月 9 日-12 日. 於：東京都
15) 池田公史、奥坂拓志. 肝細胞癌に対する分子標的治療薬－最近の動向.（シンポジウム 15：消化器癌に対する分子標的治療薬－最近の動向　消 S15-4 指）（日本消化器病学会雑誌, 110, 臨時増刊号（第 55 回大会）, A625, 2013.）第 55 回日本消化器病学会大会. 2013 年 10 月 9 日-12 日. 於：東京都

H.知的所有権の出願・登録状況

①特許取得；なし
②実用新案登録；なし
③その他

ヒト肝組織を用いた線維化/脱線維化の解析

研究分担者　原田憲一　金沢大学形態機能病理学

研究要旨:HCV 関連の前硬変肝/肝硬変患者の肝実質では、組織学的正常肝に較べて活性化星細胞, TGF-β, MMP, Wnt 経路の関連分子の発現パターンが異なっており、また好中球や CD11b 陽性細胞の減少も見られ、肝線維化進展との関連性が示唆された。また、HCV 肝硬変の門脈域には細胆管反応を伴う interface 肝炎を認めるが、胆管細胞は自然免疫応答や炎症性サイトカイン刺激にて肝星細胞走化性分子 MCP1 を産生し、胆管細胞が直接肝星細胞を誘導し肝線維化に加担することが示唆された。PRI-724 投与による抗線維化機序を解明するため、Wnt シグナル伝達系の変化に加えて、肝星細胞, 細胞外マトリックス分解酵素, 炎症細胞および胆管細胞の観点からも解析する必要がある。

A.研究目的

PRI-724 は CREB -binding protein(CBP)/β-カテニンの複合体形成を選択的に阻害する低分子化合物であり、Wnt シグナルが異常亢進している癌細胞に対して細胞増殖抑制作用を示す。一方、Wnt シグナル伝達経路は肺線維症などの線維化にも関与しており、PRI-724 が HCV 慢性肝炎モデルマウスにおいて抗線維化作用を示すことも報告されている。我々の研究目的は、HCV 関連の肝硬変患者に関して PRI-724 が肝線維化を軽減することを確認し、更に PRI-724 による抗線維化機序の基礎的解析を行うことである。本年度、我々は HCV 関連肝硬変における肝線維化機序の病理学的解析ならびに Monocyte chemoattractant protein-1(MCP-1)を介した肝線維化における胆管細胞の関与について解析を行い、PRI-724 にて期待される抗線維化の機序解明に向けての予備的研究を施行した。

B.研究方法

B-1:肝組織における肝線維化関連因子の検出

対象：HCV 感染患者より診断目的に施行された肝生検材料で病理学的に F3～F4 相当の進行性肝疾患(前硬変肝～肝硬変)と診断された 10 例の肝針生検を対象とした。また、対照群として原因不明の肝障害患者より診断目的に肝生検が施行され、組織学的に特異的な所見が見られなかった症例(組織学的正常肝) 5 例を用いた。

方法：肝線維化および脱線維化に関与する細胞や分子として、①活性化星細胞マーカーである αSMA、②好中球マーカーである好中球エラスターゼ、③単球/マクロファージマーカーで NK 細胞, 顆粒球にも発現する CD11b、④M2 マクロファージのマーカーである CD163、⑤主要な profibrogenic factor である TGF-β、⑥細胞外マトリックス分解酵素である MMP1, MMP8、⑦Wnt 経路のシグナル伝達分子である β カテニン, CBP, P300 の免疫組織化学的解析を施行した。

B-2:肝線維化進展における胆管細胞の関与

対象：培養ヒト胆管細胞２株、ヒト肝星細胞株(LI90, HSRRB より分与)および HCV 関連慢性肝炎の他、各種肝疾患患者より得られた肝針生検。

方法：ヒト培養胆管細胞における profibrogenic factor (MCP-1, PDGF-B, CTGF, endothelin 1, TGF-β)の mRNA を PCR 法にて検出し、さらに poly(I:C) (TLR3 リガンド), Pam3CSK4(TLR1/2 リガンド), LPS (TLR4 リガンド)または炎症性サイトカイン(IL-1β, IL-4, IL-6, IFNγ, TNFα)刺激による profibrogenic factor の動態を解析するため、Real-time PCR および一部 ELISA 法にて検出した。また、ヒト肝星細胞株における MCP-1 受容体(CCR2)の発現および TLR リガンドや炎症性サイトカイン刺激による動態を検討した。最後に、肝組織切片を対象に、MCP-1, αSMA, CCR2 の免疫組織化学的染色を施行した。

(倫理面への配慮)

本研究は、金沢大学ヒトゲノム・遺伝子解析研究倫理審査委員会にて承認済みである。課題名：「胆道系炎症性疾患および胆道系腫瘍の病理学的解析　－病理検体の研究目的での使用について－」(審査番号 305)。

C. 結果

C-1: 肝組織における肝線維化関連因子の検出

組織学的正常肝では、好中球エラスターゼ陽性好中球および CD11b 陽性細胞を実質内に散見したが、HCV 前硬変/肝硬変症例ではこれらの陽性細胞はごく少数のみであり、正常肝に較べて減少していた。CD163 陽性の M2 マクロファージは正常肝、HCV 症例ともに実質内に多数の細胞を認め、両群間で明らかな差違は認めなかった。αSMA 陽性の活性化肝星細胞は主に HCV 症例の interface 肝炎を伴う門脈域周囲に多数認めた。TGF-β 陽性細胞は、HCV 症例の類洞内細胞に散見し、また実質内の壊死部の肝細胞にも陽性所見を認めた。MMP1, MMP8 は好中球等の多核白血球の他、肝細胞に弱い発現を認め、MMP1 は胆管にも弱い発現を認めた。Wnt 経路の関連分子である β カテニン, CBP, P300 のうち、活性型を示唆する β カテニン, CBP の核発現は、HCV 症例の interface 肝炎部周囲の肝細胞に発現を認めた。しかし、P300 の発現は正常肝の実質肝細胞にびまん性に発現を認めるものの、HCV 症例では全体的に P300 の発現が低下していた。

C-2: 肝線維化進展における胆管細胞の関与

培養ヒト胆管細胞を用いた検討にて、胆管細胞から MCP-1, PDGF-B, CTGF, endothelin 1, TGF-β のすべての profibrogenic factor の mRNA を検出できた。また、TLR リガンド (Pam3CSK4, poly(I:C) , LPS) および炎症性サイトカインの IL-1β, TNFα の刺激にて MCP1 発現の亢進が見られ(図 1)、ELISA 法でも MCP1 蛋白の分泌および産生亢進が確認出来た(培養上清中で 8-10ng/ml)(図 2)。また、ヒト肝星細胞は MCP1 リガンドである CCR2 を発現していなかったが、poly(I:C)および TNFα 刺激にて CCR2 発現の誘導が見られた。また、肝組織切片を用いた免疫組織化学的検討にて、MCP1 の発現は門脈域周囲の増生細胆管に発現を認め、同部では αSMA および CCR2 陽性の活性化肝星細胞も見られた(図 3)。

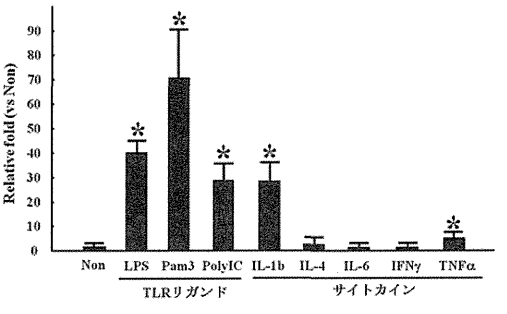


図 1　培養胆管細胞における TLR リガンド,炎症性サイトカイン刺激による MCP-1 mRNA 発現の変化。Real time PCR 法。*<0.01.

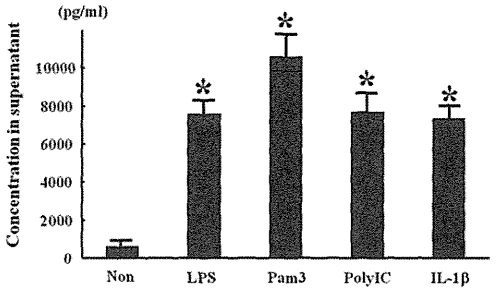
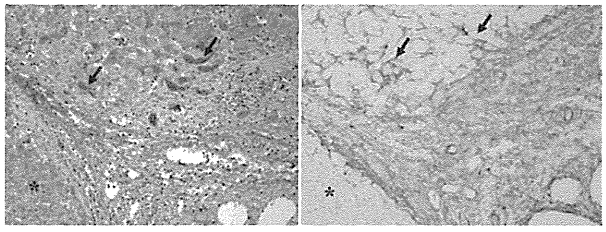


図 2　培養胆管細胞における TLR リガンド,炎症性サイトカイン刺激による培養上清中 MCP-1 蛋白濃度。ELISA 法。*<0.01.



A　　B

図 3　HCV 関連肝硬変。MCP1(A), αSMA(B)の免疫染色。MCP1 陽性の細胆管増生部位にはαSMA 陽性活性化肝星細胞を散見するが(矢印)、細胆管増生を認めない部位ではαSMA 陽性活性化肝星細胞は認めない(＊)。

D.考察

肝線維化の発生および進展に、活性化肝星細胞から筋線維芽細胞への分化と細胞外マトリックスの異常増加が重要である。今回の検討により αSMA 陽性の活性化肝星細胞(筋線維芽細胞)は HCV 前硬変肝/肝硬変の interface 肝炎部を中心に多数出現し、静止期の線維芽細胞に対して活性化した線維芽細胞の遺伝子発現を引き起こす TGFβ の発現も見られた。また、HCV 症例では組織学的正常肝に較べて好中球, CD11b 陽性細胞が減少していた。好中球は MMP1, MMP8 を産生する細胞であり、HCV 前硬変肝/肝硬変では MMP 関連の線溶系機能不全が起こっていることが示唆された。HCV トランスジェニックマウスを用いた検討では、PRI-724 投与による抗線維化の作用機序として単球/マクロファージ, 好中球などの炎症性細胞の増加および肝内 MMP-8 の上昇が指摘されており、ヒト HCV 患者でも PRI-724 投与による同様な抗線維化機構が期待される。また、活性型 β カテニン, CBP の発現を interface 肝炎部周囲の肝細胞に認め、PRI-724 の有効性が期待される所見と考えられた。なお、CD163 陽性細胞はいわゆる M2 マクロファージと解されているが、今回の検討では正常肝、HCV 肝ともに多数の陽性細胞を類洞内に認め、CD163 陽性クッパー細胞の明らかな増減は認めなかった。

MCP-1 は単球/マクロファージの走化性因子および活性化因子として作用するケモカインで、また肝星細胞の走化性因子として肝線維化にも関与する(Marra et al., *Hepatology*, 1999)。今回の検討により、MCP1 産生細胞として interface 肝炎部に出現する増生細胆管を見出し、特に TLR を介した自然免疫応答および IL-1β 等による炎症性サイトカインによる MCP1 発現の誘導が見られた。細胆管増生は種々の病的肝で出現する非特異的反応であるが、慢性進行性肝疾患では特に目立つ組織反応であり、病期進展に大きく関連することが推測されている。しかし、病期進展の直接的な関与の機序については不明であったが、今回の検討により、増生細胆管の胆管細胞は自然免疫応答や炎症性反応により MCP1 を産生し、肝星細胞の動員に関与することが示唆された。一方、肝星細胞は、通常の培養状態では MCP1 の受容体である CCR 2 の発現を認めなかったが、自然免疫応答および炎症性サイトカインにて CCR2 の発現誘導が見られた。これらの所見より、通常の生理的環境では胆管細胞からの MCP1 産生や肝星細胞の CCR2 発現はないものの、感染や炎症に伴って胆管細胞からの MCP1 産生および肝星細

胞の CCR2 発現が惹起され、肝星細胞の誘導、TGF-β 作用により線維化を来すことが示唆され、増生細胆管の組織反応が直接肝線維化に関わることが明らかとなった。

E.結論

HCV 肝硬変患者に対する PRI-724 投与の有効性を確認し、さらにその機序を解明するためには、Wnt シグナル伝達系の変化に加えて、肝星細胞，細胞外マトリックス分解酵素，炎症細胞および胆管細胞の観点からも解析する必要があると考えられた。

F.健康危険情報

特になし

G.研究発表

1. 論文発表

1) Harada K, Kakuda Y, Sato Y, Ikeda H, Shimoda S, Yamamoto Y, Inoue H, Ohta H, Kasashima S, Kawashima A, Nakanuma Y. Alteration of energy metabolism in the pathogenesis of bile duct lesions in primary biliary cirrhosis. J Clin Pathol. 2013 Nov 29.

2) Harada K, Kakuda Y, Nakamura M, Shimoda S, Nakanuma Y. Clinicopathological significance of serum fractalkine in primary biliary cirrhosis. Dig Dis Sci 2013 Jun 14.

3) Harada K, Sato Y, Ikeda H, Hsu M, Igarashi S, Nakanuma Y. Notch1-Hes1 signalling axis in the tumourigenesis of biliary neuroendocrine tumours. J Clin Pathol 2013;66:386-391

4) Harada K, Chiba M, Okamura A, Hsu M, Sato Y, Igarashi S, Ren XS, Ikeda H, Ohta H, Kasashima S, Kawashima A, Nakanuma Y. Monocyte chemoattractant protein-1 derived from biliary innate immunity contributes to hepatic fibrogenesis. J Clin Pathol 2011 Aug;64(8):660-5

2. 学会発表

1) Harada K, et al. IgG4 reaction in biliary intraepithelial neoplasia and cholangiocarcinoma arising from sclerosing cholangitis. The 102nd USCAP ANNUAL MEETING (2013. Mar 2- 8, Baltimore, USA)

2) Harada K, et al. Energy metabolic change induced by ERRα-PGC-1α axis in chronic non-suppurative destructive cholangitis of primary biliary cirrhosis. The 102nd USCAP ANNUAL MEETING (2013. Mar 2- 8, Baltimore, USA)

3) Harada K, et al. Pathological Differences between IgG4-Related Sclerosing Cholangitis with and without Autoimmune Pancreatitis. The 102nd USCAP ANNUAL MEETING (2013. Mar 2- 8, Baltimore, USA)

4) Harada K, et al. Clinicopathological significance of serum fractalkine and its regulation by ursodeoxycholic acid in primary biliary cirrhosis. AASLD, The Liver Meeting 2013 (2013.Nov 1-5, Washington, DC)

5) Harada K, et al. Alteration of energy metabolism and subsequent oxidative stress in the pathogenesis of cholangiopathy in primary biliary cirrhosis. AASLD, The Liver Meeting 2013 (2013.Nov 1-5, Washington, DC)

6) 原田憲一，中沼安二．自己免疫性膵炎合併の有無からみた IgG4 硬化性胆管炎の病

態の違い．第 99 回日本消化器病学会総会（2013 年 3 月 21～23 日、鹿児島）

7）原田憲一，中沼安二．原発性胆汁性肝硬変の障害胆管におけるエネルギー代謝の変化．第 49 回日本肝臓学会総会（2013 年 6 月 6～7 日、東京）

8）原田憲一，佐藤保則，中沼安二．IgG4 関連硬化性胆管炎に出現する Biliary intraepithelial neoplasia（BiIIN）病変．第 49 回日本肝癌研究会（2013 年 7 月 11～12 日、東京）

9）原田憲一，池田博子，佐藤保則，中沼安二．胆管細胞腺腫における glucose transporter 1 発現　－悪性ポテンシャルの解析―．第 49 回日本肝癌研究会（2013 年 7 月 11～12 日、東京）

10）原田憲一，中沼安二．原発性胆汁性肝硬変の胆管障害機序：エネルギー代謝の変化と細胞死の関与．第 50 回日本消化器免疫学会総会　（2013 年 8 月 1～2 日，東京）

11）原田憲一，中沼安二．原発性胆汁性肝硬変の胆管傷害とエネルギー代謝との関連性．第 45 回日本臨床分子形態学会総会・学術集会　（2013 年 9 月 13～14 日、福岡）

12）原田憲一，佐藤保則，中沼安二．IgG4 関連硬化性胆管炎に見られる BilIN 病変の解析．第 49 回日本胆道学会学術集会（2013 年 9 月 19～20 日、千葉）

13）原田憲一，中沼安二．原発性胆汁性肝硬変の障害胆管における endocytosis 機能の低下．第 17 回日本肝臓学会大会　（2013 年 10 月 9～10 日、東京）

14）原田憲一，中沼安二．原発性胆汁性肝硬変の障害胆管におけるエネルギー代謝と細胞死との関連．第 17 回日本肝臓学会大会　（2013 年 10 月 9 日～10 日、東京）

15）原田憲一，中沼安二．胆道系自然免疫応答による胆道閉鎖症の病態形成機序．第 40 回日本胆道閉鎖症研究会　（平成 25 年 11 月 16 日、茨城）

16）原田憲一，中沼安二．原発性胆汁性肝硬変の障害胆管におけるエネルギー代謝の変化―脂肪酸代謝系への偏位による胆管細胞アポトーシス感受性の亢進―．第 40 回日本肝臓学会西部会　（平成 25 年 12 月 6～7 日、岐阜）

17）原田憲一，中沼安二．原発性胆汁性肝硬変の障害胆管における endocytosis 関連分子の解析．第 40 回日本肝臓学会西部会　（平成 25 年 12 月 6～7 日、岐阜）

18）原田憲一，池田博子，佐藤保則，中沼安二．胆管癌および biliary intraepithelial neoplasia（BilIN）における glucose transporter1 の発現．第 40 回日本肝臓学会西部会　（平成 25 年 12 月 6～7 日、岐阜）

H.知的所有権の出願・登録状況

①特許取得　特になし

②実用新案登録　特になし

③その他

肝線維化における腫瘍壊死因子(TNF-α)の役割

研究協力者　大澤陽介　岐阜大学大学院医学系研究科

研究要旨:肝線維化における腫瘍壊死因子（TNF-α）の役割を胆管結紮肝障害モデルと TNF-α欠損マウスを用いて検討した。TNF-αは肝細胞障害、炎症細胞浸潤、肝再生には関与せずに肝線維化を促進することが示唆され、その機序として肝星細胞の TIMP-1 発現が関与することが示唆された。

A.研究目的

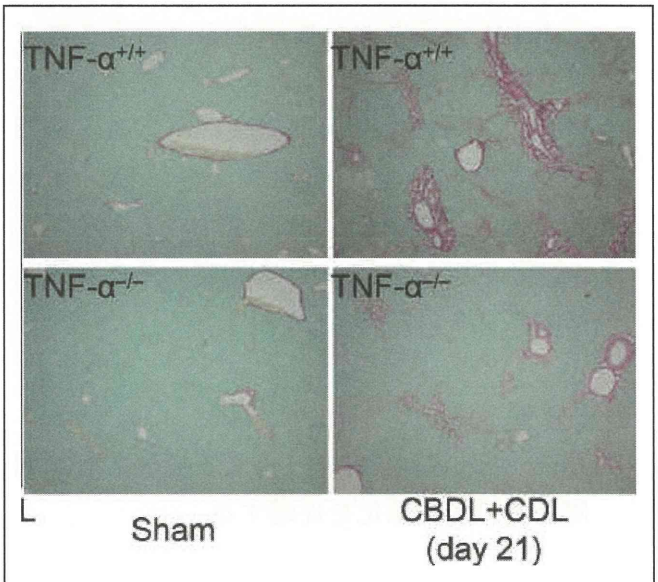
腫瘍壊死因子(TNF-α)は炎症性サイトカインの一つであり、C型肝炎をはじめとした種々の肝障害に深く関与している。動物実験モデルでは、TNF-α欠損マウスやその受容体の欠損マウスで、肝障害や肝線維化が抑制されることが報告されており、TNF-αは肝障害・肝線維化に促進的に働くと考えられている。しかし TNF-αが肝線維化を促進する機序については不明な点が多い。そこで、これらにおける TNF-αの役割について、胆管結紮肝障害モデルと TNF-α欠損マウスを用いて検討した。

B.研究方法

TNF-$\alpha^{+/+}$および TNF-$\alpha^{-/-}$マウスの総胆管と胆のう管を結紮することにより、肝細胞障害および肝線維化を誘導し、肝障害・線維化、マクロファージ・リンパ球浸潤、肝再生を比較した。肝星細胞（HSCs）のコラーゲン産生における TNF-αの影響を検討するためラットより HSCs を分離し、TNF-α添加後のコラーゲン mRNA を測定した。さらに、肝線維化における TIMP-1 の役割を検討するため TIMP-1 mRNA 発現を比較し、肝組織における TIMP-1 の局在を検討した。また、TIMP-1 欠損の肝線維化におよぼす影響を検討した。

C.研究結果

胆管結紮により血清中 TNF-αは増加した。TNF-$\alpha^{-/-}$マウスでは胆管結紮後の肝線維染色（下図）およびヒドロキシプロリンの増加が抑制された。



TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice received bile duct ligation (CBDL+CDL). The animals were sacrificed on 21 days after the surgery. Collagen deposition was assessed by Sirius red staining.

一方、血清 ALT, T-BIL, 肝組織中 F4/80 陽性細胞、CD3 陽性細胞、Ki67 陽性細胞、および Cyclin E 発現は、TNF-$\alpha^{+/+}$と TNF-$\alpha^{-/-}$マウスで差が無かった。このことから、TNF-

αは肝細胞障害、炎症細胞浸潤、肝再生には関与せずに肝線維化を促進することが示唆された。初代培養 HSCs に TNF-α を添加するとコラーゲン mRNA の発現が低下し、TNF-α が直接コラーゲン発現を促進させる効果は無かった。MMP 阻害作用をもつ TIMP-1 は胆管結紮後の肝組織で発現が増加し、免疫染色では desmin 発現細胞で強く発現が認められた。また、初代培養 HSCs に TNF-α を添加すると TIMP-1 mRNA の発現が増強した。さらに TIMP-1$^{-/-}$マウスでは胆管結紮後の肝線維化が抑制された。

D.考察

TNF-α は肝細胞障害、炎症細胞浸潤、肝再生には関与せずに肝線維化を促進することが示唆された。これまで、TNF-α は肝細胞障害を介して肝線維化を誘導すると考えられていたが、この結果から、TIMP-1 を介して肝線維化に直接的な促進効果があることが明らかとなった。今後、肝星細胞や TIMP-1 欠損マウスを用いて Wnt シグナルとの関連に関する解析を進め、肝線維化に対する新規治療の開発を目指す。

E.結論

TNF-α は HSCs の TIMP-1 発現を介して肝細胞障害後の肝線維化を促進する。

F.健康危険情報

なし

G.研究発表

1. 論文発表

1) Osawa Y, Hoshi M, Yasuda I, Saibara T, Moriwaki H, Kozawa O. Tumor necrosis factor-α promotes cholestasis-induced liver fibrosis in the mouse through tissue inhibitor of metalloproteinase-1 production in hepatic stellate cells. PLoS One. 8, e65251, 2013.

2) Osawa Y, Suetsugu A, Matsushima-Nishiwaki R, Yasuda I, Saibara T, Moriwaki H, Seishima M, Kozawa O. Liver acid sphingomyelinase inhibits growth of metastatic colon cancer. J Clin Invest. 123, 834-843, 2013.

2. 学会発表

1) 大澤陽介　森脇久隆：ワークショップ「胆汁酸研究の最近の進歩」胆汁酸による肝細胞障害および肝線維化における TNF-α の役割　第49回日本肝臓学会総会，東京，2013.

2) 大澤陽介　森脇久隆：パネルディスカッション3「機能性食品や補助食品の消化器疾患における役割」アミノ酸の肝脂肪化に対する影響.　第55回日本消化器病学会大会，東京，2013

3) 大澤陽介　森脇久隆：肝線維化における腫瘍壊死因子（TNF-α）の役割.　第40回日本肝臓学会西部会　岐阜　2013

H.知的所有権の出願・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

Ⅲ　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kimura K.	①Should we try antiviral therapy for hepatitis C virus infection with pyoderma-gangrenosum-like lesions?	Hepatol Res	44	173-175	2014 (in press)
Aoki J, Kowazaki Y, Ohtsuki T, Kimura K. et al.	②Kinetics of Peripheral Hepatitis B Virus-specific $CD8^+$ T Cells in Patients with Onset of Viral Reactivation.	J gastroenterology	48	728-37	2013
Sekiguchi S, Kimura K, et al.	③Immunization with a Recombinant Vaccinia Virus That Encodes Nonstructural Proteins of the Hepatitis C Virus Suppresses Viral Protein Levels in Mouse Liver	PLOS ONE	Vol.7 No.12	e51656(1-12)	2012
Sakata, K. Kojima S, et al.	④HCV NS3 protease enhances liver fibrosis via binding to and activating TGF-β type I receptor	Sci. Rep.	3	3243	2013
Qin Xian-Yang, Kojima S, et al.	⑤The Effect of Acyclic Retinoid on the Metabolomic Profiles of Hepatocytes and Hepatocellular Carcinoma Cells	PLOS ONE	Vol.8 No.12	e82860(1-11)	2013
Sakata, K. Kojima S et al.	⑥Neovessel forma- tion promotes liver fibrosis via providing latent transforming growth factor-β	Biochem. Biophys. Res. Commun.:	443(3)	950-956.	2014
Yamagata H, Ikejima K, et al.	⑦Altered expression and function of hepatic natural killer T cells in obese and diabetic KK-A^y mice.	Hepatol Res	43(3)	276-288	2013

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hosoya S, Ikejima K, et al.	⑧Innate immune responses involving natural killer and natural killer T cells promote liver regeneration after partial hepatectomy in mice.	Am J Physiol Gastrointest Liver Physiol.	304(3)	G293-G299	2013
Fukuo Y, Ikejima K, et al.	⑨Abnormality of autophagic function and cathepsin expression in the liver from patients with non-alcoholic fatty liver disease.	Hepatol Res.	doi: 10.1111/hepr.12282.	1-11	2013
Okusaka T, Ojima H, Morizane C, Ikeda M, Shibata T	⑩Emerging drugs for biliary cancer.	Expert Opin Emerg Drugs.	(Vol.19 No.1 (2014))		2013
Valle JW, Furuse J, Jitlal M, Bridgewater J, Okusaka T et al.	⑪Cisplatin and gemcitabine for advanced biliary tract cancer: a meta-analysis of two randomised trials.	Ann Oncology	25(2)	391-398	2014
Arai Y, Totoki Y, Hosoda F, Okusaka T, et al.	⑫Fibroblast growth factor receptor 2 tyrosine kinase fusions define a unique molecular subtype of cholangiocarcinoma.	Hepatology			2013
Ueno H, Ioka T, Okusaka T, et al.	⑬Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study.	J Clin Oncol.	31(13)	1640-1648	2013

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Harada et al.	⑭Alteration of energy metabolism in the pathogenesis of bile duct lesions in primary biliary cirrhosis.	J Clin Pathol			2014 (in press)
Harada et al.	⑮Clinicopathological significance of serum fractalkine in primary biliary cirrhosis.	Dig Dis Sci	58	3037-3043	2013
Harada et al.	⑯Notch1-Hes1 signalling axis in the tumourigenesis of biliary neuroendocrine tumours.	J Clin Pathol	66	386–391	2013
Harada et al.	⑰Monocyte chemoattractant protein-1 derived from biliary innate immunity contributes to hepatic fibrogenesis.	J Clin Pathol	64	660-665	2011
Osawa Y, Hoshi M, Yasuda I, Saibara T, Moriwaki H, Kozawa O.	⑱Tumor necrosis factor-αpromotes cholestasis-induced liver fibrosis in the mouse through tissue inhibitor of metalloproteinase-1 production in hepatic stellate cells.	PLOS One.	Vol.8 No.6	e65251(1-10)	2013
Osawa Y, Suetsugu A, Matsushima-Nishiwaki R, et al.	⑲Liver acid sphingomyelinase inhibits growth of metastatic colon cancer.	J Clin Invest	123	834-843	2013

Ⅳ　研究成果の刊行物・別刷

Hepatology Research

Hepatology Research 2014; 44: 173–175 doi: 10.1111/hepr.12158

Editorial

Should we try antiviral therapy for hepatitis C virus infection with pyoderma gangrenosum-like lesions?

See article in *Hepatology Research* 44: 238–245
Eradication of hepatitis C virus could improve immunological status and pyoderma gangrenosum-like lesions
Yasuteru Kondo, Tomoaki Iwata, Takahiro Haga, Osamu Kimura, Masashi Ninomiya, Eiji Kakazu, Takayuki Kogure, Tatsuki Morosawa, Setsuya Aiba and Tooru Shimosegawa

Pyoderma gangrenosum (PG) is a rare, ulcerative and painful neutrophilic dermatosis without any infectious signs. PG usually begins with pustules, red papules, plaques or nodules that rapidly grow to ulcerations with undetermined purple borders.[1] Five clinical variants have been characterized: classic, bullous, pustular, vegetative and peristomal types. Although PG is idiopathic in 25–50% of cases, approximately 50% of clinical cases are associated with underlying systemic diseases such as inflammatory bowel disease, rheumatological disease, paraproteinemia, hematological malignancies, and HIV and hepatitis virus infection.[2] PG may be due to neutrophilic dysfunction, autoinflammation, genetic factors and/or inflammatory cytokines.[3] PG is diagnosed by excluding other cutaneous ulcerative diseases by biopsy and identification of clinical features, because PG has no specific histopathological features. There is no gold standard for treatment of PG, therefore, therapy should focus on the associated underlying systemic disease. In addition to local wound management and pain control, topical or systemic agents have been considered. For mild and superficial lesions, topical agents (e.g. corticosteroids, tacrolimus and cyclosporin) are administrated. When more effective treatment is required, corticosteroids and cyclosporin are considered as first-line systemic agents. Recently, the use of tumor necrosis factor (TNF)-α inhibitors for PG has emerged, and infliximab is the only efficacious systemic agent.[4,5]

Hepatitis C virus (HCV) infection may present with various cutaneous manifestations such as urticaria, pruritus, cryoglobulinemia, erythema multiforme, granuloma annulare, porphyria cutanea tarda, vasculitis, lichen planus, vitiligo, erythema nodosum, pityriasis rubra pilaris and perniosis-like lesions.[6] PG is thought to be an extrahepatic cutaneous manifestation of HCV infection, although its frequency is low.[7] Only few cases of PG associated with chronic hepatitis C have been reported. Smith *et al.* reported the first case of chronic hepatitis C and PG.[8] Several skin biopsies were performed, but leukocytoclastic vasculitis in cutaneous ulcerative lesions on the lower legs, with typical clinical manifestations of PG, was not observed. Therefore, it was suggested that the PG-like lesion was not associated with mixed cryoglobulinemia (MC) because of the absence of histological findings. Treatment with interferon (IFN)-α-2a was started and the lesion improved within 5 weeks. Keane *et al.* reported another case, in which biopsies of ulcerative lesions in the lateral aspect of the calf showed a dense mixed lymphocytic infiltration in the dermis and vasculitis in the erythematous ulcer rim. Treatment with topical clobetasol and systemic minocycline resulted in improvement within several weeks, and there was no recurrence during 4 months of follow-up.[9] Currently, no specific treatment regimen for PG associated with chronic hepatitis C has been established, because there are few case reports and the pathogenic mechanism is still unknown.

In general, PG is also associated with the administration of drugs such as granulocyte colony-stimulating factor,[10] antipsychotic agents and IFN-α.[11] Indeed, IFN-α treatment has some well-known cutaneous side-effects such as dry skin, hair loss and vitiligo.[12,13] Furthermore, cutaneous ulcerations, indurated erythema and leukocytoclastic vasculitis may occur at IFN-α injection sites. The pathogenesis of local cutaneous reactions against IFN-α is still unknown. Several case reports have suggested that PG results from IFN-α treatment during the course of hematological malignancies. One case was improved by treatment with cyclosporin A and prednisone.[14] In addition, serum levels of TNF-α, interleukin

(IL)-6 and soluble IL-2 receptor increased when the cutaneous lesions appeared and returned to normal levels when the lesion healed. This indicated that the onset of PG was required for elevated inflammatory cytokine levels.[14] Furthermore, Yurci *et al.* reported the first PG case resulting from pegylated (PEG)-IFN-α-2a during the treatment of chronic hepatitis C.[15] Four weeks after starting PEG-IFN-α-2a treatment, cutaneous ulcerative lesions were clinically suspected as PG appeared, and treatment was discontinued after 8 weeks. After withdrawal of PEG-IFN-α-2a, PG-like lesions improved. Thus, PEG-IFN-α-2a may trigger cutaneous side-effects because pegylation allows stable serum levels of drugs.

Thus, the pathogenesis of PG with chronic hepatitis C is still unclear, suggesting that PG related to chronic hepatitis C is not associated with MC, but with other immunological mechanisms. In a study reported in this issue, Kondo *et al.* examined whether peripheral blood mononuclear cells (PBMC), including activated B cells, T-helper (Th)1 cells, Th2 cells, Th17 cells and $CD4^+CD25^+IL7R^-$ regulatory T cells (Treg) were involved in PG disease activity.[16] They demonstrated that Th17 cell numbers were markedly higher in patients with chronic hepatitis C and PG compared with those without extrahepatic immunological complications. In addition, after clearance of HCV following IFN therapy, the abnormal immunological status returned to normal and the PG-like lesions recovered without immunosuppressive therapy. These findings suggest that Th17 cells have an important role in the onset of PG, although it is still unknown whether these cells are HCV specific. As reported in this issue, HCV-infected CD4 T cells are required for disease progression, suggesting that HCV-specific Th17 cells are responsible for development of PG.

Th17 immunity has been identified during HCV infection, and PBMCs from HCV antibody positive patients secrete IL-17, IFN-γ, IL-10, and transforming growth factor (TGF)-β in response to stimulation with HCV non-structural protein (NS)4. The authors suggested that both HCV-specific Th1 and Th17 cells were suppressed by NS4-induced production of the innate anti-inflammatory cytokines, IL-10 and TGF-β.[17] This suggests that HCV infection suppresses antigen-specific Th17 cell functions and is responsible for induction of persistent chronic infection.

Although it has not been demonstrated that PG-like lesions are not associated with cryoglobulinemia, Kondo *et al.* have suggested an alternative hypothesis in which the pathogenesis of PG-like lesions with chronic hepatitis C is associated with Th17-related immunity. To the best of our knowledge, this report is the first evidence that the onset of PG is related to the number and cytokine production of Th17 cells.

According to the study reported in this issue and other recent reports, PG may be induced by IFN treatment as well as HCV infection. Kondo *et al.* have demonstrated that IFN treatment is more effective than immunosuppressants for the treatment of PG in the case of HCV infection. Thus, when a case presents with HCV and PG it may be useful to consider IFN treatment. The validity of IFN for PG should be verified by accumulating a greater number of clinical cases.

Kiminori Kimura
Hepatology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan

REFERENCES

1 Wollina U, Haroske G. Pyoderma gangraenosum. *Curr Opin Rheumatol* 2011; **23**: 50–6.
2 Miller J, Yentzer BA, Clark A, Jorizzo JL, Feldman SR. Pyoderma gangrenosum: a review and update on new therapies. *J Am Acad Dermatol* 2010; **62**: 646–54.
3 Ruocco E, Sangiuliano S, Gravina AG, Miranda A, Nicoletti G. Pyoderma gangrenosum: an updated review. *J Eur Acad Dermatol Venereol* 2009; **23**: 1008–17.
4 Adisen E, Oztas M, Gurer MA. Treatment of idiopathic pyoderma gangrenosum with infliximab: induction dosing regimen or on-demand therapy? *Dermatology* 2008; **216**: 163–5.
5 Hughes AP, Jackson JM, Callen JP. Clinical features and treatment of peristomal pyoderma gangrenosum. *JAMA* 2000; **284**: 1546–8.
6 Mehta S, Levey JM, Bonkovsky HL. Extrahepatic manifestations of infection with hepatitis C virus. *Clin Liver Dis* 2001; **5**: 979–1008.
7 Crowson AN, Nuovo G, Ferri C, Magro CM. The dermatopathologic manifestations of hepatitis C infection: a clinical, histological, and molecular assessment of 35 cases. *Hum Pathol* 2003; **34**: 573–9.
8 Smith JB, Shenefelt PD, Soto O, Valeriano J. Pyoderma gangrenosum in a patient with cryoglobulinemia and hepatitis C successfully treated with interferon alfa. *J Am Acad Dermatol* 1996; **34**: 901–3.
9 Keane FM, MacFarlane CS, Munn SE, Higgins EM. Pyoderma gangrenosum and hepatitis C virus infection. *Br J Dermatol* 1998; **139**: 924–5.
10 Farina MC, Requena L, Domine M, Soriano ML, Estevez L, Barat A. Histopathology of cutaneous reaction to granulocyte colony-stimulating factor: another pseudomalignancy. *J Cutan Pathol* 1998; **25**: 559–62.

11 Mir-Bonafe JM, Blanco-Barrios S, Romo-Melgar A, Santos-Briz A, Fernandez-Lopez E. Photoletter to the editor: localized pyoderma gangrenosum after interferon-alpha2b injections. *J Dermatol Case Rep* 2012; **6**: 98–9.
12 Berk DR, Mallory SB, Keeffe EB, Ahmed A. Dermatologic disorders associated with chronic hepatitis C: effect of interferon therapy. *Clin Gastroenterol Hepatol* 2007; **5**: 142–51.
13 Hadziyannis SJ. Skin diseases associated with hepatitis C virus infection. *J Eur Acad Dermatol Venereol* 1998; **10**: 12–21.
14 Montoto S, Bosch F, Estrach T, Blade J, Nomdedeu B, Nontserrat E. Pyoderma gangrenosum triggered by alpha2b-interferon in a patient with chronic granulocytic leukemia. *Leuk Lymphoma* 1998; **30**: 199–202.
15 Yurci A, Guven K, Torun E *et al.* Pyoderma gangrenosum and exacerbation of psoriasis resulting from pegylated interferon alpha and ribavirin treatment of chronic hepatitis. C. *Eur J Gastroenterol Hepatol* 2007; **19**: 811–5.
16 Kondo Y, Iwata T, Haga T *et al.* Eradication of hepatitis C virus could improve immunological status and pyoderma gangrenosum-like lesions. *Hepatol Res* 2014; **44**: 238–45.
17 Lemmers A, Moreno C, Gustot T *et al.* The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatology* 2009; **49**: 646–57.

J Gastroenterol (2013) 48:728–737
DOI 10.1007/s00535-012-0676-y

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Kinetics of peripheral hepatitis B virus-specific CD8$^+$ T cells in patients with onset of viral reactivation

Jun Aoki · Yuka Kowazaki · Takahiro Ohtsuki · Rumiko Okamoto · Kazuteru Ohashi · Seishu Hayashi · Hisashi Sakamaki · Michinori Kohara · Kiminori Kimura

Received: 16 May 2012 / Accepted: 21 August 2012 / Published online: 26 September 2012

Abstract
Background Patients with resolved hepatitis B virus (HBV) infection undergoing chemotherapy or immunosuppressive therapy are potentially at risk of HBV reactivation. However, it remains unclear how liver disease develops after HBV reactivation. To compare the host immune response against HBV, we performed immunological analyses of six HBV reactivation patients.
Methods The numbers of peripheral HBV-specific CD8$^+$ T cells were investigated longitudinally in six HLA-A2- and/or A24-positive patients with HBV reactivation. In addition, 34 patients with resolved HBV, 17 patients with inactive chronic hepatitis B (ICHB), 17 patients with chronic hepatitis B (CHB) and 12 healthy controls were analyzed. The number and function of HBV-specific CD8$^+$ T cells were assessed by flow cytometry using tetramer staining and intracellular IFN-γ production. Furthermore, the numbers of CD4$^+$CD25$^+$ or CD4$^+$Foxp3$^+$ T cells and serum inflammatory cytokine levels were analyzed.
Results The frequency of HBV-specific CD8$^+$ T cells was significantly increased in HBV reactivation patients compared with ICHB and CHB patients. In addition, the number of HBV-specific CD8$^+$ T cells was increased in resolved HBV patients compared with ICHB patients. PD-1 expression was decreased in HBV reactivation patients compared with ICHB and CHB patients. The numbers of HBV-specific CD8$^+$ T cells and CD4$^+$CD25$^+$ or CD4$^+$Foxp3$^+$ T cells were negatively correlated following onset of HBV reactivation.
Conclusions During HBV reactivation, the frequency of HBV-specific CD8$^+$ T cells increased even though the administration of immunosuppressive drugs and interactions with CD4$^+$ regulatory T cells may be important for the onset of liver disease.

Keywords Hepatitis B virus reactivation · Cytotoxic T lymphocyte · Regulatory T cell · PD-1

Abbreviations

HBV	Hepatitis B virus
CTL	Cytotoxic T lymphocyte
sALT	Serum alanine aminotransferase
TNF	Tumor necrosis factor
IFN	Interferon
MIP	Macrophage inflammatory protein
MCP	Monocyte chemotactic protein

Electronic supplementary material The online version of this article (doi:10.1007/s00535-012-0676-y) contains supplementary material, which is available to authorized users.

J. Aoki · K. Ohashi · H. Sakamaki
Division of Hematology, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, 18-22-3 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan

Y. Kowazaki · S. Hayashi · K. Kimura (✉)
Division of Hepatology, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, 18-22-3 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan
e-mail: kkimura@cick.jp

T. Ohtsuki · M. Kohara · K. Kimura
Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan

R. Okamoto
Division of Chemotherapy, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, 18-22-3 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan

J Gastroenterol (2013) 48:728–737

Introduction

More than 300 million people worldwide suffer from persistent hepatitis B virus (HBV) infection, making the virus a common cause of morbidity and mortality [1, 2]. Each year, an estimated one million people die of complications associated with chronic HBV infection, including cirrhosis, end-stage liver disease and hepatocellular carcinoma [3, 4]. Viral reactivation in hepatitis B surface antigen (HBsAg) carriers undergoing immunosuppressive therapy is well documented, as some immunosuppressive therapies can enhance HBV replication in hepatocytes at the same time as they curb host immune responses, resulting in detectable viremia followed by clinical hepatitis [1]. In general, although the development of surface and core antibodies and loss of surface antigen following HBV infection are thought to represent clearance of the virus, evidence exists to support the possibility that the virus may remain latent within the liver [5, 6]. The course and outcome of HBV infection are modulated by the host immune response [7, 8], and the loss of immune surveillance can cause reactivation of viral replication and exacerbation of disease activity. HBV reactivation is a well-characterized syndrome marked by the abrupt reappearance or elevation of HBV DNA in the serum of a patient with previously inactive or resolved HBV infection [1, 9]. Although the mechanisms of reactivation and associated liver damage remain unclear, they may include a rebound increase in the lymphocyte number following cessation of immunosuppressive and myelosuppressive chemotherapy, leading to a rapid destruction of infected hepatocytes with subsequent severe hepatitis. As one of the viral factors, it has been reported that the fulminant outcome of HBV reactivation can be associated with genotype Bj, which exhibits high replication owing to the A1896 mutation [10]. Therefore, although there are increasing reports regarding viral factors for the mechanism of HBV reactivation, it is still premature to conclude how the host immune response affects the liver injury and viral load.

It has been demonstrated that chronic persistent HBV infection is manifested by cytotoxic T lymphocytes (CTLs) that are functionally impaired or exhausted [7]. Recent reports have indicated that PD-1 is markedly upregulated on the surface of exhausted virus-specific $CD8^+$ T cells in mice with lymphocytic choriomeningitis virus infection [11] and humans with human immunodeficiency virus (HIV) infection [12, 13] or hepatitis C virus (HCV) infection [14]. Based on these observations, we evaluated the hypothesis that HBV-specific $CD8^+$ T cells can restore their function during HBV reactivation, by analyzing PD-1 expression on HBV-specific $CD8^+$ T cells. In a previous report, we demonstrated that fulminant hepatitis in HBV patients was responsible for high amounts of interferon (IFN)-γ production by $CD8^+$ T cells from peripheral blood mononuclear cells (PBMCs) [15]. Thus, HBV-specific $CD8^+$ T cells may be responsible for the liver disease at the onset of HBV reactivation, although a causal relationship among these events has not been defined.

In this study, we found that the frequency of HBV-specific $CD8^+$ T cells was increased and the interactions between these $CD8^+$ T cells and $CD4^+$ regulatory T cells (Tregs) showed a negative correlation at the onset of HBV reactivation.

Patients and methods

Patients

Six patients with HBV reactivation from resolved HBV infection before chemotherapy or immunosuppressive therapy, 34 patients with resolved HBV, 17 patients with inactive chronic hepatitis B (ICHB), 17 patients with chronic hepatitis B (CHB) and 12 healthy controls gave informed consent to participate in the study. The study was performed at Tokyo Metropolitan Komagome Hospital after receiving institutional review board approval (approved ID number: 714). We defined resolved HBV infection as serum HBsAg (−), anti-HBcAb (+) and/or anti-HBsAb (+), ICHB as serum HBsAg (+), HBV DNA (+) and normal serum alanine aminotransferase (sALT; 5–40 IU/mL), and CHB as serum HBsAg (+), HBV DNA (+) and continuous serum ALT elevation (>2 × normal level). The study protocol and procedures were conducted in accordance with the ethical guidelines of the Declaration of Helsinki. The patient characteristics are summarized in Table 1. The healthy controls comprised six males and six females, who ranged in age from 32 to 63 years. There were no significant differences in age between the HBV reactivation patients and the other groups. The patients were human leukocyte antigen (HLA)-A2- or A24-positive and negative for HCV and HIV-1/2.

Sample preparation

PBMCs were isolated from whole blood using Lymphoprep™ (Axis-Shield, Oslo, Norway). The isolated cells were washed twice in phosphate-buffered saline (Gibco, Auckland, NZ) and used immediately. The cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 50 μg/mL gentamicin and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA) at 37 °C in a humidified 5 % CO_2 incubator, as previously described [15]. Plasma preparation tubes (BD Biosciences, San Jose, CA) were used to isolate plasma from whole

Table 1 Characteristics of the respective patient groups

	Number	Sex (M/F)	Age	ALT (U/L)	HBV DNA (log/mL)
Healthy volunteer	12	6/6	57 ± 11	25 ± 11	–
Resolved HBV	34	18/16	59 ± 15	33 ± 20	–
ICHB	17	8/9	55 ± 14	33 ± 14	3.9 ± 1.8
CHB	17	8/9	53 ± 11	75 ± 19	5.5 ± 1.9
Reactivation	6	3/3	66 ± 12	82 ± 66	5.8 ± 3.3

Results are shown as mean ± SD

ALT alanine transaminase, *ICHB* inactive chronic hepatitis B, *CHB* chronic hepatitis B, *HBV* hepatitis B virus, *M* male, *F* female

blood. The plasma samples were frozen and subsequently thawed for viral load and genotype testing.

Synthetic peptides

Three HBV peptides; HLA-A*0201 core 18–27 (FLPSDFFPSV), envelope 183–191 (FLLTRILTI), polymerase 575–583 (FLLSLGIHL), HLA-A*2402 core 117–125 (EYLVSFGVW), polymerase 756–764 (KYTSFPWLL) and were synthesized by Sigma Aldrich (Hokkaido, Japan).

Major histocompatibility complex (MHC) class I tetramer staining

Patients expressing HLA-A2 or A24 were assessed for antigen-specific responses to HBV by tetramer staining. For the staining, phycoerythrin (PE)-conjugated HLA-A*0201-restricted HBV core (FLPSDFFPSV) and HLA-A*2402 HBV core (EYLVSFGVW) HBV polymerase (KYTSFPWLL) were purchased from MBL (Nagoya, Japan).

FACS analysis

HLA typing was performed by staining PBMCs with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A2 and PE-conjugated anti-HLA-A24 antibodies (MBL) according to the manufacturer's instructions. PBMCs were surface-stained on ice for 20 min with the following monoclonal antibodies: FITC-conjugated anti-human CD62L, anti-PD-1 and anti-CD25 (BD Biosciences); PE-conjugated anti-human Foxp3 (eBioscience, San Diego, CA); and PE-Cy5-conjugated anti-human CD4 and anti-human CD8 (BD Biosciences). For intracellular staining, isolated PBMCs were incubated with peptides (10 μl/mL) in the presence of human recombinant IL-2 (50 U/mL) and 1 μl/mL of BD GolgiPlug protein transport inhibitor (BD Bioscience) for 4 h. After incubation (37 °C, 5 % CO_2), cells from each well were stained with PE-Cy5-conjugated anti-human CD8 (BD Biosciences). Prior to staining with intracellular antibodies against PE-conjugated anti-human IFN-γ, cells were fixed and permeabilized by adding Cytofix-Cytoperm (BD Pharmingen). Cells were acquired by FACS scan (BD Biosciences), and the data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Cytokine and chemokine profiles

Bio-Plex Cytokine Assay Kits (Bio-Rad Laboratories, Hercules, CA) were used to measure the amounts of cytokines and chemokines in sera in accordance with the manufacturer's instructions. Specifically, we used the Bio-Plex Human Cytokine 17-Plex Panel. The resulting samples were analyzed in a 96-well plate reader using a Bio-Plex Suspension Array System and Bio-Plex Manager software (all from Bio-Rad Laboratories).

Serum HBV assay

The serum HBV DNA concentrations were quantified using the COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Diagnostics, Basel, Switzerland). The four major HBV genotypes (A–D) were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed against distinct epitopes on the preS2-region products using commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co. Ltd., Tokyo, Japan). HBV DNA sequences bearing the core promoter and precore or core regions were amplified by PCR with hemi-nested primers. The PCR products were directly sequenced by the di-deoxy-chain termination method using a Big Dye Terminator Kit and an ABI PRISM 3100-Avant Analyzer (both from Applied Biosystems, Foster City, CA).

Statistical analysis

Data are shown as mean ± SD. The data were analyzed by the nonparametric Mann–Whitney or Kruskal–Wallis tests or ANOVA using Prism 5 for Macintosh software (GraphPad, San Diego, CA). Values of $P < 0.05$ were considered to indicate statistical significance.

Table 2 Characteristics of HBV reactivation patients

Patient	Sex	Disease	HBsAg	HBsAb	HBcAb	Chemotherpy/ Immunosuppressant	HBV genotype	HBVcore/precore mutation
#1	Female	ALL, BMT	−	+	+	PSL, FK	C	−
#2	Male	AML, BMT	−	+	+	PSL, CsA	C	−
#3	Male	Eso.ca	−	+	+	5FU + CDDP	C	−
#4	Male	B cell lymphoma	−	+	+	R-CHOP	B	−
#5	Male	B cell lymphoma	−	+	+	CHOP	B	−
#6	Female	T-LBL, BMT	−	+	+	Anthracycline + AraC	B	−

ALL acute lymphoblastic leukemia, *BMT* bone marrow transplantation, *PSL* prednisolone, *FK* tacrolimus, *CsA* ciclosporin A, *CDDP* cisplatin, *5FU* 5-fluorouracil, *R-CHOP* rituximab, cyclophosphamide, doxorubicin hydrochloride (hydroxydaunorubicin), vincristine (oncovin), and prednisone, *AraC* cytarabine

Results

HBV reactivation patient profiles

We identified six patients with HBV reactivation (Table 2). Patients with malignant lymphoma and bone marrow transplantation for leukemia were observed, and treatment with rituximab, which is a well-known inducer of HBV reactivation, was encountered in the case of one patient (patient #4). There was no sex difference regarding the incidence of HBV reactivation. All patients were negative for HBsAg and positive for anti-HBc and anti-HBs antibodies as HBV-related markers. In addition, three patients were HBV genotype C and one patient was genotype B. No core and precore promoter mutations were detected in the patients. All patients received entecavir (0.5 mg/day) when serum HBV DNA was initially detected or sALT elevation was observed.

Serum inflammatory cytokine and chemokine levels

To determine whether the serum cytokine and chemokine levels correlated with the development of HBV reactivation, we examined the concentrations of various cytokines and chemokines. We measured serum cytokine/chemokine levels at the time of diagnosis of HBV reactivation. In the groups, serum was isolated prior to chemotherapy or antiviral therapy.

The data for the serum cytokine and chemokine levels are shown in Fig. 1. Serum IL-1β was significantly higher in resolved HBV patients (median, 0.32 pg/mL; range 0.10–0.75 pg/mL) than in healthy controls (median, 0.17 pg/mL; range 0.04–0.43 pg/mL) or ICHB patients (median, 0.17 pg/mL; range 0.09–0.24 pg/mL). Serum IL-7 was elevated in resolved HBV patients (median, 6.4 pg/mL; range 0.54–28.12 pg/mL) compared with ICHB patients (median, 3.2 pg/mL; range 0.54–12.6 pg/mL) or CHB patients (median, 3.13 pg/mL; range 0.29–5.16 pg/mL). There were no significant differences in serum IL-6 among resolved HBV, ICHB, CHB and HBV reactivation patients. Serum IL-8 and MCP-1 were significantly increased in resolved HBV patients (IL-8: median, 12.5 pg/mL; range 5.15–34.2 pg/mL; MCP-1: median, 75.2 pg/mL; range 0.7–300.23 pg/mL) compared with healthy controls (IL-8: median, 5.24 pg/mL; range not detected–17.46 pg/mL; MCP-1: median, 23.3 pg/mL; range not detected–48.51 pg/mL) and CHB patients (IL-8: median, 7.25 pg/mL; range 1.13–23.84 pg/mL; MCP-1: median, 21.8 pg/mL; range 3.49–58.66 pg/mL). Although circulating IFN-γ was detected at very low levels in all samples, ICHB patients exhibited significant suppression of serum IFN-γ. Regarding HBV reactivation, serum G-CSF was slightly increased compared with healthy controls.

Comparison of HBV-specific $CD8^+$ T cell frequencies

The development of hepatitis with HBV infection is mediated by antigen-specific CTLs [7]. To compare the frequencies and phenotypes of HBV-specific $CD8^+$ T cells from PBMCs, 16 HLA-A2- and 19 HLA-A24-positive resolved HBV patients, 9 HLA-A2- and 11 HLA-A24-positive ICHB patients, 11 HLA-A2- and 13 HLA-A24-positive CHB patients, and four HLA-A2- and four HLA-A24-positive HBV reactivation patients were examined using a panel of three MHC class I tetramers containing frequently detected HBV epitopes (A2 core, amino acids 18–27; A24 core, amino acids 117–125; and A24 polymerase, amino acids 756–764) (Fig. 2a). The frequency of peripheral HLA-A2 core-specific $CD8^+$ T cells was significantly higher in HBV reactivation patients than in ICHB and CHB patients (Fig. 2b, upper panels). Interestingly, the frequencies of HLA-A2 core-specific $CD8^+$ cells were also significantly higher in resolved HBV patients than in ICHB patients, indicating that HBV-specific $CD8^+$ T cells are circulating even though serum HBV DNA was not detected. Consistent with these data,