] using the DMRIE-C reagent, and luciferase activity (relative light units [RLU]) was measured 24 and 72 h after transfection. Shown are the activities at 24 and 72 h. Error bars indicate the standard deviations of the results from at least three independent experiments. (C) Activity at 24 h was used as an indication of each transfection. Shown are the ratios of activity (percent) at 72 h relative to that at 24 h. Error bars indicate the standard deviations of the results from at least three independent experiments.

To rule out the cytotoxic effects of the suppression of endogenous nucleolin, we transfected pGL3 control, with or without each siRNA, and measured luciferase activity 48 and 72 h after transfection. We found that cotransfection of each siRNA did not inhibit luciferase activity at both 48 and 72 h (Fig. 6), indicating that both suppression of nucleolin and transfection of siRNA did not have detrimental effects on transfected cells.

DISCUSSION

HCV replication has been found to take place in a distinctly altered membrane structure, or membranous web, of the endoplasmic reticulum (11). When HCV NS proteins are coexpressed in stable cell lines harboring replicons, they colocalize to these membrane structures, indicating that they might form a complex (16, 39, 47). These nonstructural proteins, together with host factors, form the viral replicase, the complex in which viral replication is thought to take place. The in vitro level of the RdRp activity of NS5B is low (12), indicating that cofactors, whether viral and/or host proteins and/or the appropriate cellular environment, are necessary for optimal activity of HCV RdRp. HCV NS5B has been reported to interact with NS3, NS4A, NS4B, NS5A, and NS5B itself (9, 48, 57, 65). Using an HCV subgenomic replicon, we previously reported the critical role of the interaction between NS5A and NS5B and the oligomerization of NS5B itself in HCV replication (36, 56). NS3 and NS4B have been shown to be positive and negative regulators, respectively, of NS5B in the replication complex (46).

In addition to interacting with HCV nonstructural proteins, NS5B has been reported to interact with many host proteins, including a SNARE-like protein (62); eIF4AII, an RNA-dependent ATPase/helicase; a component of the translation initiation complex (30), protein kinase C-related kinase 2, which specifically phosphorylates NS5B (27); and p68, a human RNA helicase I (15). The suppression of protein kinase C-related kinase 2 has been reported to reduce the phosphorylation of NS5B and to inhibit HCV RNA replication (27), and the suppression of p68 has been reported to inhibit the synthesis of negative-strand HCV RNA from the positive strand (15).

Several host proteins have been shown to interact with RdRp of other RNA viruses. For example, in poliovirus, an RdRp and an RdRp precursor interact with human Sam68 (38) and heterogeneous nuclear ribonucleoprotein C1/C2 (5), respectively, and modulate RdRp activity directly or indirectly. Bromo mosaic virus RdRp and tobacco mosaic virus RdRp interact with eukaryotic initiation factor 3 and eukaryotic initiation factor 3-related factor, altering RdRp activity (45, 50).

Here and in a previous report, we identified and characterized the interaction between nucleolin and HCV NS5B (20). Nucleolin was originally identified as a common phosphoprotein of growing eukaryotic cells, although its function is not completely understood. Nucleolin is a multifunctional protein that shuttles between the nucleus and cytoplasm. In addition, it is expressed on the surface of various cells, acting as a receptor for various ligands, including lipoproteins (55), cytokines, growth factors (6, 52, 60), the extracellular matrix (10, 18, 25), bacteria (58), and viruses (4, 8, 21, 41–44).

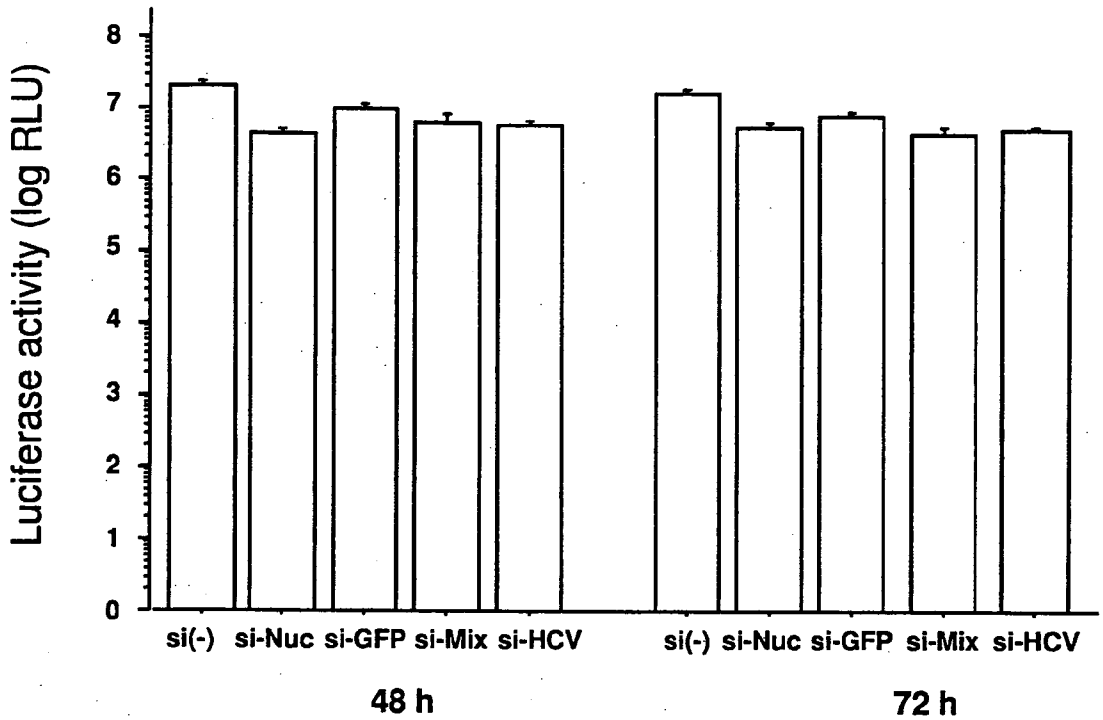


FIG. 6. Effect of suppression of endogenous nucleolin on cell proliferation. The plasmid pGL3 control, encoding the luciferase gene under the control of the CMV promoter/enhancer, was cotransfected with 2 μM of si-Mix, si-GFP, si-Nuc, si-HCV, or no siRNA [si(−)] using DMRIE-C reagent, and luciferase activity was measured 48 and 72 h after transfection. The error bars indicate the standard deviations of the results from at least three independent experiments.

We found that recombinant C-terminal nucleolin proteins can bind NS5B and inhibit its RdRp activity in a dose-dependent manner (20), suggesting that nucleolin may affect HCV replication by interacting with NS5B. The direct interaction of nucleolin with HCV NS5B in vivo and in vitro was shown to require two critical stretches of NS5B. Here, we showed that within one of these regions, aa 208 to 214, the W208 residue was critical for both binding of nucleolin and HCV replication. Transient down-regulation of endogenous nucleolin by siRNA considerably inhibited HCV replication in Huh7 cells. These results strongly indicate that nucleolin has an important role in HCV replication through its direct interaction with NS5B.

Our finding of an important positive role for nucleolin in HCV replication is apparently inconsistent with previous findings of an inhibitory role for nucleolin. It was previously reported that purified C-terminal nucleolin proteins inhibited the RdRp activity of NS5B in vitro. The latter result, however, may have been due to the use of recombinant truncated nucleolin proteins, because recombinant full-length nucleolin was not available (70). Taken together, however, these results indicate that N-terminal nucleolin may be important for the positive function of nucleolin in HCV replication, although the NS5B-binding region is within the RGG domain and RNA-binding domain 4 is at the C terminus.

Transfection of the mutant replicon containing NS5B W208A, which could not bind nucleolin, led to almost no HCV replication. By contrast, the suppression of nucleolin by siRNA moderately inhibited HCV replication, a result also observed with the transient assay using luciferase reporter replicon and G418-resistant colony formation. While HCV replication was completely inhibited by MA/W208A, replication was only partially inhibited by si-Nuc, indicating that si-Nuc can transiently suppress, but cannot eliminate, expression of endogenous nucleolin. Recently, nucleolin was reported to inhibit cell cycle progression after heat shock and genotoxic stress by increasing complex formation with human replication protein A (26). When pGL3 control or pCI-Neo was cotransfected with si-Nuc, the luciferase activity or the number of G418-resistant colonies was not reduced, strongly suggesting that the moderate inhibition of nucleolin expression did not have severe cytotoxic effects on siRNA-transfected cells. More efficient suppression of nucleolin may result in more severe inhibition of HCV RNA replication. It is therefore important to determine whether nucleolin is dispensable in mammalian cells as it is in *Saccharomyces pombe* (17) and *Saccharomyces cerevisiae* (31), since nucleolin may constitute a putative therapeutic target to inhibit HCV replication.

Using a clustered alanine substitution mutant library (CM) of NS5B, we previously showed that two stretches of NS5B amino acids, aa 208 to 214 and 500 to 506, were critical for nucleolin binding. According to the crystal models of NS5B, the former stretch is in the palm and the latter stretch is in the bottom of the thumb domain. We focused on identifying residues in aa 208 to 214 that are essential for nucleolin binding and HCV replication, as the CM mutant of aa 500 to 506 was defective in RdRp activity in vitro and HCV replication in vivo (36, 48, 49). We found that the W208 residue was critical for

both nucleolin binding and HCV replication. This residue is exposed to solvent at the edge of the palm and is not close to the catalytic pocket.

Nucleolin may stabilize monomeric NS5B, making it ready for oligomerization to NS5B, or it may facilitate the formation of a complex between NS5B and template RNA. In both cases, a substoichiometric amount of nucleolin may be required transiently at a step prior to the catalytic RdRp reaction of NS5B. Efforts to determine the contribution of amino acid residues 500 to 508 to nucleolin binding and HCV replication in vivo are ongoing and may reveal further correlations. We found that another mutant replicon, MA/K211A, reduced the number of G418-resistant colonies compared with the wild type and the other mutants. Because K211A of NS5Bt is close to the pocket of catalytic activity and did not affect binding to nucleolin, K211 may contribute to the structural integrity of the pocket or the heat-stable property of RdRp as reported previously (36).

Efficient HCV replication and infection in tissue-cultured cells by using full-length HCV RNA replicons have been reported previously (32, 63, 72). HCV replication occurs in differentiated subcellular fractions and involves dynamic complexes of structural proteins, nonstructural proteins, and HCV RNA demarcated by membrane structures. It is therefore of great interest to determine whether nucleolin is involved in such HCV-replicating intermediates in compartmented subcellular structures.

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Virological effects and safety of combined double filtration plasmapheresis (DFPP) and interferon therapy in patients with chronic hepatitis C: A preliminary study

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Abstract

Purpose: In patients with chronic genotype 1b hepatitis C and a high viral load, the viral load was reduced by double filtration plasmapheresis (DFPP), followed by combined interferon and ribavirin therapy. The safety and virological effects of this treatment method were preliminarily investigated.
Methods: In nine patients with chronic hepatitis C, DFPP was performed three times on days 1, 2, and 4, and the administration of interferon and ribavirin was initiated immediately after DFPP on day 1.
Result: The HCV RNA was undetectable in all patients after the plasma was passed through a plasma fractionator (second filter) in the DFPP circuit. After 2 weeks, the HCV RNA tended to decrease in the DFPP group more than in the control group (-2.45 ± 1.12 versus -1.57 ± 0.95, $P = 0.073$). However, this decrease was not attributable to a sustained virological response (SVR) (22.2% versus 18.2%, $P = 0.822$). Most of the adverse events were caused by the interferon and ribavirin combination therapy.
Conclusion: DFPP can be safely performed concomitantly with interferon and ribavirin combination therapy in chronic hepatitis C patients. The combination may contribute to an early virological response. The effect of DFPP on the SVR and its significance remain to be clarified.

Keywords: Chronic hepatitis C; Interferon therapy; Double filtration plasmapheresis

1. Introduction

Hepatitis C virus (HCV) infection induces acute hepatitis, and approximately 70–80% of these cases progress to chronic hepatitis. The course of the disease is stable in approximately 30% of chronic hepatitis cases; however, the remaining 70% of cases progress to liver cirrhosis after approximately 30–40 years. Further, the cases that progress to liver cirrhosis develop hepatocellular carcinoma at an annual rate of approximately 8% [1].

From the viewpoint of viral eradication, interferon therapy is the only radical therapy for chronic hepatitis C. Once complete viral elimination is achieved by interferon therapy, liver fibrosis improves, and the risk of liver carcinogenesis is reduced [1].

In interferon therapy for chronic hepatitis C, administration of $(3–6) \times 10^6$ IU interferon alone, three times a week for 24 and 48 weeks, has been reported to produce a sustained virological response (SVR) in approximately 6% and 16% of patients, respectively [2]. Treatment with interferon in combination with ribavirin for 24 and 48 weeks has been reported to produce an SVR in 21% [3] and 41% [2,4,5] of patients, respectively.

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A combination of pegylated interferon (PEG-IFN) – a recently marketed long-acting interferon for once-a-week administration – and ribavirin induced an SVR in 47% of patients with chronic genotype 1b HCV infection and high viral loads in whom previous interferon therapy failed to produce a response [6,7].

However, even the combination therapy with PEG-IFN and ribavirin for 48 weeks, which is currently the most promising therapy, does not improve viremia in the other patients for whom there is no appropriate therapy for the eradication of HCV viremia other than time-course observation and the administration of liver-protective drugs. An NS3 protease inhibitor has been developed as a novel antiviral agent, and clinical studies have been performed using this agent; however, the clinical application of this drug will take time because of the occurrence of certain adverse events [8]. The development of an NS5 polymerase inhibitor and an internal ribosome entry site (IRES)-targeting agent is in progress [9–11].

The presence or absence of concomitant ribavirin therapy, the duration of therapy, the virus genotype, the viral load before administration, the grade of liver fibrosis, and the gender and age of the patient are all factors that influence the therapeutic effect of interferon therapy. An early virological response (EVR) is defined as a decline of more than 2 $\log_{10}$ units in the viral load 8 or 12 weeks after the initiation of treatment; it has also been reported to be one of the important factors for an SVR [12,13].

There have been several studies in which the HCV level was investigated in HCV-positive patients during extracorporeal circulation therapies, such as plasmapheresis and hemodialysis. In these studies, the HCV RNA level decreased transiently by approximately 50–90% immediately after plasmapheresis or hemodialysis; however, it returned to either the pre-treatment level or to a higher level within approximately 4–6 h [14–17].

In double filtration plasmapheresis (DFPP), which is a plasmapheresis therapy, the patient's whole blood is separated into plasma and blood cell components by using a plasma separator (first filter). The separated plasma components are further separated into high and low molecular weight components by using a plasma fractionator (second filter); the high molecular weight components including immunoglobulins are removed, and the low molecular weight components including albumin are returned to the body. Although this technique using two filters is relatively more complicated than the normal simple plasma exchange, its advantage is that supplemental plasma transfusion is not necessary.

The diameter of the HCV particle is approximately 55–65 nm [18]. In theory, these viruses are unable to pass through the second filter that has a pore size smaller than the diameter of the viral particle; they are therefore eliminated from the plasma.

In this study, we attempted to reduce the viral loads of patients with chronic genotype 1b hepatitis C and a high viral load by using DFPP. We focused on HCV RNA levels both before plasmapheresis therapy and before interferon and ribavirin combination therapy, and then investigated the early virological effects and also the safety of the treatment.

2. Experimental/materials and methods

2.1. Patients

The study involved nine patients (four males and five females, mean age: 51.7 ± 11.3 years) with chronic genotype 1b hepatitis C that was histologically diagnosed at our department between December 2002 and July 2004. In these patients, the HCV RNA level determined by reverse transcriptase-polymerase chain reaction (RT-PCR) was not less than 100 KIU/ml.

The inclusion criteria were as follows. (1) Minimum age: 20 years; maximum age: 70 years. (2) Blood test values before therapy: hemoglobin, 12 g/dl or higher; platelet count, 100,000 mm^{-3} or higher; white blood cell count, 3000 mm^{-3} or higher; neutrophil count, 1500 mm^{-3} or higher.

The exclusion criteria were as follows. (1) Pregnancy or possible pregnancy, and lactation; (2) depression; (3) serious complications, particularly uncontrollable hypertension and impaired function of the bone marrow, kidneys, or the lungs; (4) autoimmune diseases or suspicion of the same; (5) diabetes or suspicion of the same; (6) allergic predispositions; (7) history of hypersensitivity to interferon or nucleic acid analogues; (8) history of hypersensitivity to biological products such as vaccines; (9) suspicion of alcoholic liver injury, autoimmune hepatitis, or drug-induced liver injury; (10) multiple infections with hepatitis B virus within 48 weeks before the initiation of therapy or suspicion of the same; (11) previous hepatic encephalopathy, rupture of the esophageal varix, orascites; (12) complications of hepatic cirrhosis or hepatocellular carcinoma on examination within 4 weeks before the initiation of therapy or ongoing treatment for the same; (13) treatment with drugs having antiviral actions, immunoregulatory actions, or bone marrow-inhibiting actions such as interferon, Ara-A, zidopudine, glucocorticoid, interleukin 2, or Shosaikoto within 12 weeks of the initiation of therapy or administration of injections containing glycyrrhizin as the main ingredient, theophylline, antipyrine, or warfarin within 4 weeks before therapy.

2.2. Study design

This study was conducted in accordance with the Good Clinical Practice guidelines, conforming to the Helsinki Declaration. It was approved by the Ethics Committee of Kanazawa University Clinical Study Center, and written informed consent was obtained from the patients before their participation in the study.

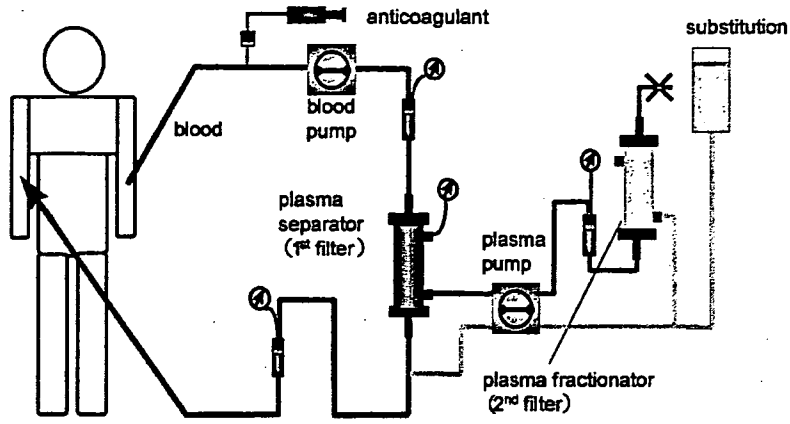

Fig. 1. Mechanism of double filtration plasmapheresis (DFPP). The blood is separated into plasma and blood corpuscles by the plasma separator (first filter), and then filtered using a plasma fractionator (second filter) which separates the plasma into low- and high-molecular weight components.

2.2.1. Treatment schedule

DFPP was performed on day 1 of the therapy to decrease the viral load, and the administration of interferon and ribavirin was initiated 1 h after the completion of DFPP. DFPP was performed three times, on days 1, 2, and 4, and a blood test was performed before each treatment to determine the efficacy of the treatment.

2.2.2. Double filtration plasmapheresis

In order to access the blood during DFPP, a Soft-Cell double-lumen catheter (GamCath catheter N®; Gambro, Stockholm, Sweden) was inserted and indwelled in the right femoral vein for 5 days.

For DFPP, a Plasmaflo KM8800 (Kuraray Medical Inc., Tokyo, Japan) was used as the dialysis apparatus. The plasma separator and plasma fractionator (first and second filters, respectively) used were Plasmaflo OP-08W® and Cascadeflo EC-50W®, respectively (Asahi Kasei Medical Co. Ltd., Tokyo, Japan) (Fig. 1). With regard to the frequency of treatment, in a previous study it was observed that the level of fibrinogen decreased to less than 100 mg/dl when DFPP was continuously performed twice. Thus, DFPP was discontinued on day 3 to prevent fibrinogen-associated complications, such as a tendency to bleed, and the applicability of DFPP on day 4 was determined based on laboratory test results.

In order to process 50 ml/kg of blood in a single round of DFPP, DFPP was performed for approximately 3 h at a blood flow rate of approximately 80 ml/min. The potent protease inhibitor, Nafamostat mesilate (Naotamin®; Asahi Kasei Pharma Co. Ltd., Tokyo, Japan), was used as an anticoagulant because heparin is considered to influence RT-PCR when this procedure is used for HCV RNA measurement. For fluid replacement, we used either 50 or 100 ml of 25% albumin (Kenketsu Albumin-Wf®; Mitsubishi Pharma Co., Tokyo, Japan) that was diluted with 200 ml of saline.

Blood tests were performed before each DFPP to ensure that the plasmapheresis could be performed without the occurrence of any adverse events; DFPP was not performed if the platelet count was 50,000 mm^{-3} or less, or if the fibrinogen level was 100 mg/dl or less. The DFPP was resumed after the recovery of these test values was confirmed.

2.2.3. Interferon therapy

For the interferon therapy, interferon (IFN) α-2b (Intron A®; Schering-Plough KK, Kenilworth, NJ) and ribavirin (Rebetol®; Schering-Plough KK) were concomitantly administered. IFN α-2b was administered intramuscularly 1 h after the completion of DFPP on day 1; the oral administration of ribavirin was initiated after the completion of DFPP on day 1. IFN α-2b was administered six times a week for 2 weeks at a daily dose of 6×10^6 IU, followed by three times a week for 22 weeks or three times a week intermittently for 46 weeks. The ribavirin dose was determined based on the body weight measured at the time of patient registration. The dose used was 600 and 800 mg for body weights of less than 60 and 60 kg or higher, respectively. The daily dose was divided into two doses and administered orally for 24 weeks.

2.2.4. Evaluation

The HCV RNA was measured during and after therapy by using an RT-PCR assay (Amplicor HCV RNA Monitor®; BML, Tokyo, Japan: measurement sensitivity, 0.5 KIU or higher); the sample was diluted when the HCV RNA level was higher than the upper quantification limit (850 KIU/ml). When the HCV RNA was less than the lower quantification limit, a qualitative method was used (Amplicor HCV RNA®, BML; measurement sensitivity, 50 IU).

The HCV RNA was measured after 2, 4, 8, and 12 weeks of therapy, either before DFPP or every morning on days 1–6. It was measured after 24 and 48 weeks of therapy as well as at 24 weeks after the completion of the interferon therapy. Whenever possible, the HCV RNA was also measured immediately after DFPP completion. The HCV RNA in the plasma before and after the second filtration in the DFPP circulation was also measured 1 h after the initiation of the DFPP and immediately before the completion of the DFPP. A negative

viral detection at 24 weeks after the interferon administration was defined as an SVR.

As a control group for a comparison of the decrease in HCV RNA during the 2-week therapy period, we used the HCV RNA data of 11 patients with chronic genotype 1b hepatitis C and a high viral load who underwent IFN and ribavirin combination therapy without DFPP at our department during the same period.

In all patients, a liver biopsy was performed immediately before therapy, and fibrosis and inflammation were evaluated based on the New Inuyama classification. The inflammatory activity in the portal vein and the periportal area as well as the degrees of intralobular inflammation and hepatocyte degeneration were graded from A0 to A3 (0: none, 1: mild, 2: moderate, and 3: severe) based on the "degree of inflammatory activity". Fibrosis was also graded from F0 to F4 (0: none, 1: mild without septa, 2: moderate with few septa, 3: numerous septa without cirrhosis, and 4: cirrhosis) [19].

For the blood tests, we performed white blood cell, red blood cell, platelet, and differential leukocyte (neutrophils, eosinophils, basophils, lymphocytes, and monocytes) counts as well as the zinc sulfate turbidity test (ZTT). Further, the percentage of hemoglobin, hematocrit, and reticulocytes as well as the levels of fibrinogen, total protein, albumin, γ-globulin, and total cholesterol were all measured.

Adverse events were evaluated in accordance with the WHO guidelines, and these were classified into mild, moderate, severe, and life-threatening events.

The significance of the differences was analyzed using the χ^2-test, Fisher's exact test, a *t*-test, and logistic regression analysis.

3. Results

3.1. Patients' backgrounds

The patients' backgrounds are listed in Table 1. In the DFPP group, there were four male and five female patients with a mean age of 51.7 ± 11.3 years. The HCV RNA before therapy was at least 100 KIU/ml and less than 500 KIU/ml in three patients and 500 KIU/ml or higher in six patients, indicating that many of the patients had a high viral load. Three patients had previously undergone IFN therapy, which was virologically ineffective. For the remaining six patients, this was their first experience of IFN therapy. With regard to liver histology, the fibrosis was graded as F2 or lower in all patients, and in all patients there was no evidence of advanced chronic hepatitis. Compared with the control group, gender, age, serum HCV RNA, previous IFN treatment, liver histology, and blood biochemical data for the treatment group were statistically not significantly different.

3.2. Virological changes after DFPP

To confirm the elimination of the virus by DFPP, HCV RNA was measured before and after the second filtration. One hour after the initiation of DFPP, once the DFPP exchange blood flow had stabilized, the HCV RNA in the blood after it had passed through the second filter was quantitatively undetectable in all nine patients. This confirmed the elimination of the virus by the second filter (Fig. 2). Further, at the completion of DFPP – approximately 3 h after its initiation – the HCV RNA was undetectable in all nine patients (data not shown). These findings confirmed that DFPP is capable of

Table 1
Baseline characteristics of chronic genotype 1b hepatitis C patients with a high viral load, treated with a combination of DFPP with interferon (IFN) and ribavirin therapy (DFPP group) and IFN and ribavirin therapy without DFPP (control group)

	DFPP + IFN-R	IFN-R	*P*-value
Gender (M/F)	4/5	7/4	0.684
Age	51.7 ± 11.3	50.6 ± 10.6	0.856
Serum HCV RNA (KIU/ml)			
Mean (minimum–maximum)	2162 (224–12000)	818 (340–1700)	0.254
100–500/500≦	3/6	3/8	
Previous IFN treatment			
Naïve/retreatment	6/3	5/6	0.343
Liver histology			
Stage (F0/F1/F2/F3/F4)	1/1/7/0/0	0/5/6/0/0	0.167
Grade (A0/A1/A2/A3)	0/5/3/1	1/7/3/0	0.541
ALT (IU/ml)	88.4 ± 47.7	89.5 ± 34.5	0.957
Hemoglobin (g/dl)	14.0 ± 1.7	14.2 ± 1.2	0.728
Platelet count ($\times 10^4\ \mu l^{-1}$)	16.9 ± 5.2	15.7 ± 3.4	0.551
Fibrinogen (mg/dl)	231.0 ± 38.9	222.7 ± 92.3	0.804
ZTT (IU)	12.4 ± 4.9	14.2 ± 6.4	0.496
Total protein (mg/dl)	6.9 ± 0.4	6.9 ± 0.4	>0.999
Albumin (g/dl)	4.2 ± 0.3	4.1 ± 0.4	0.373
γ-Globulin (g/dl)	1.3 ± 0.2	1.4 ± 0.3	0.492
Total cholesterol (mg/dl)	178.6 ± 28.3	172.0 ± 45.2	0.714

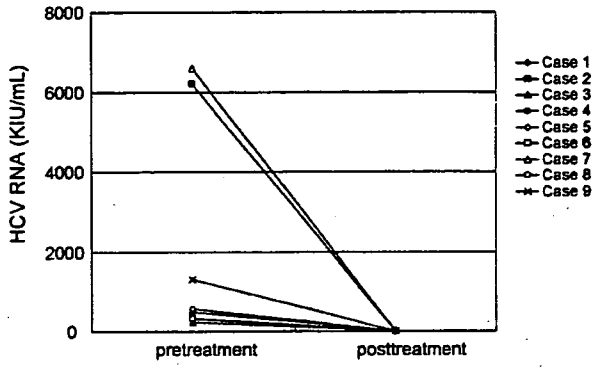

Fig. 2. Change in plasma HCV RNA pre- and post-plasma fractionator (second filter). In all nine cases, HCV RNA was not detected in the plasma of the post-plasma fractionator filtrate 1 h after starting the DFPP.

eliminating the HCV particles and that the elimination efficiency does not decrease with time.

3.3. Early virological response

In order to investigate the EVR, the HCV RNA was measured 2 weeks after the initiation of therapy. It was quantitatively undetectable in four of the nine patients (44.4%). In the control group treated with IFN and ribavirin combination therapy without DFPP, the HCV RNA was quantitatively undetectable in two patients (18.2%). The number of patients with undetectable HCV RNA 2 weeks after the initiation of therapy was higher in the DFPP group, but the difference was not significant ($P = 0.201$).

The EVR is defined as a viral load decline of 2 $\log_{10}$ units or more from the baseline level at an early stage in the therapy, i.e., 2 weeks after the initiation of the therapy in the case of this study. An EVR was achieved in 6 of the 9 patients in the DFPP group (66.7%) and in 4 of the 11 patients in the control group (36.4%), indicating that the HCV RNA tended to decrease earlier during concomitant DFPP and interferon therapy; however, the difference was not statistically significant ($P = 0.178$, Fig. 3A).

The change in HCV viral load also tended to decline more in the DFPP group than in the control group (-2.45 ± 1.12 versus -1.57 ± 0.95; $P = 0.073$, Fig. 3B).

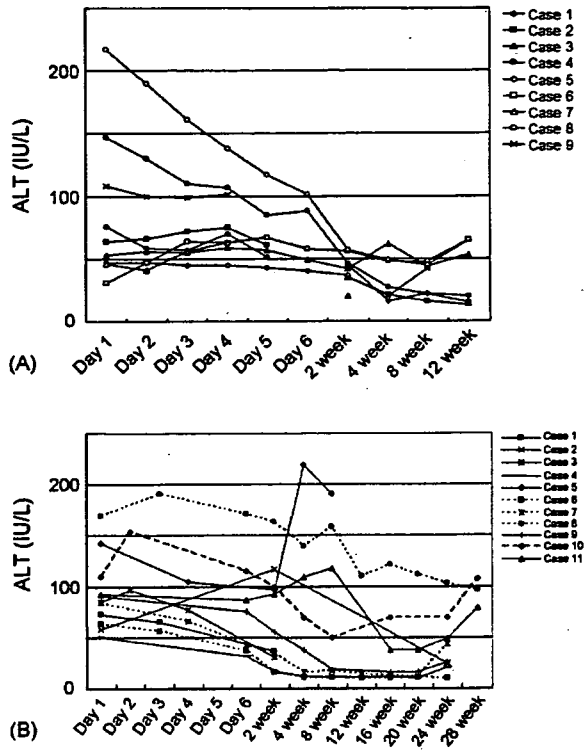

Fig. 4. Change in the serum ALT level during treatment. The ALT level was normalized in eight cases in the second week of treatment.

3.4. Biochemical response

The time-course changes in alanine aminotransferase (ALT) are shown in Fig. 4. The ALT level was normalized in seven of the nine patients (77.8%) by 4 weeks of the therapy. In the control group, it was normalized in 7 of the 11 patients (63.6%). The difference was not statistically significant ($P = 0.845$).

3.5. Sustained virological response

An SVR was observed in two of the nine patients (22.2%) treated with concomitant DFPP and interferon therapy (an intent-to-treat approach). Of these, one patient received IFN

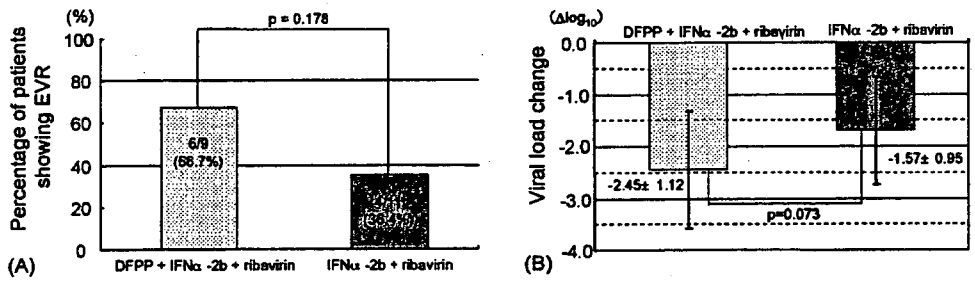

Fig. 3. Change of HCV RNA load 2 weeks after treatment. (A) An EVR is defined as a viral load decline of 2 $\log_{10}$ units or more from the baseline level after 2 weeks of treatment. The numbers in each column indicate the ratio of EVR cases/all treatment cases. (B) Viral load change after 2 weeks. Viral load change was calculated by the formula; $\log_{10}$ (HCV RNA load after 2 weeks/HCV RNA load at pre-treatment).