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

慢性C型肝炎患者由来HCV株感受性正常肝細胞による病原性発現機構の解明および薬剤評価系の構築

平成23～24年度　総合研究報告書

研究代表者　伊藤　昌彦

平成25（2013）年3月

別紙１

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総合　研究報告書

慢性C型肝炎患者由来HCV株感受性正常肝細胞による病原性発現機構の解明および薬剤評価系の構築

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研究要旨

C型肝炎ウイルス(HCV)感染により、その多くが慢性肝炎から肝硬変、肝細胞癌へと移行し、肝癌での年間死亡者は我が国で3万人を超える。C型肝炎ウイルスに関する研究は、これまで限られたヒト肝癌細胞株とHCV株の組み合わせにより行われてきた。しかしながら、HCVの病原性発現の分子機構を解析するためには、本来の肝細胞機能を有する正常ヒト肝細胞株に様々な患者由来HCV株が感染増殖することのできるモデルが必要である。本研究では、慢性C型肝炎感染患者由来のHCV株を高効率に分離培養できる正常肝細胞株の樹立し、これにより、C型肝炎感染患者の有する様々なHCV株の病原性発現機構の解明、さらには薬剤効果や新規治療薬開発のための実験モデル系の樹立を試みた。

平成23年度においては、(1) 正常ヒト肝臓細胞から得た肝前駆細胞を種々の条件下で培養することにより成熟肝細胞へと誘導し、HCV JFH1株の感染性、複製能にもっとも適した分化培養条件を確立した。(2) ヒト肝癌細胞株であるHuh-7をリプログラムすることにより低分化細胞を樹立した。樹立した細胞株はその形質から、肝実質細胞および胆管上皮細胞への分化能を有するオーバル細胞に近い細胞株であることを明らかにした(HuH7-derived oval-like cell; Hdo細胞と命名)。平成24年度では、(2) Huh-7とは異なり、樹立したHdo細胞はHCV感染性、複製能が欠損していることを明らかにした。(3) Hdo細胞を肝分化誘導することにより、成熟肝細胞(induced Hdo細胞; iHdo細胞と命名)を得た。この細胞はHCV複製能が回復することを見出した。(4) Huh7、Hdo細胞、iHdo細胞のmRNA, miRNAの発現をマイクロアレイによって網羅的な解析を行った。また、肝組織特異的遺伝子の発現、肝細胞機能を明らかにし、HCV感染・複製に関与する新規因子の同定を行った。(5) Hdo 細胞の腫瘍原性の変化を調べ、Hdo細胞は、腫瘍原性が低下していることを明らかにした。(6) 患者HCV株の病原性解析のための完全長HCV RNAを簡便に分離するための手法を構築した。

本研究において、ヒト肝細胞癌株をエピジェネティックにリプログラムすることでC型肝炎ウイルス感受性や腫瘍原性が変化することを明らかにした。このことは、慢性C型肝炎感染患者の治療において、エピジェネティックな変化を誘導することによりC型肝炎ウイルスの排除もしくは肝癌への進行を遅らせることできることを示唆する。また、成熟肝細胞への分化の過程で獲得するHCV生活環に必須な分子の同定は新たな創薬のターゲットとなることが期待される。

A．研究目的

国内のおけるC型肝炎ウイルス(HCV)感染者は約300万人とされ、その多くが慢性肝炎から肝硬変、肝細胞癌へと移行し、肝癌での年間死亡者は3万人を超える。肝硬変、肝癌の発症を予防するためには、慢性肝炎の段階でHCVを体内から排除することが重要である。研究開始時の慢性肝炎に対する標準的な治療法では治癒率は50%を少し上回る程度であり、遺伝子型やHCVゲノムの変異による薬剤耐性から、新たなHCV特異的抗ウイルス剤の開発が望まれている。HCVに関する研究は、これまで限られたヒト肝癌細胞株とHCV株の組み合わせにより行われてきた。本研究では、様々な患者由来HCV株の感染増殖機構・病原性発現機構を明らかにするため、本来の肝細胞機能を有する正常ヒト肝細胞で患者由来のHCV株が感染増殖することのできるモデルを構築する。

本研究によっては、患者個々のHCV株の病原性発現の解析のみならず、既存の薬剤の効果の検証、さらには新規治療薬の評価法としても有用となりえる。より効果的な薬剤の選択は肝炎患者にとって大きな福音になるものと思われる。ひいては他の肝炎克服対策事業の研究推進に活用できるだけでなく、保健、医療、福祉の向上、医療費の低減を期待することができる。

B．研究方法

(1) 肝幹細胞の分化誘導によるHCV感染増殖細胞の樹立

フローサイトメトリーとモノクローナル抗体等を用いた高精度細胞分離法により、正常ヒト肝臓細胞から肝幹細胞を分離することができる(横浜市立大学医学研究科谷口先生より提供)。この肝幹細胞は多分化能を有したまま6ヵ月以上にわたりin vitroで増殖可能であり、幹細胞の性質として重要な自己複製能を有している。この細胞を様々な条件下(培地組成, 添加物, 支持担体など)で培養することにより分化させた成熟肝細胞および分化中間段階の肝細胞をHCV J6/JFH1株のウイルス粒子を含む培養液(HCVcc)に暴露し、HCV感染性を調べた。また、サブゲノムレプリコンRNAを導入しLucfiraseの活性を指標にHCV RNA複製能を調べた。さらに、これらの肝細胞の分化状態および機能を肝細胞特異的遺伝子の発現により解析した。以上より、HCV感受性正常肝細胞株への分化条件の検討を行った。

(2) HuH7由来低分化細胞株の樹立

これまで、ヒト間葉系幹細胞(human Mesenchymal Stem Cell; hMSC)やヒト胚性幹細胞(human Embryonic Stem Cell; hESC)から肝細胞への分化誘導の報告は多くあるが、ヒトiPS細胞から肝細胞への報告は少なく(Inamura M, Mol Ther 2011; Sullivan GJ, Hepatol 2010; Song Z, Cell Res 2009,)、誘導肝細胞のHCV感染性についてはあまり調べられていない。一方、がん細胞からiPS細胞を作成するとがん抑制遺伝子p53, p16の機能が回復し、腫瘍形成能のない正常な細胞を誘導することができる(Miyoshi N, PNAS 2010)。そこで、HCV感受性のあるヒト肝癌細胞株HuH-7にOCT3/4, SOX2, KLF4 LIN28, NANOGを発現するレトロウイルスを感染させることで、低分化細胞(Hdo細胞)の樹立を試みた。

(3) Hdo細胞のHCV感受性の解析

HCVcc (J6/JFH1株およびJFH1株)をmoi=0.5でHuH-7, Hdo細胞に感染

させ、細胞内のHCVコピー数をリアルタイムPCR法で定量することでHdo細胞のHCV感染性を調べた。また、Glucを発現するHCVサブゲノムレプリコンRNA(JFH1株およびcon1株)を作成し、in vitro transcriptionによりRNA合成を行った。このRNAをエレクトロポレーション法によりHuH-7, Hdo細胞に遺伝子導入し、培養上清に分泌されたLuciferase活性を測定することでHCV複製能を検討した。

(4) induced Hdo細胞の樹立

Hdo細胞を成熟肝細胞に分化誘導する実験を試みた。誘導培地は、DMEM, 10%FBS, NEAA, 10-7M DEX, 10ng/mL OsM, 10ng/mL HGF, 10ng/mL FGF4, ITS supplementを用いた。誘導することにより得られた細胞の成熟肝細胞マーカーALB、肝芽細胞マーカーAFP、オーバル・胆管上皮細胞マーカーCK19, EpCAMの発現をリアルタイムPCR法、ELISA法およびイムノブロットにより調べた。誘導細胞のHCV感染性および複製能は、HCVcc(J6/JFH1株およびJFH1株)およびHCVサブゲノムレプリコンRNA(JFH1株およびcon1株)の遺伝子導入により調べた。

(5) HCV感染および複製に関与する新規因子の探索

樹立した細胞株における遺伝子発現を、マイクロアレイ(mRNA; ヒト25k Ver 2.10, miRNA; Human miRNA V16.1.0.0, 東レ)による網羅的なトランスクリプトーム解析により調べた。HCV複製能が欠損したHdo細胞で顕著に発現低下する遺伝子について、HCVサブゲノムレプリコンRNAを持続的に複製する細胞株(5-15)を用いてsiRNAによるノックダウンを試みた。HCV感染受容体として報告のあるLDLR, CD81, SR-BI, CLDN1, OCLN, NPC1L1, EGFRなどのmRNA、タンパク質の発現をリアルタイムPCR、イムノブロット、FACS解析、免疫染色法により調べた。また、一過的にCD81を発現させたHdo細胞にHCVpvを感染させ、Luciferase活性を測定することで、HCV感染能の回復を検討した。

(6) Hdo細胞の胆管上皮細胞への分化能

Hdo細胞がオーバル細胞としての形質である、肝実質細胞および胆管上皮細胞への両方向性の分化能を有しているかを明らかにするために、胆管様細胞へと誘導を行った。胆管誘導培地を用いて、Cell culture insert上でmatrigel/コラーゲンIに包埋して長期培養を行った。得られた細胞の肝細胞分化マーカーの発現をリアルタイムPCRにより調べた。また、CK19およびF-actionの免疫染色を行い、胆管構造にみられる極性の存在を検討した。

(7) Hdo細胞の腫瘍原性の解析

これまでに消化器癌細胞などでiPS化することにより腫瘍原性が低下することが報告されている。そこで、Hdo細胞の腫瘍原性の変化を調べた。癌関連の遺伝子の発現として、MYC, MYCN, AURKB, KLB, TP53, RB1, CDKN2A, CDKN1Bなどの発現をリアルタイムPCR法により調べた。また、免疫不全マウスの背部にHuH-7, Hdo細胞を移植し、腫瘍径の経時的変化と形成された腫瘍におけるAFP、p53、Ki67の免疫染色を行い、その悪性度を調べた。

(8) 完全長HCV RNA濃縮法の開発

多検体の患者血清から全長HCV

RNA を簡便に分離するための方法の構築を行った。HCV ゲノム 3'NTR に存在する保存された領域に相補的な 9 種類のビオチン化プライマーを設計した。このビオチン化プライマーと HCV RNA をアニーリングし、非特異吸着を低減させたアビジンビーズによって精製を行った。HCV RNA としては JFH1 株、con1 株、患者 HCV 株(1b)を用い、ビオチン化プライマーの結合領域の検討、最適なプライマー量やビーズ量、アニーリングや RNA 解離のための溶液組成、温度などの条件検討を行った。

（倫理面への配慮）　肝炎患者等からの試料提供を受ける場合には、試料提供者、その家族、および同様の肝炎患者の人権、尊厳が保護されるよう十分に配慮した。厚生労働省等により定められた「ヒトゲノム・遺伝子解析研究に関する倫理指針」に準拠し当該研究機関の医学研究倫理審査委員会に申請し、インフォームドコンセントに係る手続きを実施し、提供試料、個人情報を厳格に管理、保存した。取り扱うすべての DNA および病原性微生物に関しては適切な封じ込めレベルの実験施設で取り扱われた。取り扱う DNA に関しては組換え DNA 実験計画を提出し承認を得、また一部の実験で第二種使用等拡散防止措置確認申請を文部科学大臣に申請し確認を得た上で実験を行った。なお、組換え HCV の作製は遺伝子組換え生物等の第二種使用等にあたるため「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律」（平成 15 年法律第 97 号）の規定に従って申請を行い承認を得ている（大臣確認通知番号　平成 22 年 10 月 8 日付 22 受文科振第 1850 号）。

C．研究結果

(1) 肝幹細胞の分化誘導による HCV 感染増殖細胞の樹立

肝幹細胞を分化誘導するための支持基質（TypeI、TypeIV コラーゲン、マトリゲルなど）の比較を行い、TypeIV コラーゲンが HCV 感染受容体の発現、HCVcc (J6/JFH1)感染能が高いことを明らかにした。また、10-7M DEX, 10ng/mL OsM, 20ng/mL HGF, 10ng/mL FGF4, 100ng/mL FGF-18, 1ng/mL Insulin, 20ng/mL HB-EGF, 2mM Ascorbic acid, 30ug/mL L-Proline を添加した培地が、最も高い HCV 複製能であることを明らかにした。

(2) HuH7 由来低分化細胞株の樹立

ヒト肝癌細胞株である HuH-7 に OCT3/4, SOX2, KLF4 LIN28, NANOG を発現するレトロウイルスを感染させることにより、2 種類の細胞株 (HuH-7 derived oval-like cell; Hdo-17, Hdo-23)を樹立した。この細胞はアルカリフォスファーター弱陽性で ALB+, AFP+, CK19+, EpCAM+のオーバル細胞に近い遺伝子発現であり、低分化状態にあると結論された。また肝細胞機能に関わる分子の発現が認められた。

(3) Hdo 細胞の HCV 感受性の解析

HCVcc (J6/JFH1 株および JFH1 株)に暴露することにより Hdo 細胞の HCV 感染性を調べた結果、感染感受性が欠損していることを明らかにした。また、Gluc を発現する HCV サブゲノムレプリコン RNA (JFH1 株および con1 株)を遺伝子導入することにで、HCV 複製能も欠損していることを明らかにした。

(4) induced Hdo 細胞の樹立

Hdo 細胞を肝細胞へと分化誘導することにより、成熟肝細胞(induced Hdo; iHdo)を得た。iHdo 細胞では、成熟肝細胞マーカーである ALB の発現亢進、肝芽細胞マーカーである AFP の発現低下していた。iHdo 細胞の HCV 感染性および複製能を調べたところ、Hdo-23 細胞で HCV 複製能が回復することが明らかになった。しかしながら、Hdo 細胞の HCVcc の感染性は欠損したままであり、HCV 感染受容体の発現が十分でないことが明らかになった。

(5) HCV 感染および複製に関与する新規因子の探索

樹立した細胞株(HuH-7, Hdo, iHdo)における遺伝子発現を、マイクロアレイにより解析した結果、既知の HCV 複製への関与する遺伝子の発現に有意な変動は認められなかった。このことから、HCV 複製に関与する新規の宿主遺伝子が存在することが示唆された。そこで、Hdo 細胞で発現が顕著に低下する遺伝子について、HCV サブゲノムレプリコン RNA を持続的に複製する細胞株(5-15)を用いて siRNA によるノックダウンを試みた結果、CES1, NUPR1, GADD45B, HERPUD1, ARHGAP8, KDELR3 のノックダウンにより HCV 複製が有意に低下した。このうち、GADD45B, HERPUD1, KDELR3 は HCV 複製能のある iHdo で発現が亢進することが明らかになった。感染感受性については、これまで HCV 感染受容体として報告のある LDLR, SR-BI, CLDN1, OCLN, NPC1L1, EGFR などの mRNA、タンパク質の発現に変化はなかったが、CD81 の発現が顕著に低下していることが明らかになった。しかしながら、HCVpv の感染によって CD81 過剰発現 Hdo 細胞は HuH-7 と同程度にまで HCV 感染性が回復しなかったことから、他の要因もしくは新規の感染受容体の関与が示唆された。miRNA については、Hdo 細胞で HuH-7 と比べて miR-200b, -141, -145, -146a, -200c, -200a, -451, -146b, -1290, -711, -4294 の発現が亢進し、miR-152, -483, -1274a の発現が低下していた。iHdo-23 では、このうち miR-200b, -141, -146a, -200c, -200a, -451, -4294 が発現亢進することから、この miRNA の中に HCV 複製もしくは感染に関与する新規の miRNA が存在していること考えられる。

(6)Hdo 細胞の胆管上皮細胞への分化能

Hdo 細胞がオーバル細胞としての形質である、肝実質細胞および胆管上皮細胞への両方向性の分化能を有しているかを明らかにするために、胆管様細胞へと誘導を行った。その結果、胆管上皮系マーカー遺伝子 EpCAM, CK19 mRNA の発現亢進とともに、CK19 と F-actin の免疫染色から極性を持つ管腔様の構造を有する細胞塊ができることが明らかにした。これらのことから、Hdo 細胞はオーバル細胞の特性を持つ細胞株であることを示された。

(7) Hdo 細胞の腫瘍原性の解析

樹立した Hdo 細胞の腫瘍原性の変化を調べた。Hdo 細胞は増殖能が低下をしており、癌原遺伝子である Myc や KLB の発現の低下、癌抑制遺伝子の Rb の発現亢進がみられた。また、免疫不全マウスにおける腫瘍形成能も HuH-7 と比べて低下するなど、悪性細胞としての性質が低下していることが明らかになった。悪性度の示す腫瘍マーカーである AFP の発現も低下しており、肝癌細胞

をリプログラミング因子により低分化にすることで癌としての悪性度を緩和できることを明らかにした。

(8)完全長 HCV RNA 濃縮法の開発

多検体の患者血清から全長 HCV RNA を簡便に分離するための方法を検討した。HCV ゲノム 3'NTR に存在する保存された領域に相補的なビオチン化プライマーのうち 2 種類を組み合わせることで効果的に HCV RNA を単離精製できることが分かった。また、アリーニングの塩濃度および解離するための EDTA 濃度および温度を決定した。最終的により 1b および 2a クローンで完全長 HCV RNA を特異的に捕捉できる系を構築した。

D．考察

ウイルス研究におけるウイルス増殖細胞系は、ウイルスの生活環、病原性の解明だけでなく抗ウイルス薬のスクリーニングにおいて非常に重要となる。これまで国内外で多くの試みがなされ、細胞内で自律的に HCV ゲノムが複製増殖できる HCV レプリコンシステム(Lohmann V et al., Science 1999;285;110-113)や感染性 HCV 粒子を持続的に産生する感染細胞系(Wakita T et al., Nat Med 2005;11;791-796)が開発され、HCV に関する研究は大きく進展した。しかしながら、患者由来の HCV 株を効率よく感染増殖できる細胞株はこれまで樹立されていない。

本研究において、ヒト肝細胞癌株をエピジェネティックにリプログラムすることで C 型肝炎ウイルス感受性や腫瘍原性が変化することを明らかにした。このことは、慢性 C 型肝炎感染患者の治療において、エピジェネティックな変化を誘導することにより C 型肝炎ウイルスの排除もしくは肝癌への進行を遅らせることできることを示唆する。また、成熟肝細胞への分化の過程で獲得する HCV 生活環に必須な因子は新たな創薬のターゲットとなりうる。一方、完全長の患者 HCV 株を単離する手法の樹立は、今後の患者 HCV 株の病原性解析のための有用な方法として活用できる。

本研究課題によって、変異ウイルスを生じさせないような宿主因子をターゲットとした効果的な薬剤の開発、個々の HCV クローンに対して適切な薬剤の選択が可能となることが期待される。

E．結論

C 型肝炎ウイルスに関する研究は、これまで限られたヒト肝癌細胞株と HCV 株の組み合わせにより行われてきた。しかしながら、HCV の病原性発現の分子機構を解析するためには、本来の肝細胞機能を有する正常ヒト肝細胞に様々な患者由来 HCV 株が感染増殖することのできるモデルが必要である。

平成 23 年度では、慢性 C 型肝炎感染患者由来の HCV 株を高効率に分離培養できる正常肝細胞株を得るために、肝幹細胞からの分化誘導の条件を検討した。また、肝癌由来細胞株 Huh7 から iPS 細胞誘導により低分化細胞株を樹立した。平成 24 年度においては、HCV 感染および複製に関与する新規因子の同定、慢性 C 型肝炎感染患者由来の多種の HCV 株の病原性発現機構の解明のための基盤を構築した。これ

により HCV 感染に伴う肝発癌、代謝異常などの病原性発現機構、また持続感染の成立に関与する分子機構の解明や治療のための薬剤の選択など多岐にわたる応用が期待される。

F．健康危険情報

該当なし

G．研究発表

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H．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

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
Mizuochi T, Ito M, Takai K, Yamaguchi K.	Peripheral blood memory B cells are resistant to apoptosis in chronic hepatitis C patients	Virus Res	155	349-351	2011
Ito M, Kusunoki H, Mizuochi T.	Peripheral B cells as reservoirs for persistent HCV infection	Front Microbiol	2	177-177	2011
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Virus Research 155 (2011) 349–351

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Peripheral blood memory B cells are resistant to apoptosis in chronic hepatitis C patients

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ARTICLE INFO

Article history:
Received 9 July 2010
Received in revised form
21 September 2010
Accepted 21 September 2010
Available online 25 September 2010

Keywords:
HCV
Memory B cell
Apoptosis

ABSTRACT

Our recent study indicated that peripheral B cells in chronic hepatitis C (CHC) patients were infected with hepatitis C virus (HCV). It was also demonstrated that the frequency of $CD27^+$ B cells, i.e. memory phenotype, was significantly reduced in the peripheral blood of CHC patients. An assumption was made by these findings that the $CD27^+$ B cells are susceptible to apoptosis when infected with HCV. Therefore, in this study, the susceptibility of $CD27^+$ B cells to apoptosis in CHC patients was analyzed. Contrary to our assumption, it was found that $CD27^+$ B cells are more resistant to apoptosis than the counterpart subset, i.e. $CD27^-$ B cells. The rationale for this finding is discussed with regard to the possible role for memory B cells as an HCV reservoir for persistent infection in CHC patients.

Hepatitis C virus (HCV) infection has been recognized as one of the major causes of chronic liver diseases, including chronic hepatitis, cirrhosis and, eventually hepatocellular carcinoma, affecting nearly 200 million people worldwide (Lauer and Walker, 2001). The liver is considered to be the primary and main target of HCV infection. However, extrahepatic manifestations, such as mixed cryoglobulinemia, a systemic immune complex-mediated disorder characterized by B cell proliferation that may evolve into overt B cell non-Hodgkin's lymphoma, have been demonstrated (Agnello et al., 1992; Zuckerman et al., 1997). The occurrence of B cell abnormalities often noticed among patients persistently infected with the HCV has suggested the possibility that HCV infects not only hepatocytes but also peripheral B cells. Recent studies including ours have demonstrated that peripheral B cells are in fact infected with HCV (Inokuchi et al., 2009; Ito et al., 2010), which suggest the unprecedented role for B cells in HCV pathogenesis.

Two major human peripheral B cell subsets have been identified based on the expression of CD27, a member of the tumor necrosis factor receptor family. Functional differences between the two subsets have been extensively investigated and it is now generally accepted that CD27 is a memory B cell marker (Agematsu et al., 2000). Our previous study demonstrated that the frequency of peripheral $CD27^+$ memory B cell subset in chronic hepatitis C (CHC) patients is significantly reduced (Mizuochi et al., 2010). To elucidate the reason of this reduction, in this study, we compared the susceptibility of the peripheral $CD27^+$ and $CD27^-$ B cell subsets to apoptosis in CHC patients. Our results demonstrated that $CD27^+$ memory B cells in CHC patients are more resistant to apoptosis than $CD27^-$ B cells. The rationale for this finding is discussed with regard to the possible role for memory B cells in HCV pathogenesis.

A total of 26 CHC patients were enrolled in this study (male/female: 15/11, mean age: 59.6 ± 6.9 years old, mean serum ALT levels: 65.5 ± 31.7 IU/L, mean serum AST levels: 53.2 ± 24.4 IU/L, HCV genotype: 1b = 23, 2a = 3, mean HCV RNA: 2493 ± 959 KIU/mL). All of them were confirmed to be negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The study protocols were approved by the Review Board at the National Institute of Infectious Diseases. All donors gave written informed consent. The controls were 15 healthy blood donors at the Tokyo Red Cross Blood Center (Tokyo, Japan), who were confirmed to be negative for HCV, HBV, and HIV. HCV genotype was determined by PCR of the core region with genotype-specific primers (Ohno et al., 1997). HCV RNA was quantified by the Roche Amplicor assay (Roche Diagnostics, Branchberg, NJ), and results were standardized to international units (IU). Determination of serum levels of ALT and AST was performed using standard methods.

The following fluorescence-conjugated antibodies (Abs) were used for flow cytometry: Allophycocyanin-anti-CD19 (Cat. MHCD1905; Invitrogen, Carlsbad, CA); PE-anti-CD27 (Cat. IM2578; Beckman Coulter, Fullerton, CA); and FITC-anti-CD27 (Cat. 555440; BD Biosciences, San Jose, CA). Cells were washed twice with cold PBS containing 0.2% BSA, followed by incubation with an appro-

Abbreviations: CHC, chronic hepatitis C patients; HCV, hepatitis C virus.

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doi:10.1016/j.virusres.2010.09.017

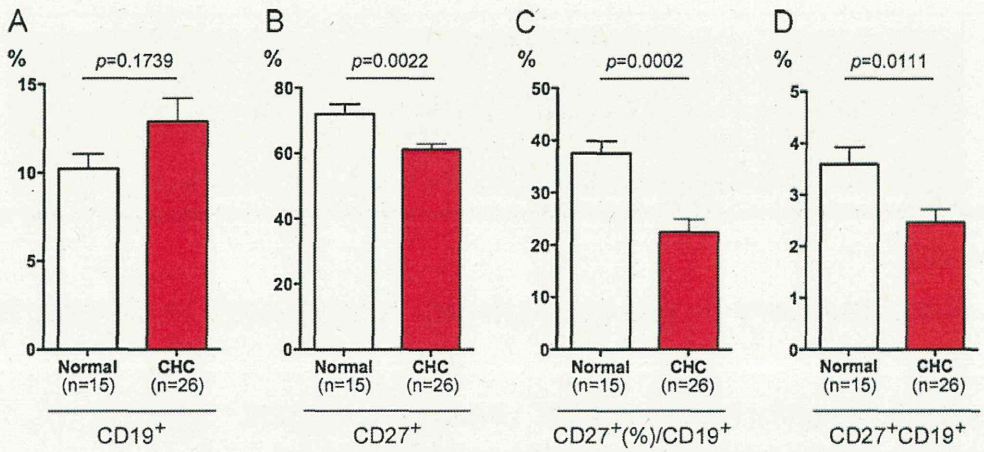

Fig. 1. Flow cytometric analysis of PBMC from normal subjects and CHC patients. Lymphocytes were identified by forward and orthogonal light scatter characteristics. Percentages of $CD19^+$ cells (A), $CD27^+$ cells (B), $CD27^+$ (%) in $CD19^+$ cells (C) and $CD27^+CD19^+$ cells (D) in normal ($n=15$) and CHC patients ($n=26$) are shown with SEM bars and p-values.

priate combination of directly conjugated Abs for 30 min on ice. Stained cells were analyzed by FACSCallibur (Becton Dickinson, San Jose, CA). Data were collected using CellQuest software (Becton Dickinson, San Jose, CA) and were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Levels of Annexin V binding to both $CD27^+$ and $CD27^-$ B cells were assessed with a commercially available Annexin V apoptosis detection kit Annexin V-FITC (PN IM3546, Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

Unpaired (two-tailed) Student's t-test was applied at the 95% confidence level ($p<0.05$) using Prism ver.4 (GraphPad Software, Inc., San Diego, CA) in all cases.

We first analyzed the frequencies of peripheral blood $CD19^+$ cells, i.e. B cells. They were not statistically different ($p=0.1739$) between normal subjects and CHC patients as shown in Fig. 1A. When the percentages of peripheral $CD27^+$ cells were analyzed, a statistically significant ($p=0.0022$) decrease was noticed in CHC patients when compared to normal subjects (Fig. 1B). The percentages of peripheral $CD27^+$ cells in $CD19^+$ cells were then analyzed. A significant ($p=0.0002$) decrease was noticed in CHC patients when compared to normal subjects (Fig. 1C). It was also verified that the frequencies of peripheral $CD27^+CD19^+$ cells were significantly ($p=0.01111$) reduced in CHC patients (Fig. 1D). These results are in good agreement with those of Racanelli et al. (2006). In their report, patients with higher plasma HCV loads had lower percentages of $CD27^+$ B cells, thus suggesting that high HCV replication is associated with a reduction in $CD27^+$ B cells. They hypothesized that, under conditions of persisting HCV antigenemia, memory B cells not receiving specific B cell receptor triggering before having T-cell help would be pushed to enhance immunoglobulin production and prone to apoptosis (Racanelli et al., 2006), which may explain the reduction of $CD27^+$ memory B cells in HCV-infected patients. We next examined this possibility by analyzing apoptosis in both peripheral blood $CD27^+$ and $CD27^-$ B cells.

The levels of spontaneous apoptosis among peripheral blood $CD27^+$ and $CD27^-$ B cells in both normal subjects and CHC patients were analyzed using three-color flow cytometry by staining with allophycocyanin-anti-CD19, PE-anti-CD27 and Annexin V-FITC. As shown in Fig. 2A, $CD27^-$ B cells bound to much larger amounts of Annexin V than $CD27^+$ B cells in CHC patients. In contrast, the pat-

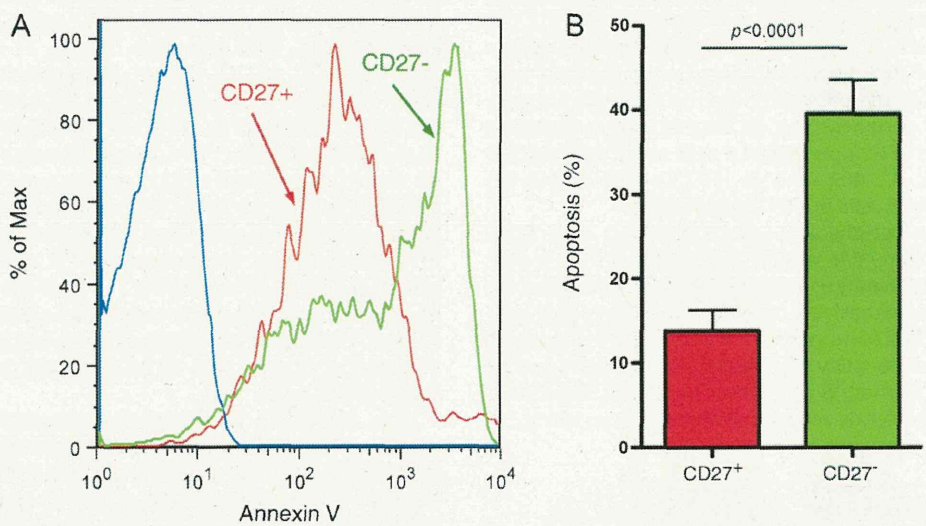

Fig. 2. Annexin V binding to $CD27^+$ and $CD27^-$ B cells. Representative staining patterns for Annexin V binding to $CD27^+$ (red line) and $CD27^-$ (green line) B cells are shown in CHC patients (A). Blue lines indicate background (bkg) staining in negative controls. Summary of data on Annexin V binding to $CD27^+$ (red bar) and $CD27^-$ (green bar) B cells in normal subjects ($n=8$) and CHC patients ($n=9$) are shown with SEM bars and p-value (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

terns of Annexin V binding were similar between $CD27^+$ and $CD27^-$ B cells in normal subjects (data not shown). The percentages of each cell subset bound to large amounts of Annexin V are shown in Fig. 2B (the cut-off point was tentatively set at a fluorescence intensity of 2000). It was concluded that, $CD27^-$ B cells were more vulnerable to apoptosis than $CD27^+$ B cells upon HCV infection; in other words, $CD27^+$ B cells were apparently resistant to apoptosis.

Hepatocytes have long been recognized as main cellular sites for HCV infection. However, this does not necessarily imply that hepatocytes are the exclusive targets for HCV infection. It would be of benefit for HCV to seek other cellular compartments as reservoirs in the event that the liver becomes unsuitable for HCV replication, possibly due to cellular destruction caused by the host immune response. Our recent study verified that peripheral $CD19^+$ B cells in CHC are in fact infected with HCV, thus suggesting a new viral reservoir during the course of natural HCV infection in humans (Ito et al., 2010). Interestingly, another recent study of ours demonstrated that $CD27^+$ B cells are recruited from peripheral blood to the inflammatory site of the liver of CHC patients (Mizuochi et al., 2010). The present study thus may offer new insights into the role of memory B cells in HCV pathogenesis. We assume that memory B cells are the main extrahepatic reservoir of HCV infection because of their long life span which may be correlated with their apparent resistance to apoptosis. This would be a robust strategy for HCV in order to secure sites for persistent infection.

In conclusion, the present study demonstrated that $CD27^+$ B cell subsets in CHC patients are resistant to apoptosis. The long-life of memory $CD27^+$ B cells may be suitable for persistent infection of HCV. Therefore, elimination of peripheral $CD27^+$ B cells in CHC patients with anti-B cell monoclonal antibodies, such as rituximab, would be effective for HCV clearance in CHC patients. Additional study with large sample number and infection with distinct HCV genotype may offer further information.

Acknowledgments

We would like to thank Drs. Miho Suzuki and Kenji Ikebuchi for providing the CHC blood samples used in this study. This study was supported by Grants-in-Aid from the Ministry of Health, Labour, and Welfare.

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frontiers in
MICROBIOLOGY

OPINION ARTICLE
published: 31 August 2011
doi: 10.3389/fmicb.2011.00177

Peripheral B cells as reservoirs for persistent HCV infection

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Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus of approximately 9.6 kb that belongs to the Flaviviridae family (Suzuki et al., 2007). HCV infection is a global health problem affecting nearly 200 million people (Lauer and Walker, 2001). The infection causes prolonged and persistent disease in over half of viral carriers that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Afdhal, 2004). Recent studies have suggested that HCV infects not only hepatocytes but also peripheral mononuclear lymphocytes, particularly B cells, which express CD81, a widely expressed tetraspanin molecule. CD81 has been shown to interact with the E2 region of HCV envelope proteins (Pileri et al., 1998) and is thus regarded as one of the key molecules involved in HCV infection. HCV infection of B cells is the likely cause of various B cell dysregulation disorders. Herein, we propose that HCV uses peripheral B cells as reservoirs for persistent infection, which are in turn responsible for HCV pathogenesis.

Although the liver is considered the primary and main target of HCV infection, extrahepatic manifestations such as mixed cryoglobulinemia, a systemic immune complex-mediated disorder characterized by B cell proliferation that may evolve into overt B cell non-Hodgkin's lymphoma (B-NHL), are often recognized among patients persistently infected with HCV (Agnello et al., 1992; Zuckerman et al., 1997). Epidemiological evidence strongly suggests a close association between chronic HCV infection and B-NHL occurrence (Turner et al., 2003; de Sanjose et al., 2008). A pathogenic role of HCV in B cell disorders has been further demonstrated by studies in which the clinical resolution of B cell dysfunctions was observed after successful regimens of anti-HCV treatment (Mazzaro et al., 1996; Agnello et al., 2002). Based on the aforementioned data, Antonelli et al. (2008) postulated a role of B cells in HCV pathogenesis. In accordance with this notion, our recent study clearly demonstrated that HCV infects and may replicate in the peripheral CD19$^+$ B cells of chronic hepatitis C (CHC) patients (Ito et al., 2010a). In order to determine how HCV evades antiviral innate immune responses that are normally induced in B cells, we analyzed expression levels of IFN-β in peripheral B cells of CHC patients because type I IFN plays a critical role in the antiviral innate immune response. We found that HCV infection failed to trigger antiviral immune responses, such as IFN-β production, in B cells of CHC patients (Ito et al., 2010b). This suggests that HCV evades antiviral innate immune responses in peripheral B cells and uses these cells as reservoirs for its persistent infection in the host.

The idea that B cells may serve as HCV reservoirs was advocated by Muller et al. (1993). Several subsequently published papers also favored the notion of HCV lymphotropism (Ducoulombier et al., 2004; Blackard et al., 2006; Pal et al., 2006). **Figure 1** illustrates the possible process of HCV infection in B cells based on previous studies using human hepatocyte-derived cell lines (Burlone and Budkowska, 2009; Georgel et al., 2010). Among B cell subsets, memory B cells are assumed to be the main reservoirs of HCV infection primarily because of their long lifespans. In support of this notion, our recent study indicated that CD19$^+$ CD27$^+$ cells (i.e., memory B cell phenotype) express a high amount of CXCR3, a chemokine receptor, and are recruited to the inflammatory site in the liver of CHC patients where IFN-γ-inducible protein-10, a CXCR3 ligand, is highly produced (Mizuochi et al., 2010; **Figure 1**). This unique strategy seems to

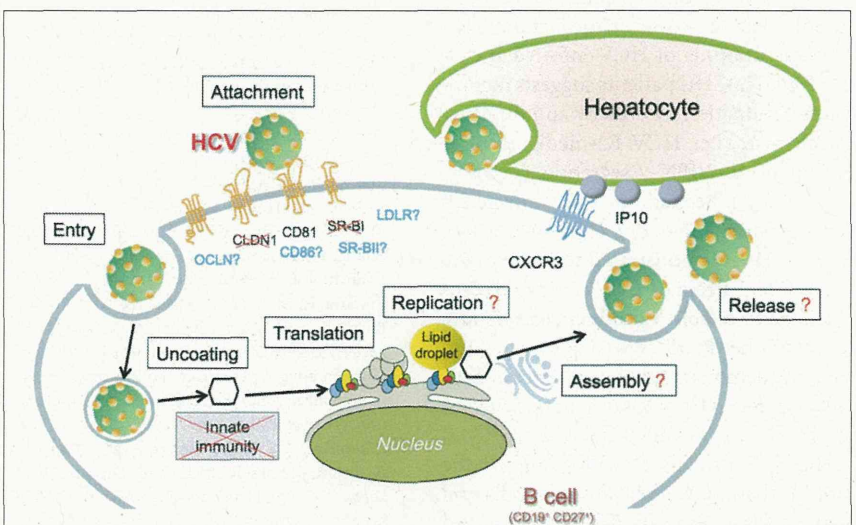

FIGURE 1 | Proposed lifecycle of HCV in B cell. The life cycle of the hepatitis C virus (HCV) has several specific steps. Virus entry is mediated by the direct interaction of envelope glycoproteins with co-receptors, presumably E2–CD81 binding. CLDN1 and SR-BI are not expressed in B cell. Innate immunity response against HCV is suppressed by HCV infection. Some HCV-encoding proteins, i.e., core and NS3, have been proven to be expressed in B cells. HCV RNA replication was examined by nested PCR and *in situ* hybridization. Mechanisms of virus assembly, vesicle fusion, and virion release in B cell remain unknown. LDLR, low-density lipoprotein receptor; SR-BI, scavenger receptor class B type I; SR-BII, scavenger receptor class B type II; CLDN1, claudin-1; OCLN, occludin; CXCR3, chemokine (C-X-C motif) receptor 3; IP-10, interferon gamma-induced protein-10.

be beneficial for HCV in securing sites for persistent infection. HCV may search for reservoir sites in cellular compartments other than hepatocytes in case the liver becomes unsuitable for HCV replication, perhaps because of cellular destruction caused by the host immune response and/or by irrelevant conditions for successful virus replication, such as the development of cirrhosis or hepatocellular carcinoma.

At least two important issues remain to be investigated. First, how do HCV-infected B cells evade "acquired/adaptive" immune responses represented by cytotoxic T cells (CTL)? In peripheral blood, the frequencies of HCV-specific $CD8^+$ lymphocytes with persistent HCV infection are lower than those with acute HCV infection. Furthermore, the CTL response to the HCV antigen is impaired in chronic HCV patients (Lechner et al., 2000). Interestingly, the percentage of CTL in peripheral blood is lower than that in the liver, which may be advantageous for persistent HCV infection in B cells. Because the peripheral B cells of CHC patients express the HCV core as well as NS3 antigens (Ito et al., 2010a), both of which encode functional CTL epitopes (Hiroishi et al., 2010), it is possible that HCV-infected B cells are eliminated by CTL to some extent. However, the fact that substantial amounts of HCV-infected B cells are found in CHC patients suggests incomplete elimination by CTL by an inhibitory mechanism, i.e., HCV E2-mediated inhibition of IL-2/IFN-γ secretion (Petrovic et al., 2011). Second, do HCV-infected B cells produce infectious HCV? Stamataki et al. (2009) demonstrated that the infectious JFH-1 strain of HCV can bind B cells but fails to establish productive infection. On the other hand, Inokuchi et al. (2009) recently demonstrated the presence of negative-stranded HCV RNA, a marker of viral replication, in B cells from 4 of 75 (5%) CHC patients. These results support the notion that HCV replicates in B cells and suggest that infectious HCV are produced in B cells. We have currently been investigating this intriguing issue by using an *in vitro* assay system.

In conclusion, lymphoid reservoirs of HCV infection may play a role in viral persistence and thereby be involved in its pathogenesis. Infection and replication of HCV in peripheral B cells should be regarded as a considerable impediment to the treatment of CHC patients undergoing various antiviral regimens. From a therapeutic viewpoint, it may be beneficial to eliminate peripheral B cells in CHC patients by administering anti-B cell antibodies, such as rituximab, along with combination chemotherapy of peg-IFN-α and ribavirin, which eliminate circulating HCV in the blood. Together, this could lead to a synergistic effect on HCV clearance in CHC patients.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid from the Ministry of Health, Labour, and Welfare, Japan.

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Received: 04 July 2011; accepted: 11 August 2011; published online: 31 August 2011.

Citation: Ito M, Kusunoki H and Mizuochi T (2011) Peripheral B cells as reservoirs for persistent HCV infection. Front. Microbio. 2:177. doi: 10.3389/fmicb.2011.00177 This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

Hindawi Publishing Corporation
Advances in Hematology
Volume 2011, Article ID 835314, 8 pages
doi:10.1155/2011/835314

Review Article

HCV Infection and B-Cell Lymphomagenesis

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Received 7 March 2011; Revised 14 June 2011; Accepted 17 June 2011

Academic Editor: Daniele Vallisa

Hepatitis C virus (HCV) has been recognized as a major cause of chronic liver diseases worldwide. It has been suggested that HCV infects not only hepatocytes but also mononuclear lymphocytes including B cells that express the CD81 molecule, a putative HCV receptor. HCV infection of B cells is the likely cause of B-cell dysregulation disorders such as mixed cryoglobulinemia, rheumatoid factor production, and B-cell lymphoproliferative disorders that may evolve into non-Hodgkin's lymphoma (NHL). Epidemiological data indicate an association between HCV chronic infection and the occurrence of B-cell NHL, suggesting that chronic HCV infection is associated at least in part with B-cell lymphomagenesis. In this paper, we aim to provide an overview of recent literature, including our own, to elucidate a possible role of HCV chronic infection in B-cell lymphomagenesis.

1. Introduction

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus that belongs to the *Flaviviridae* family [1]. HCV infection is a worldwide problem affecting nearly 200 million people [2] and causes prolonged and persistent diseases in virus carriers, often leading to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [3]. Although the liver is considered to be the primary target of HCV infection, extrahepatic manifestations, such as mixed cryoglobulinemia, which is a systemic immune complex-mediated disorder characterized by B-cell proliferation that may evolve into overt B-cell non-Hodgkin's lymphoma (B-NHL), are often recognized among patients persistently infected with HCV [4, 5]. In fact, epidemiological evidence strongly suggests a close link between chronic HCV infection and B-NHL [6, 7]. The pathogenic role of HCV in B-cell disorders has been suggested in reports wherein a clinical resolution of the B-cell dysfunctions, stated above, was observed after successful anti-HCV treatment using interferon (IFN) [8–10]. Based on such evidences, a possible role of B cells in HCV pathogenesis has been postulated but not yet conclusively demonstrated.

The objective of this paper is to summarize recent literature focused on the possible involvement of HCV infection in B-cell lymphomagenesis, which could offer new insights into the role of B cells in the pathogenesis of HCV infection.

2. Does HCV Infect and Replicate in Peripheral B Cells of Chronic Hepatitis C (CHC) Patients?

HCV, as the name indicates, has been regarded as a hepatotropic virus. However, the possibility that HCV infects cells other than hepatocytes cannot be excluded. In the early 1990s, the existence of HCV RNA was demonstrated by PCR in not only serum/plasma [11] and liver tissues [12] but also in peripheral blood mononuclear cells (PBMCs) of patients infected with HCV [13, 14]. Muller et al. first reported in 1993 that HCV RNA could be found in B cells [15]. They predicted that PBMC, particularly B cells, could be sites for HCV replication and may serve as reservoirs of HCV infection. Moldvay et al. demonstrated that negative-strand HCV RNA, a replicative intermediate of HCV, was observed in PBMC of

patients with CHC (6 of 11) by *in situ* hybridization [16]. Muratori et al. reported negative-strand HCV RNA within PBMC detected by fluorescein-tagged *in situ* RT-PCR (12 of 14 patients with CHC) [17]. Further evidence suggested that HCV replicates in B cells. For example, Morsia et al. demonstrated the replication of HCV in $CD19^+$ B cells by detecting the negative-strand RNA although their sample size was very small (1 of 3 patients with CHC was positive) [18]. Around the same time, Pileri et al. demonstrated that the HCV envelope protein E2 binds the CD81 molecule that is expressed on not only hepatocytes but also various cell types including B cells [19]. This finding thus provided a rationale for the notion that HCV infects and replicates in B cells. Several years later, Gong et al. confirmed the existence of negative-strand HCV RNA in PBMC of patients with CHC (14 of 35) [20]. Some argued that the negative-strand HCV RNA in PBMC may be due to mere contamination or passive absorption by circulating HCV in peripheral blood. They successfully excluded this possibility by demonstrating the expression of HCV-encoding protein, NS5, which indicates that HCV not only replicates but also produces HCV protein in PBMC. Their results are in agreement with an earlier study by Sansonno et al. in which HCV core and NS3 proteins were detected in PMBC of patients with CHC [21].

Occult HCV infection is characterized by the presence of HCV RNA in the liver and the absence of both HCV RNA and anti-HCV antibodies in serum. Castillo et al. detected HCV RNA in PBMC of 40 of 57 (70%) patients with occult HCV infection [22]. In a subsequent report, they confirmed the replication of HCV in PBMC of patients with occult HCV infection by detecting both positive and negative strands of HCV RNA using a strand-specific RT-PCR and *in situ* hybridization techniques [23]. Meanwhile, Januszkiewicz-Lewandowska et al. demonstrated the presence of HCV RNA in PBMC of patients who underwent antiviral chemotherapy and therefore were HCV-serum negative [24]. Collectively, these findings not only favor the notion that PBMC, particularly B cells (discussed later), infected with HCV can serve as reservoirs for persistent HCV infection but are also an alert that PBMC of patients with CHC, including patients with occult CHC, could be potentially infectious even when HCV RNA is negative in their sera. There has been a debate over which cell population in PBMC is the main target for HCV infection. An array of evidence suggests that HCV replicates in various cell types of PBMC, including peripheral dendritic cells, monocytes, and macrophages [25–27]. A recent study by Kondo et al. demonstrated that lymphotropic HCV (SB strain) could infect not only established T-cell lines and B-cell lines but also primary naïve $CD4^+$ T cells, suggesting that HCV replication in such T cells suppressed their proliferation and development in Th1 commitment [28]. Under these circumstances, a number of reports have indicated that HCV infects CD81-positive lymphocytes, preferentially B cells [18, 29–31]. Our recent study also clearly demonstrated that HCV RNA and HCV core and NS3 proteins are detected in $CD19^+$ but not in $CD19^-$ PBMC [32]. Furthermore, Inokuchi et al. confirmed that negative-strand HCV RNA, regarded as a marker of viral replication, was detected in B cells of patients with CHC [33]. Considering this evidence, it can be concluded that HCV infects and replicates in PBMC, particularly in the $CD19^+$ B-cell subset, of patients with CHC. An intriguing question has emerged as to whether different HCV variants or B-tropic HCV cause HCV infection in the $CD19^+$ B cells of patients with CHC or not. When cDNA sequences derived from RNA isolated from plasma and $CD19^+$ B cells of randomly selected patients with CHC were compared, limited variations were found in the internal ribosome entry site (IRES) region (our unpublished data). However, as predicted by a computer program named mfold, these nucleotide substitutions did not affect RNA secondary structure or thermodynamic stability of IRES region [34]. Furthermore, the amino acid sequences in the hypervariable region 1 (HVR1), which directly reflect clonal variations of HCV, did not show any distinct differences between plasma and $CD19^+$ B cells of patients with CHC. These results indicate that HCV RNA isolated from CHC B cells is indistinguishable from RNA isolated from plasma of the same patient with CHC (our unpublished data). Sequence polymorphisms located at IRES and HVR1 of E2 were observed in lymphoid cells of individuals with persistent HCV infection, strongly favoring the concept of HCV lymphotropism. Recently, HCV variants observed in B cells showed poor translational activity in hepatocytes but not in B-cell lines, indicating that adaptive mutations had occurred in B cells [35]. However, our results do not support the concept of lymphotropism or B-tropism of HCV in patients with CHC [30] but instead are in good agreement with studies by Muller et al. in which the PCR products obtained from serum and PBMC specimens of an HCV-positive individual were found to have nearly identical sequences [15]. Although the number of clones analyzed was limited, our conclusion that HCV RNA isolated from $CD19^+$ B cells is indistinguishable from RNA isolated from the plasma of the same patient with CHC is inconsistent with the concept of B-tropic HCV RNA. Further investigation involving a large number of HCV patients would be necessary to support this conclusion.

Overall, the data accumulated to date strongly suggest that HCV infects and replicates in the peripheral B cells of patients with CHC. However, currently it is not known whether a novel HCV strain, B-tropic HCV RNA, preferentially infects peripheral B cells or not. The role of B cells in the pathogenesis of HCV infection is examined in the next section.

3. Peripheral B Cells May Serve as Reservoirs for Persistent Infection of HCV

As described in the previous section, evidence indicates that peripheral B cells in patients with CHC were infected with HCV and thus may serve as HCV reservoirs. This evidence posed a logical question as to how HCV evades the innate antiviral immune responses in B cells. However, this important issue has so far not been formally investigated.

Sensing mechanisms for invading viruses in host immune cells consist of toll-like-receptor (TLR-) mediated [36] as well as retinoic-acid-inducible-gene-I-(RIG-I-) mediated [37] pathways. Both pathways culminate in

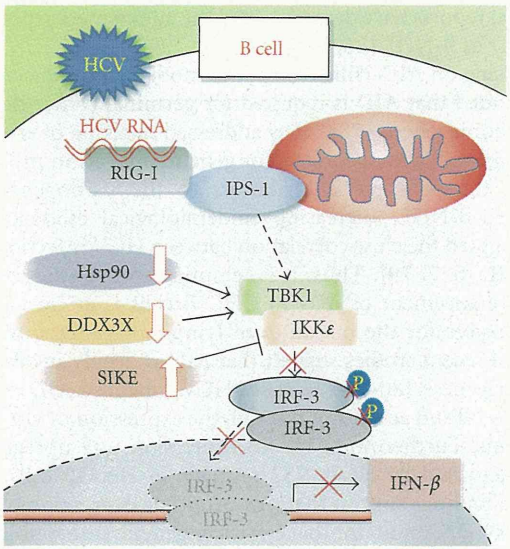

FIGURE 1: Impaired innate antiviral immunity in B cells of patients with chronic hepatitis C.

the translocation of IFN regulatory factor-3 (IRF-3) to the nucleus to transcribe the IFN-β gene. Type-I IFN, for example, IFN-β, plays a critical role in the innate antiviral immune response [38, 39]. In our recent study, it was found that the expression levels of RIG-I and its adaptor molecule, IFN promoter-stimulator 1 (IPS-1), were substantially enhanced in CHC B cells. However, dimerization and the subsequent nuclear translocation of IRF-3 were almost undetectable in CHC B cells. It has been demonstrated that TANK-binding kinase-1 (TBK1) and IκB kinase-ε (IKKε) are essential for the phosphorylation of IRF-3 [40]. The constitutive expression levels of both kinases were found to be markedly enhanced in CHC B cells. However, the reduced expression of TBK1 stabilizers, including Hsp90 [41] and DDX3X [42], and the enhanced expression of the IKK suppressor SIKE [43], were observed in CHC B cells, suggesting that IRF-3 phosphorylation was downregulated. Hence, transcription of the IFN-β gene was not augmented. These results strongly suggest that HCV infection circumvents innate antiviral immune responses, that is, type I IFN production in B cells, and (Figure 1) thus, takes advantage of B cells for persistent infection.

It can be assumed that, among B-cell subsets, memory B cells are the main reservoirs of HCV infection primarily because of their long lifespans. Supporting this notion, our recent study indicated that $CD19^+$ $CD27^+$ cells (memory B cells [44]) are recruited to the liver of patients with CHC through the interaction between CXCR3 expressed on $CD19^+$ $CD27^+$ cells and IP-10 (IFN-γ-inducing protein 10 kD) produced in the liver [45]. This strategy would be beneficial for HCV in securing sites for long-lasting infection. HCV infection of hepatocytes has long been considered an a *priori* assumption. However, this assumption does not necessarily mean that hepatocytes are the exclusive target of HCV infection. HCV may search for reservoir sites in other cellular compartments if the liver becomes unsuitable for replication, perhaps due to cellular destruction caused by the host immune response and/or by the development of conditions such as cirrhosis and hepatocellular carcinoma.

Lymphoid reservoirs of HCV infection could play a role in viral persistence [29, 46–48]. Several maneuvers are employed for persistent infection of HCV [49]. Viral modulation is an effective strategy to escape host immune responses [50]. Another strategy is the suppression of the innate immunity of host by viral components. These components include HCV E2 protein, which acts as a decoy target of protein kinase R (PKR) [51]; HCV NS3/4A protein, which cleaves the adaptor molecules TRIF and IPS-1 and thereby blocks TLR3 and RIG-I signaling, respectively [52, 53]; HCV NS5A protein, which inhibits IFN-stimulated genes expression [54] and PKR function [55]; HCV core protein, which interferes with JAK/STAT signaling [56, 57]. Regardless of the mechanisms, the infection and replication of HCV in peripheral B cells should be considered barriers to the treatment of patients with CHC with antiviral regimens. Based on the notion that peripheral B cells serve as reservoirs for persistent HCV infection and from a therapeutic perspective, it may be beneficial to eliminate peripheral B cells in patients with CHC by the administration of anti-B-cell antibodies, such as rituximab, along with combination therapy with peginterferon and ribavirin to eliminate circulating HCV in the blood, leading to a synergistic effect on HCV clearance in patients with CHC.

4. HCV Infection and B-Cell Lymphomagenesis

The striking association between HCV infection and type II mixed cryoglobulinemia (MC) has been well documented [4, 58, 59]. MC is a benign lymphoproliferative disorder and is regarded as a variant of low-grade B-NHL. Therefore, lymphotropism of HCV suggests that HCV could play a pathogenic role in the clonal proliferation of B cells [60, 61]. Because HCV RNA genomic sequences are not able to integrate into the host genome, indirect mechanisms of malignant transformation should be considered. In this regard, the persistent stimulation of B cells by viral antigens and/or the enhanced expression of lymphomagenesis-related genes could be responsible for leading to polyclonal and later to monoclonal expansion of B cells. Furthermore, the occurrence of a subsequent transformation may lead to B-NHL.

A number of epidemiological studies regarding the association between HCV infection and the occurrence of B-NHL have been carried out [5, 7, 62–65]. A substantial geographic as well as demographic variation exists in the association between HCV infection and risk of B-NHL. A positive association was found in Italy, Japan, and USA. A recent case-control study with a large number of subjects from the International Lymphoma Epidemiology Consortium based in Europe, North America, and Australia further confirmed the association between HCV infection and NHL and specific B-NHL subtypes, that is, diffuse large B-cell lymphoma (DLBCL), marginal zone lymphoma, and lymphoplasmacytic lymphoma [6]. In contrast, other studies from Northern Europe, UK, and Canada failed to show the