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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年度 総括研究報告書

研究代表者 伊藤 昌彦

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目 次

I. 総括研究報告

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解明および薬剤評価系の構築

伊藤 昌彦

1

II. 研究成果の刊行に関する一覧表

4

III. 研究成果の刊行物・別刷

5

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
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研究代表者 伊藤 昌彦 浜松医科大学医学部 助教

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、肝臓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臓細胞株と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を目指す。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を構築する。

平成23年度では、新規のHCV感受性正常肝細胞株を得るために、肝幹細胞からの分化誘導条件の検討を行った。また肝臓由来細胞株Huh-7からiPS細胞誘導により低分化細胞株を樹立した。これらの樹立細胞ではHCV感染能・複製能が異なっていたことから、網羅的な遺伝子発現解析による比較を行った。平成24年度では、これらの知見をもとに慢性C型肝炎感染患者由来のHCV株を感染、複製できる細胞株を樹立し、患者由来HCV株の病原性発現機構、薬剤感受性および耐性獲得機構の解明を行う。

A. 研究目的

国内における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、肝臓での年間死亡者は3万人を超える。肝硬変、肝臓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。現在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臓細胞株と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。これまでに、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Luciferase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) iPS 細胞の肝細胞誘導による HCV 感染増殖細胞の樹立

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 Huh7 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、iPS 細胞 (低分化細胞) を樹立した。

(3) 樹立細胞株の特性の解析

マイクロアレイによるトランスクリプトーム解析によって、樹立した HCV 感染感受性株と非感染感受性株との間での mRNA および miRNA の発現解析を行った。また、樹立した細胞株の肝組織特異的遺伝子の発現、肝細胞機能、HCV 感染・複製に関与する分子の発現を定量的 RT-PCR 法、免疫染色法、イムノブロットにより明らかにした。

(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、また一部の試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承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。
- (2) 成熟肝細胞特異的な遺伝子発現および HCV 感受性を指標に、肝幹細胞から適した分化誘導条件を確立した。その結果、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。
- (3) 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、iPS 細胞を樹立した。この細胞はアルカリフォスファター弱陽性で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。一方で、元株である Huh-7 とは異なり HCV 感染性、複製能を欠損していることを明らかにした。これらの細胞の mRNA、microRNA の網羅的な発現の比較検討を行い、誘導細胞で発現が顕著に亢進・低下が認められる遺伝子を数十種類見出すことができた。

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。

本年度の研究では、肝幹細胞の分化誘導および Huh-7 由来 iPS 細胞を樹立し、それらの細胞で HCV 感受性が異なることが明らかにした。細胞同士の遺伝子発現の比較により、HCV 生活環に関わる新規の宿主因子を同定することができる。目的である患者由来の様々な HCV クローンに感受性を持つ細胞株を樹立するための重要な知見となる。

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。ひきつづき、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、患者由来 HCV 株の薬剤感受性および耐性獲得機構の解明を行う。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などを解明する。

F. 健康危険情報 該当なし

G. 研究発表

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- 3) Ito M, Kusunoki H, Mochida K, Yamaguchi K, Mizuochi T: HCV infection and B-cell lymphomagenesis, *Adv Hematol*, 2011, 835314-835314, 2011

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H. 知的財産権の出願・登録状況 (予定を含む。)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

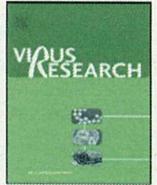
別紙4

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
<u>Mizuochi T, Ito M, Takai K, Yamaguchi K.</u>	Peripheral blood memory B cells are resistant to apoptosis in chronic hepatitis C patients	Virus Res	155	349-351	2011
<u>Ito M, Kusunoki H, Mizuochi T.</u>	Peripheral B cells as reservoirs for persistent HCV infection	Front Microbiol	2	177-177	2011
<u>Ito M, Kusunoki H, Mochida K, Yamaguchi K, Mizuochi T.</u>	HCV infection and B-cell lymphoma genesis	Adv Hematol	2011	835314-835314	2011



Short communication

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

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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 B cells, 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.

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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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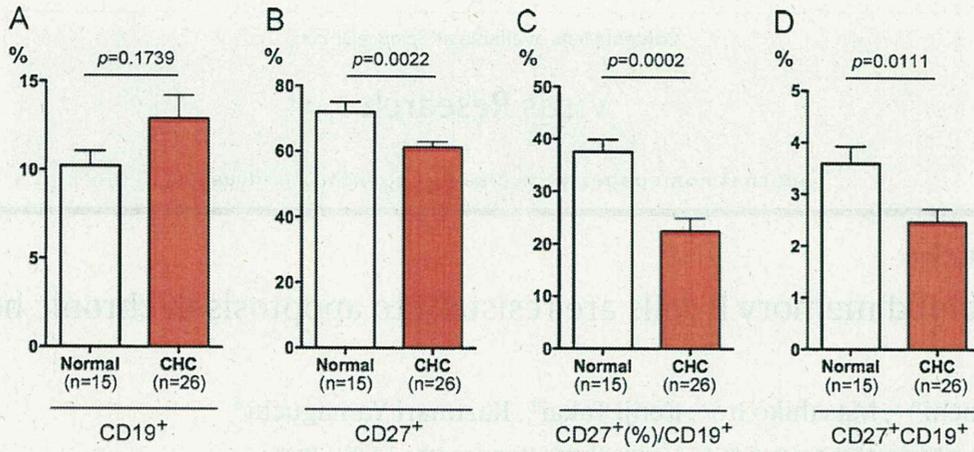


Fig. 1. Flow cytometric analysis of PBMC from normal subjects and CHC patients. Lymphocytes were identified by forward and orthogonal light scatter characteristics. Percentages of CD19⁺ cells (A), CD27⁺ cells (B), CD27⁺ (%) in CD19⁺ cells (C) and CD27⁺CD19⁺ cells (D) in normal (n = 15) and CHC patients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

appropriate 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* = 0.0111) 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-

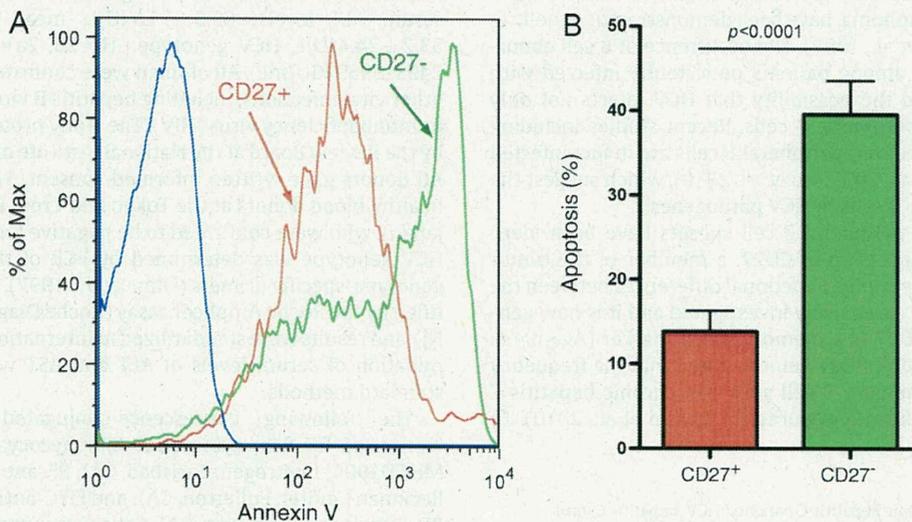


Fig. 2. Annexin V binding to CD27⁺ and CD27⁻ B cells. Representative staining patterns for Annexin V binding to CD27⁺ (red line) and CD27⁻ (green line) B cells are shown in CHC patients (A). Blue lines indicate background (bkg) staining in negative controls. Summary of data on Annexin V binding to CD27⁺ (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.

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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

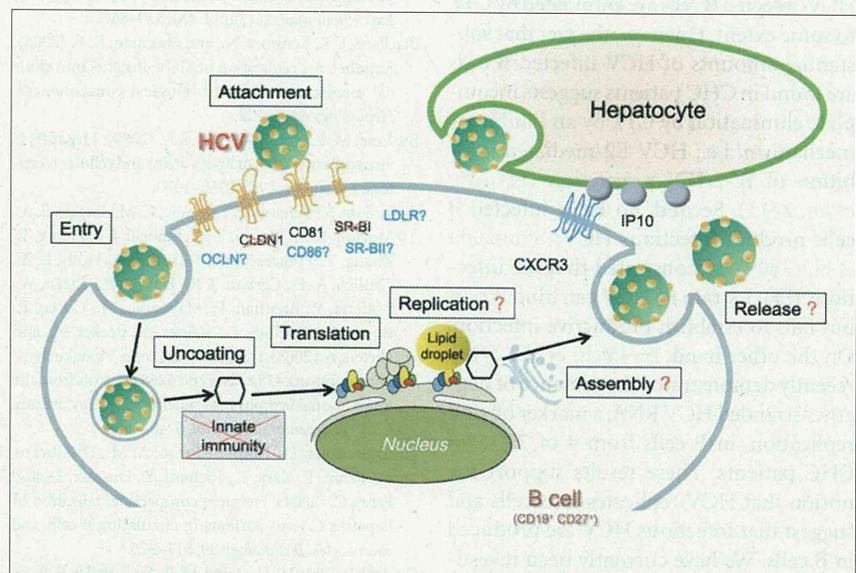


FIGURE 1 | Proposed lifecycle of HCV in B cell. The life cycle of the hepatitis C virus (HCV) has several specific steps. Virus entry is mediated by the direct interaction of envelope glycoproteins with co-receptors, presumably E2-CD81 binding. CLDN1 and SR-BI are not expressed in B cell. Innate immunity 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.

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Review Article

HCV Infection and B-Cell Lymphomagenesis

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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, extra-hepatic 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 PBMC 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 suppresses 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

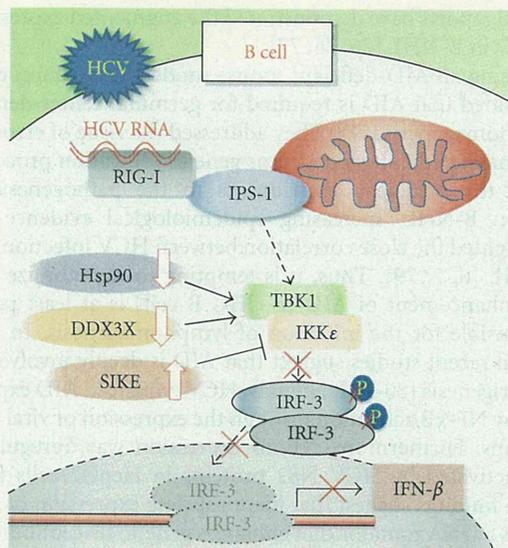


FIGURE 1: Impaired innate antiviral immunity in B cells of patients with chronic hepatitis C.

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 I κ B kinase- ϵ (IKK ϵ) 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 10kD) 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

association. Geographic differences in HCV genotype have been thought to cause these discrepancies [66] although this remains controversial. Large-scale, population-based, well-controlled studies are necessary to reach a robust conclusion. It can be concluded that, at least in areas with a high prevalence of HCV carriage, HCV is an important risk factor for B-cell lymphomagenesis.

In this paper, we propose a novel hypothesis that peripheral B cells serve as reservoirs for persistent HCV infection. We also suggest that long-lasting HCV infection in B cells may induce lymphoproliferative disorders that may eventually evolve into B-cell NHL, although little is known about the mechanism responsible for B-cell lymphomagenesis. In the remainder of this section, the possible mechanisms of B-NHL tumorigenesis induced by HCV infection will be discussed based on current knowledge of lymphomagenesis-related genes.

Activation-induced cytidine deaminase (AID) is essential for somatic hypermutation (SHM) and class switch recombination of immunoglobulin genes in B cells [67–69]. Recently, it has been proposed that AID may be instrumental in the initiation and progression of B-NHL. This is because a malfunction in either of the two processes stated above is apparently responsible for chromosomal translocations and aberrant SHM, which are the two main causes of genetic lesions associated with B-NHL [70, 71]. Several oncogenes have been demonstrated to be targets for SHM with immunoglobulin genes. In many cases, these anomalies activate the DNA damage response system that either allows DNA repair or eliminates the aberrant B-cell clones [72]. Failure of these repair systems may be a cause of B-cell malignancies. Specific features of SHM are the predominance of single-based substitution, the preference for transitions over transversion, and the specific targeting of the RGYW/WRCY motif. Pasqualucci et al. showed that hypermutation of proto-oncogenes exists in DLBCL [71]. However, in HCV-associated NHL, the number of mutations in some proto-oncogenes was lower than that already found in HCV-negative B-cell NHL patients [73]. Because there is a close association between HCV infection and the incidence of B-NHL, as described above [6, 7], analyzing the expression levels of AID in CHC B cells is of great interest.

Lai et al. established a B-cell line (SB) from HCV-infected B-NHL cells. The virus particles produced from SB cell culture could infect primary human hepatocytes, Raji cells, and PBMC in vitro [74]. They examined the expression of AID in Raji cells and PBMC after HCV infection in vitro. It was found that HCV infection activated the expression of AID in Raji cells. AID expression level was also higher in PBMC of patients infected with HCV than in uninfected individuals [75]. However, their study did not assess which cell population showed an enhancement of AID expression. It was observed in our recent study that expression levels of AID mRNA were markedly enhanced in the CD19⁺, but not in the CD19⁻ subset of patients with CHC [32]. Furthermore, the enhanced expression of AID protein was detected in the CD19⁺ B-cell subset of patients with CHC [32]. The fact that this enhancement of AID expression is confined to the B-cell subset is extremely intriguing because

several reports have demonstrated the augmented expression of AID in B-NHL [26, 76, 77].

Using an AID-deficient mouse model, Pasqualucci et al. concluded that AID is required for germinal center-derived lymphomagenesis [78]. They addressed the issue of errors in AID-mediated antigen receptor gene modification processes being the principal contributors to the pathogenesis of human B-NHL. Increasing epidemiological evidence has highlighted the close correlation between HCV infection and B-NHL [6, 7, 79]. Thus, it is tempting to hypothesize that the enhancement of AID in CHC B cells is at least partly responsible for the initiation of lymphomagenesis. In fact, several recent studies suggest that AID is deeply involved in tumorigenesis [80–84]. Notably, HCV enhanced AID expression by NF- κ B activation through the expression of viral core proteins. Furthermore, NF- κ B expression was upregulated and activated by HCV NS2 proteins in HepG2 cells [85]. These findings suggest that inappropriate expression of AID acts as a DNA mutator that enhances genetic susceptibility to mutagenesis [86].

Additionally, enhanced expression of other lymphomagenesis-related genes including cyclin D1, cyclin D2, BAL, STK15, and galectin-3 in CHC B cells is worth considering [32]. Overexpression of CCND1, which alters cell-cycle progression, is frequently observed in various tumors and may contribute to tumorigenesis [87, 88], whereas CCND2 is known to be expressed at constitutively high levels in B-NHL [89]. BAL is a novel risk-related gene in DLBCL, a typical B-NHL [90], while STK15 is a gene highly expressed in a histologically aggressive type of NHL [91]. Galectin-3 is an antiapoptotic protein, highly expressed in DLBCL [92]. Presumably, the enhanced expression of these genes in CHC B cells [32] may also correlate with B-cell lymphomagenesis.

Tumor necrosis factor alpha-induced protein 3, also called A20, was first identified in 1990 as a TNF-induced cytoplasmic protein with zinc finger motifs [93], which thereafter has been described as a key player in the negative regulation of inflammation by terminating NF- κ B signaling [94–96]. Recently, A20 has gained much attention as a novel tumor suppressor [97, 98]. Honma et al. first reported that A20 is frequently inactivated or even deleted in mantle cell lymphoma and DLBCL, and they raised the possibility that inactivation of A20 may be at least partly responsible for lymphomagenesis [99, 100]. Other investigators have subsequently supported their findings [101, 102]. Moreover, A20 also regulates antiviral signaling as well as programmed cell death [103–105]. Currently, the expression, biological activities, and mechanisms of action of A20 have been the focus of attention on a wide scale [106]. Interestingly, Ngueyn et al. reported [107] that the HCV core protein induced an increased expression of A20 in the human hepatocyte cell line HepG2, which has generated a genuine interest in the expression of A20 in peripheral B cells of patients with CHC. Our preliminary data suggests that the A20 molecule is partially cleaved in CHC B cells (Kusunoki et al.; in preparation). An intriguing possibility is that the A20 gene interacts with and is mutated by AID, the expression of which is dramatically enhanced in CHC B cells [32]. In

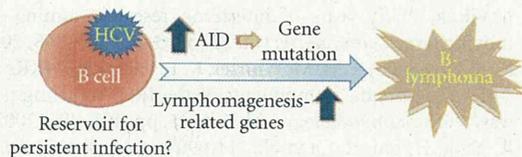


FIGURE 2: Role of HCV infection in B-cell lymphomagenesis, a hypothesis.

this regard, the expression levels of A20 in patients with B-NHL suffering from chronic HCV infection are worth investigating.

5. Conclusion

In this paper, we summarized recent studies illuminating the possible role of HCV infection in B-cell lymphomagenesis. We proposed a hypothesis that HCV utilizes B cells as reservoirs for persistent infection, which could result in the enhanced expression of lymphomagenesis-related genes, particularly AID, which is thought to be crucial for the initiation and progression of B-NHL (Figure 2). Elimination of HCV in plasma by antiviral reagents as well as in peripheral B cells by specific antibodies would be beneficial for patients with CHC to achieve a complete viral clearance. Finally, although a positive association between HCV infection and B-NHL occurrence is still being debated [108–111], it is worthwhile to investigate the possible mechanisms by which B-cell lymphoproliferative disorders, which may evolve into B-NHL, are induced in patients with CHC.

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