表. 抗線維化効果が認められた肝疾患治療

疾患名	薬剤名または治療方法	文献
C型慢性肝炎	インターフェロン単独療法	Shiratori Y. *Ann Intern Med* (2000)[1]
C型肝硬変	ペグインターフェロン・リバビリン併用療法	Poynard T. *Gastroenterology* (2002)[2]
B型慢性肝炎	ラミブジン	Dienstag JL. *Gastroenterology* (2003)[3]
	エンテカビル	Chang T-T. *Hepatology* (2010)[4]
自己免疫性肝炎	ステロイド	Dufour JF. *Ann Intern Med* (1997)[5]
	アザチオプリン	
原発性胆汁性肝硬変	ウルソデオキシコール酸	Corpechot C. *Hepatology* (2000)[6]
胆管狭窄型慢性膵炎	内視鏡的ドレナージ	Hammel P. *N Engl J Med* (2001)[7]

など，日常臨床で感じていた肝線維化の可逆性が実証された意義は大きい．

これまでに抗線維化効果が認められた肝疾患治療のうち，主なものを表にまとめた．中でも，C型慢性肝炎に対するインターフェロン単独療法[1]やC型肝硬変に対するペグインターフェロン・リバビリンの併用療法[2]，B型慢性肝炎に対するラミブジン[3]やエンテカビル[4]を用いた抗ウイルス療法など，原因療法が奏功した症例における肝線維化の改善は顕著である．また，自己免疫性肝炎に対する免疫抑制療法[5]，原発性胆汁性肝硬変症（primary biliary cirrhosis）に対するウルソデオキシコール酸治療[6]，胆管狭窄型慢性膵炎に伴う胆汁うっ滞型肝線維症に対する内視鏡的ドレナージ[7]でも，その進展要因を排除することにより線維化改善が認められている．

2. 今，なぜ肝線維化研究が注目されているのか？

これまで遅れていた肝線維化研究と線維症治療薬開発の試みが最近にわかに活発になってきたのには，いくつかの理由がある．まず第一に，ウイルス性慢性肝炎に対する治療法の進歩により，近い将来にはNASHが慢性肝疾患の主因となることが予想されるなど，肝臓病診療には大きなパラダイムシフトが起こっている．NASHに対しても体重の減量以外に特異的治療法が存在しない現在，早期から線維化進展を防ぐ薬剤，あるいは肝硬変症例に対して線維の蓄積を減少させて肝細胞機能の回復や肝発癌の抑止をもたらすような薬剤の登場が熱望されている．すなわち，肝線維症はポスト肝炎ウイルス時代の治療ターゲットと言える．

第二に，上述したように抗ウイルス治療法の進歩は，肝線維症が可逆的な病態であることを証明した．組織におけるコラーゲン含量は，合成と分解とのバランスの上に成り立っており，コラーゲン合成を抑制する，あるいは分解を適切に誘導することで，肝線維症は治療可能な病態であることが改めて認識された．

第三に，肝線維症に対する非侵襲的評価方法の開発が挙げられる．これまで検討されてきた血中線維化マーカーに加えて，超音波装置[8]やMR[9]を用いた肝の弾性度診断が可能になった．後述するように，弾性度診断も線維化治療の効果判定には必ずしも十分とは言えないが，肝線維症に対する介入試験を実施する際に，多数の症例の中から比較的均一な対象集団を設定するうえで大きな力となることが期待されている．

最後に，近年の再生医学・再生医療の進歩は，肝線維症の病態研究や治療戦略についても大きな知見をもたらした．肝の線維化と再生とは常に表裏一体の関係にあり，線維化が進行した肝臓では再生が妨げられ，逆に再生状態にある肝臓は線維化刺激の影響を受けにくい．肝線維化と再生の病態連繋に立脚した，新たな線維化治療法が模索されている．

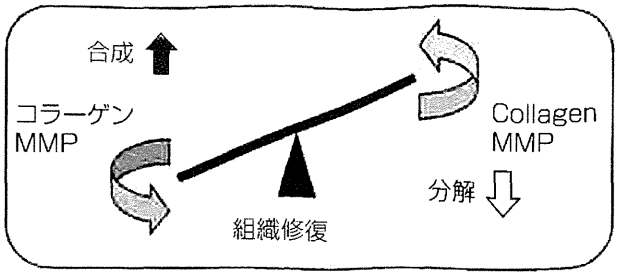

図 1. 臓器線維症におけるマトリックス代謝の変容
組織におけるコラーゲンの含量は，合成と分解のバランスの上に規定されている．その適切な発現は組織修復や創傷治癒過程において重要な働きを演じているが，調節機構が破綻を来たすと組織に過剰のコラーゲンが沈着し，諸臓器の線維化を引き起こす．MMP, matrix metalloproteinase(s)．

3. 肝線維症の治療戦略

前述したように，組織におけるコラーゲン含量は合成と分解とのバランスの上に成り立っており，その均衡が破綻して相対的な合成優位に傾くと，肝をはじめとする諸臓器の線維化を引き起こす（図 1）．端的に言ってしまえば，臓器線維症治療とはマトリックスの合成系と分解系のバランスの是正にほかならない．

肝線維化の進展と改善過程（図 2）から見た治療戦略は，①星細胞活性化の抑制，②活性化星細胞によるコラーゲン産生の抑制，③活性化星細胞に対するアポトーシスないし「脱」活性化の誘導，④Matrix metalloproteinase（MMP）によるコラーゲン線維の分解という各ステップに大別できる．中でも，線維化改善過程における活性化星細胞の脱活性化は注目に値する．肝線維化所見が改善する際には活性化星細胞は急激に減少し，これまではアポトーシスや老化に陥ると考えられてきた．最近になって，活性化した星細胞の約半数が非活性型に戻る（脱活性化）という報告がなされた[10,11]．しかしながら，肝線維化の改善に伴って脱活性化した星細胞は線維化刺激を全く受けていない静止期の星細胞と同一とは言えず，再度の線維化刺激に対する反応性も高いという．星細胞が有するこの可逆性を肝線維症の治療に応用するには，さらに詳細なメカニズムの解明が必要である．

4. 肝線維化治療薬の開発と臨床応用を妨げるものは何か？

肝線維化の進展と改善機序について多くの知見が集積されてきたにもかかわらず，前述した原因治療薬以外には，肝線維症に対する特異的かつ効果的な治療薬はいまだ存在しない．例えばNASHについては，その病態形成にperoxisome proliferator-activated receptor γ（PPAR-γ）シグナルや酸化ストレスの関与が指摘されているにもかかわらず，ピオグリタゾン投与の有効性を示した報告[12]を除くと，グリタゾン製剤やビタミンEの肝線維化抑制効果は概して否定的である[13,14]．また，C型慢性肝炎に対する抗ウイルス療法の非著効例に対して，インターロイキン 10[15]やインターフェロンγ[16]，さらにはグリタゾン製剤[17]の投与が試みられたが，いずれも組織学的には無効であった．また，アンギオテンシンII受容体拮抗薬の投与が肝組織中の酸化ストレスや線維化関連遺伝子の発現を抑制したことで，その抗線維化効果が期待されたが，長期投与では十分な線維化抑制効果は得られなかった[18]．

このように，いわゆる「線維症治療薬」が奏功しない理由としては，薬効自体の限界や副作用の懸念のみならず，臨床研究デザインの限界が挙げられる．すなわち，様々な経過をたどる多くの慢性肝疾患患者の中から比較的均一な対象集団を設定し，通常 10 年単位の長期経過をたどる肝線維症に対する薬物の投与効果を，1 年前後という短期間の介入試験で評価することの困難さがある．肝組織生検は今なお最も信頼できる肝線維症の診断手段であるが，全症例に対し

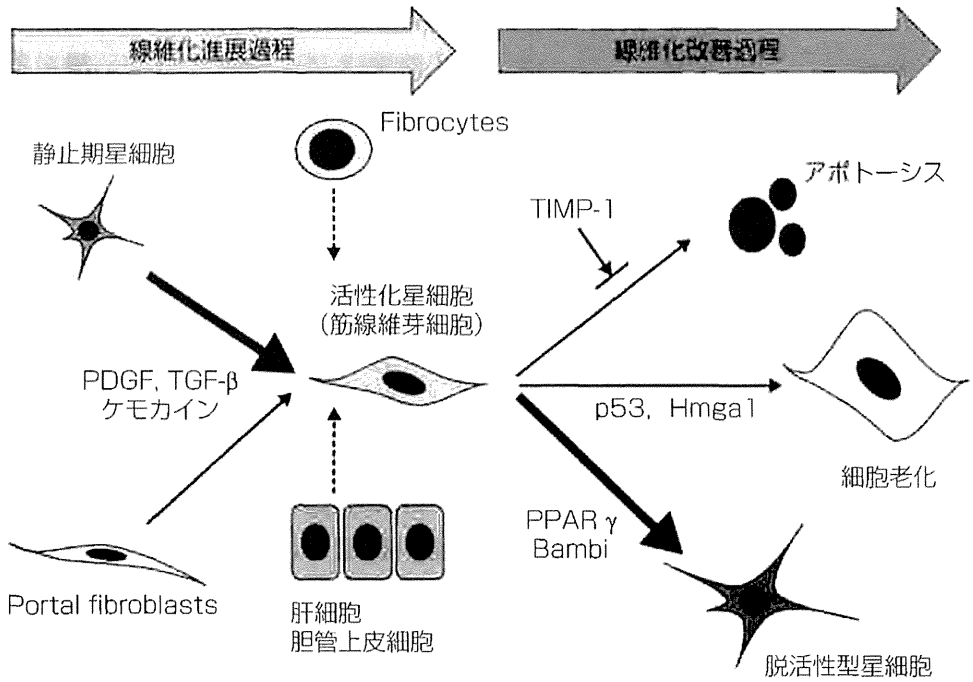

図2. 肝線維化の進展ならびに改善過程

肝実質内の星細胞や門脈域に存在するportal fibroblastsは，肝線維化刺激によって筋線維芽細胞に形質転換して，活発にコラーゲンを産生する．近年では造血細胞由来のFibrocytesや，肝細胞ないし胆管上皮細胞の上皮間葉移行（Epithelial-to-mesenchymal transition, EMT）を介したコラーゲン産生が注目されたが，その後の研究ではその関与は限局的ないし否定的とする意見が多い．一方，肝線維化の改善過程においては，活性化星細胞はアポトーシスや細胞老化に陥ることで排除されると考えられていたが，最近になってその約半数が非活性型に移行（脱活性化）することが報告された．PDGF, platelet-derived growth factor；TGF-β, transforming growth factor-β；TIMP, tissue inhibitor of metalloproteinase, PPAR γ, peroxisome proliferator-activated receptor γ.

て治療前後で実施することは現実的でない．また，肝生検のstage分類で得られる線維化情報は組織に沈着したコラーゲン線維の半定量化であり，静的な指標である．近年開発された超音波装置やMRを用いた肝の弾性度診断も，その低侵襲性や定量性には大きな利点があるものの，基本的には組織のコラーゲン蓄積量を反映している．感度および特異度に優れた肝線維化の非侵襲的診断方法，しかも線維症の程度(fibrosis)ではなく，コラーゲンの合成（fibrogenesis）と分解（fibrolysis）の動的な評価系が熱望される所以である．最近，血中タンパク質の糖鎖構造の変化が肝線維化の進展と改善を鋭敏に反映することが報告された[19]．肝線維化の新たな動的マーカーとしての有用性に大きな期待が寄せられている．

おわりに

かつての病理学や肝臓病学の教科書に「肝硬変は進行性かつ不可逆的な疾患である」と記載されていたのは，肝線維症に対する効果的な治療法が存在せず，コラーゲン産生を来たす刺激が慢性的に反復していたからにほかならない．近年の肝線維症治療薬の開発に対する産学の関心の高まりは著しい．肝線維化の可逆性を最大限活かして線維化治療薬の一刻も早い臨床応用に結び付けるには，候補薬剤の開発と並んで，

臨床的に線維化抑制効果の動的評価系の構築が必須であり，さらなる研究と工夫が求められている．

著者のCOI(conflicts of interest)開示：稲垣　豊：研究費・助成金（住友化学）

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Review Article

Stem and progenitor cell systems in liver development and regeneration

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The liver comprises two stem/progenitor cell systems: fetal and adult liver stem/progenitor cells. Fetal hepatic progenitor cells, derived from foregut endoderm, differentiate into mature hepatocytes and cholangiocytes during liver development. Adult hepatic progenitor cells contribute to regeneration after severe and chronic liver injuries. However, the characteristics of these somatic hepatic stem/progenitor cells remain unknown. Culture systems that can be used to analyze these cells were recently established and hepatic stem/progenitor cell-specific surface markers including delta-like 1 homolog (DLK), cluster of differentiation (CD) 13, CD133, and LIV2 were identified. Cells purified using antibodies against these markers proliferate for an extended period and differentiate into mature cells both *in vitro* and *in vivo*. Methods to force the differentiation of human embryonic stem and induced pluripotent stem (iPS) cells into hepatic progenitor cells have been recently established. We demonstrated that the $CD13^{+}CD133^{+}$ fraction of human iPS-derived cells contained numerous hepatic progenitor-like cells. These analyses of hepatic stem/progenitor cells derived from somatic tissues and pluripotent stem cells will contribute to the development of new therapies for severe liver diseases.

Key words: hepatic stem/progenitor cells, liver regeneration, mesenchymal cells, pluripotent stem cells

INTRODUCTION

THE LIVER, THE largest organ in the body, regulates a multitude of metabolic functions. Parenchymal cells, also known as hepatocytes, express metabolic enzymes that are necessary for mature liver functions.[1] The non-parenchymal cells of the liver, such as stellate, sinusoidal endothelial, mesenchymal and Kupffer cells, regulate the functions of mature hepatocytes through cell–cell interactions. These metabolic functions are important for maintaining homeostasis. Therefore, liver damage induced by genetic mutations, viral infection and metabolic disorders may contribute to severe liver diseases such as liver fibrosis and hepatocellular carcinoma (HCC). Liver transplantation is a radical intervention to treat these diseases; however, this approach is limited in Japan due to a shortage of donor organs. Transplantation of hepatocytes or stem/progenitor cells in the liver may serve as an alternative treatment for severe liver diseases. Herein, we review work describing the role of stem and progenitor cell systems in liver regeneration and development. In addition, we discuss the application of liver stem/progenitor cells in future regenerative therapies.

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LIVER REGENERATION

REGENERATION AFTER HEPATECTOMY or chemical-induced injury is an example of the remarkable abilities of the liver. Approximately 70% of the liver mass can be surgically removed by partial hepatectomy, and the remnants of the liver can expand and compensate its functions. Liver regeneration reportedly depends primarily on the proliferation of adult hepatocytes.[2] In the first step of liver regeneration, interleukin (IL)-6 is produced from non-parenchymal cells, priming mature hepatocytes for proliferation.[3]

Next, growth factors including hepatocyte growth factor (HGF) are induced and mature hepatocytes enter into the S-phase.[4,5] Subsequently, proliferation of non-parenchymal cells is induced and liver regeneration is completed.

During liver regeneration, hepatocytes also undergo hypertrophy. Hypertrophy was recently identified as an important process involved in regeneration induced by partial hepatectomy.[6] Recovery from 30% and 70% partial hepatectomy was shown to rely on different mechanisms. Hypertrophy of mature hepatocytes is sufficient for recovery from 30% hepatectomy. While hypertrophy is involved in the early phase of recovery from 70% partial hepatectomy, hepatocytes enter the cell cycle in the latter phase of regeneration. Therefore, the mechanisms involved in liver regeneration, hypertrophy and proliferation are regulated by the severity of liver injury.

In contrast to regeneration induced by acute liver damage, severe and chronic liver damage induces the defect of proliferation of mature hepatocytes.[7] Stem and progenitor cells in the adult liver are thought to be involved in regeneration induced by these chronic liver damage; however, the mechanisms underlying this process remain largely unknown.

STEM/PROGENITOR CELL SYSTEMS IN THE LIVER

Somatic stem cells and pluripotent stem cells

STEM CELLS, GENERALLY defined as clonogenic cells, exhibit properties of self-renewal, multipotency (producing progeny belonging two or more lineages) and long-term tissue repopulation after transplantation. Two types of stem cells exist, somatic and pluripotent stem cells. During development of several tissues, somatic stem cells give rise to non-self-renewing progenitors with restricted differentiation potential. Functionally mature cells are generated while a subpopulation of primitive stem cells is maintained. Thus, stem cells are involved in organ formation during developmental stages, and organ maintenance and repair in the adults. Pluripotent embryonic stem (ES) cells, derived from the inner cell mass of the blastocyst, give rise to tissues derived from the three primary germ layers: ectoderm, mesoderm and endoderm.[8,9] Induced pluripotent stem (iPS) cells, generated from somatic cells by simultaneously expressing Yamanaka factors (*Oct3/4*, *Klf4*, *Sox2* and *c-Myc*), have properties similar to those of ES cells.[10,11]

Stem/progenitor cell systems in the fetal liver

Two types of liver stem/progenitor cells are widely recognized. In contrast to adult liver stem/progenitor cells that are involved in regeneration, stem/progenitor cells in fetal liver are important for embryonic liver development. At the onset of liver development at approximately mouse embryonic day (E)9, hepatic progenitor cells (HPC) differentiate from foregut endoderm and expand to form the early fetal liver bud.[12,13] Characterization of HPC in fetal livers was recently advanced by the advent of a novel flow cytometry screening method.[14] Mouse fetal liver cells were dissociated and fractionated using specific cell-surface marker antibodies. Individual fractionated cells were sorted onto the wells of dishes and evaluated for their colony-forming ability and expression of hepatocytic and cholangiocytic markers (Fig. 1). Large colonies formed from single cells derived from E9.5–14.5 livers were positive for albumin and cytokeratin (CK)19, markers of hepatocytes and cholangiocytes, respectively. Mouse hepatic stem/progenitor cell-specific surface markers, such as DLK, LIV2, CD13, E-cadherin and CD133, were identified using this method (Table 1).[15–19]

Using this colony formation culture system, proliferation and differentiation of HPC in fetal livers were shown to be regulated by several transcription factors, including prospero-related homeobox 1 and its binding partner, liver receptor homolog 1, that cooperatively regulate development of HPC.[20] The transcription factor Sal-like protein 4 (SALL4) has been shown to regulate organogenesis, embryogenesis and maintenance of pluripotency. We found that SALL4 plays a crucial role in controlling the lineage commitment of HPC not only through inhibiting their differentiation into hepatocytes, but also by driving their differentiation into cholangiocytes.[21] In addition, our group and others reported that SALL4 is also important for stemness of liver cancers.[22–24] Cancers have a subpopulation of stem-like cells, or tumor-initiating cells, that are similar to somatic stem/progenitor cells. SALL4 is expressed in fetal liver stem/progenitor cells, but not normal adult hepatocytes. We demonstrated that SALL4 is also strongly expressed in human hepatocellular and cholangiocellular carcinomas. Elevated expression of SALL4 in tumors is associated with poor survival of HCC patients. Expression manipulation experiments showed that proliferation and stem cell marker expression in human HCC cells are regulated by SALL4 *in vitro* and *in vivo* (Fig. 2). Together, these results suggest that SALL4 is

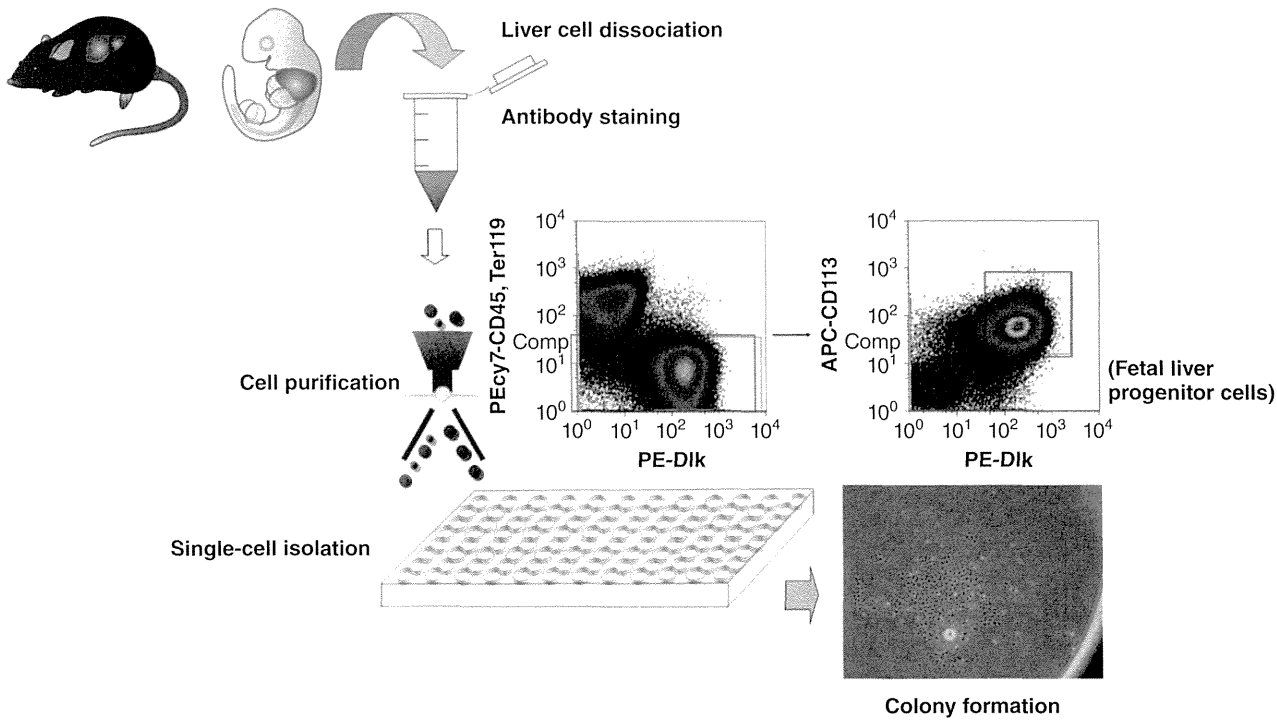

Figure 1 Colony formation assay using cells derived from fetal and adult livers. Liver cells were dissociated using collagenase and stained with stem/progenitor cell-specific antibodies. After the cells were purified using flow cytometry, individual cells were inoculated into the wells of collagen-coated culture dishes. Colonies derived from individual cells were analyzed.

a stem cell biomarker and a novel therapeutic target for liver cancers.

Regulation of fetal liver stem/progenitor cells by cell–cell interactions

During mid- to late fetal development (E11.5–16.5 in mice), hematopoietic stem cells originating from the aorta–gonad–mesonephros region migrate into the fetal liver.[25] Thus, the liver undergoes a dramatic change during development, transitioning from an embryonic hematopoietic tissue to a metabolic organ. We demonstrated that the interaction of HPC with hematopoietic cells is involved in their differentiation into mature hepatocytes.[26] HPC commence production of several metabolic enzymes involved in adult liver functions at mid- to late fetal stages. Oncostatin M (OSM), an IL-6 family cytokine, promotes hepatic maturation, as evidenced by the induction of metabolic enzymes and accumulation of glycogen. OSM is expressed in $CD45^+$ hematopoietic cells in mid-fetal livers, whereas OSM receptors are predominantly detected in hepatic cells, indicating that paracrine signaling from hematopoietic cells to HPC is important for fetal liver maturation.

In contrast to mid-fetal HPC, the *in vitro* characteristics of HPC at the onset of liver development (E9.5–10.5 in mice) are poorly elucidated, because no suitable culture system for these cells has been established. In early fetal liver development, endodermal and mesenchymal cells interact to promote liver bud growth. For example, the transcription factor H2.0-like homeobox protein, expressed in the septum transversum mesenchyme, is essential for early fetal liver development.[27] To mimic the interaction of hepatic and mesenchymal cells, we co-cultured mouse embryonic fibroblasts with HPC. Candidate HPC ($CD13^+Dlk^+$ cells) from early fetal livers that were clonally expanded using this novel culture system showed bipotency *in vitro*. Inhibition of Rho-associated protein kinase (Rock) or myosin II activity by using Y-27632 and blebbistatin, respectively, significantly enhanced the colony-forming activities of early fetal but not of mid-fetal HPC. These data suggested that purified HPC in early fetal livers have properties distinct from those in mid-fetal livers.[28]

Several mesenchymal cell types, including mesothelial, sub-mesothelial and transitional mesenchymal cells, have been identified in fetal livers.[29,30]

Table 1 Liver stem/progenitor cells in fetal and postnatal stages

Cell surface marker	Reference	Characteristics
1 Fetal stem/progenitor cells		
CD49f, c-Met	Suzuki *et al.*[14,49]	Clonal expansion of bipotent cells. *In vivo* transplantation.
Dlk	Tanimizu *et al.*[16]	Clonal expansion of bipotent cells. *In vivo* transplantation.
E-cadherin	Nitou *et al.*[19]	*In vitro* expansion.
Liv2	Watanabe *et al.*[15]	Expression in early and mid-fetal liver cells.
CD13	Kakinuma *et al.*[17]	Clonal expansion of bipotent cells. *In vivo* transplantation.
CD133	Kamiya *et al.*[18]	Clonal expansion of bipotent cells.
EpCAM	Schmelzer *et al.*[38]	Expansion of bipotent cells derived from human fetal livers. *In vivo* transplantation.
2 Postnatal stem/progenitor cells		
EpCAM	Okabe *et al.*[35]	Clonal expansion of bipotent cells derived from normal and injured livers.
EpCAM	Yovchev *et al.*[33]	Expression of oval cells in the injured rat livers. *In vivo* transplantation.
CD133	Suzuki *et al.*[34]	Clonal expansion of bipotent cells derived from injured livers. *In vivo* transplantation.
CD133	Rountree *et al.*[32]	Expansion and analyses cells derived from injured livers.
LGR5	Huch *et al.*[37]	*In vitro* organoid expansion. *In vivo* transplantation.
$CD13^+CD133^+$	Kamiya *et al.*[18]	Clonal expansion of bipotent cells derived from normal livers. *In vivo* transplantation.
$CD133^+MIC1\text{-}1C3^+$	Dorrell *et al.*[36]	Clonal expansion derived from normal and injured livers. *In vivo* transplantation
EpCAM	Schmelzer *et al.*[38]	Expansion of bipotent cells derived from human fetal livers. *In vivo* transplantation.

CD, cluster of differentiation; Dlk, delta-like 1 homolog; EpCAM, epithelial cell adhesion molecule; LGR, leucine-rich repeat-containing G-protein coupled receptor.

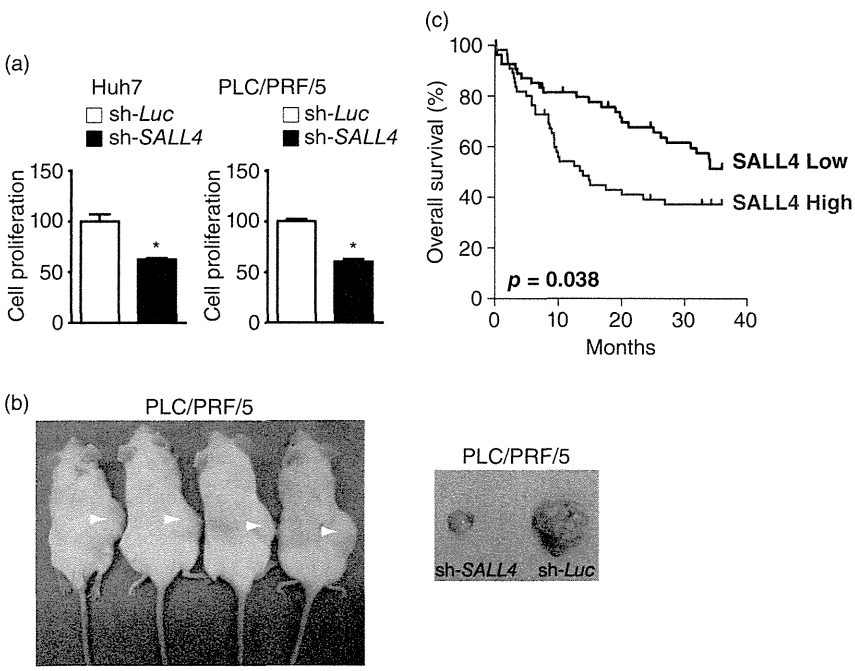

Figure 2 Sal-like protein 4 (SALL4) is a stem cell biomarker in liver cancers. (a) The effect of shRNA-mediated SALL4 downregulation on the proliferation of hepatocellular carcinoma (HCC) cell lines (Huh7 and PLC/PRF/5). sh-*Luc* was used as a negative control. (b) SALL4 downregulation affects xenograft tumor growth. (Left) Control PLC/PRF/5 and SALL4-knockdown cells were transplanted into recipient mice. Arrow heads show tumors derived from the control cells. (Right) Representative tumors derived from control and SALL4-knockdown cells. (c) Kaplan–Meier plot showing the survival of HCC patients expressing high and low levels of SALL4. (Reproduced from Oikawa *et al.* with permission).[22]

Platelet-derived growth factor receptor-α (PDGFRα) is a cell surface maker specifically expressed in fetal liver mesenchymal cells. We isolated PDGFRα$^+$ cells from fetal livers and co-cultured them with HPC using two culture systems: (i) a transwell culture system to analyze the effect of soluble factors on mesenchymal cells; and (ii) a direct co-culture system to analyze the effect of direct mesenchymal cell–HPC interactions.[31] Transwell co-culture significantly induced proliferation in HPC. These cells expressed minimal levels of albumin, a marker of hepatocyte differentiation. In contrast, expanded cells expressed significant levels of albumin when HPC were directly co-cultured, but proliferation of HPC was not increased. Therefore, the interactions between HPC and PDGFRα-expressing mesenchymal cells are important both for expansion of HPC and for induction of hepatic gene expression. Specifically, PDGFRα$^+$ cells had the potential to support HPC proliferation through secretion of soluble paracrine factors.

Stem/progenitor cell systems in the adult liver

A stem/progenitor cell system exists in normal adult livers and is thought to contribute to regeneration induced by severe liver injuries. During serious liver injury, such as that induced by retrorsine or 2-acetylaminofluorene treatment in combination with partial hepatectomy, the number of characteristic non-parenchymal oval cells increases in periportal regions. These cells express both cholangiocellular (*Ck7* and *19*) and hepatocellular marker genes (α-fetoprotein and albumin), and differentiate into both hepatocytic and cholangiocytic cells, suggesting that oval cells are candidate hepatic progenitors.[32–35]

However, the origin of oval cells is now under discussion. We recently purified CD13$^+$CD133$^+$ cells in non-injured postnatal livers and established an efficient single-cell culture system that involved the use of Rock inhibitor.[18] These CD13$^+$CD133$^+$ cell-derived colonies can expand for a prolonged period and differentiate into hepatocyte-like and cholangiocyte-like cells under appropriate culture conditions. These results show the presence of stem/progenitor cells in the CD13$^+$CD133$^+$ subpopulation of non-hematopoietic cells derived from non-injured postnatal livers. In addition, we speculate that oval cells may be transiently amplifying cells that originate from normal liver stem/progenitor cells (Fig. 3). Other postnatal cell surface markers were used for identification and purification of hepatic progenitor cells in normal and injured livers.[32–37] Hepatic progenitor cells were also identified in human livers. Schmelzer *et al.* showed that epithelial cell adhesion molecule positive cells in human fetal and adult livers can proliferate *in vitro* and expand after the transplantation into the injured mouse livers. Therefore, these

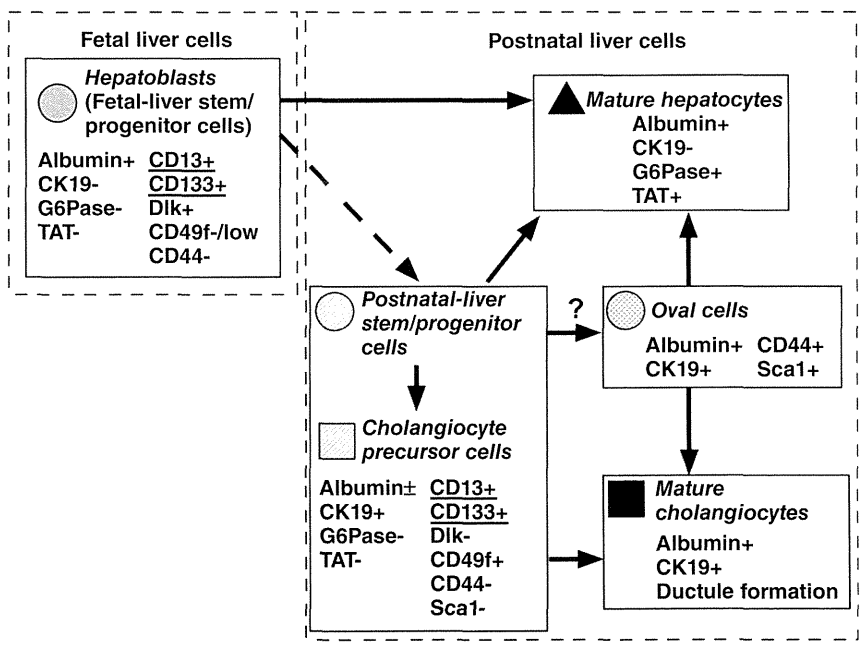

Figure 3 Embryonic and postnatal progenitor cell phenotypes during liver development. CD13 and CD133 are surface markers common to fetal-liver and postnatal-liver progenitor cells. CD, cluster of differentiation; CK, cytokeratin; Dlk, delta-like 1 homolog; G6Pase, glucose 6-phosphatase; Sca1, stem cell antigen 1; TAT, tyrosine aminotransferase. (Reproduced from Kamiya *et al.* with permission.)[18]

stem/progenitor cells will be useful for future therapies of severe liver diseases.[38]

Expansion of progenitor cells such as oval cells in injured livers is regulated by several soluble factors and cell–cell interactions. Recent studies report that fibroblast growth factor 7 (FGF7) derived from periportal mesenchymal cells is essential for the expansion of progenitor cells and survival in patients with severe liver injuries.[39] Severe and chronic liver damage induces Thy1$^+$ mesenchymal cell expansion and the expression of FGF7. Overexpression of FGF7 in livers *in vivo* induces expansion of hepatic progenitor-like cells and suppresses liver damage. In contrast, *Fgf7*-knockout mice exhibited marked suppression of progenitor cell expansion and higher mortality in the severe liver injury model.

DIFFERENTIATION OF HEPATIC STEM/PROGENITOR CELLS DERIVED FROM PLURIPOTENT STEM CELLS

MECHANISMS UNDERLYING PROLIFERATION and differentiation of human hepatic stem/progenitor cells remain largely unknown, because of the difficulty associated with analyzing cellular and molecular events *in vivo*. As mentioned previously, ES and iPS cells are multipotent and differentiate into specialized cell types of several organs. Methods to induce differentiation of mouse and human ES and iPS cells into hepatic progenitor cells and mature hepatocytes *in vitro* have been established.[40] Hepatic cells generated from patient-derived iPS cells are considered to be beneficial for the treatment of severe liver diseases, screening of drug toxicities and basic research of several hepatocytic disorders. However, generating iPS cell clones derived from many patients is problematic. These issues include the recruitment of patients, definition of guidelines to validate new clones and the diversity of iPS cell characteristics arising from different genetic backgrounds. For example, donor differences of human iPS cells affect their propensity for hepatic differentiation.[41] As recently described, novel methods to generate human iPS cells carrying genomic mutations have been established using genome editing enzymes such as zinc-finger nucleases, transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9).[42] Human cell line- or pluripotent stem cell-derived disease models carrying site-directed mutations reportedly have been established using TALEN.[43] These models are highly attractive because disease-related iPS cells carrying specific mutations can be easily and rapidly derived from control iPS cells.

Differentiation of pluripotent stem cells into hepatic lineage cells mimics the *in vivo* stepwise developmental processes, including sequential stimulation with several differentiation factors such as activin A, basic fibroblast growth factor, bone morphogenetic protein and HGF.[40] Thus, it is speculated that HPC derived from human iPS cells develop *in vitro* following the same differentiation steps and time-course as normal cells *in vivo*. Our group and others analyzed differentiation of human ES and iPS cells into fetal HPC in these culture systems.[44–46] For example, we found that highly proliferative cells exist in the CD13$^+$CD133$^+$ fraction of human iPS cells stimulated by hepatocytic differentiation factors (Fig. 4). As mentioned previously, CD13 and CD133 are cell surface markers of stem/progenitor cells in mouse fetal and adult livers. Individual CD13$^+$CD133$^+$ cells formed large colonies containing more than 100 cells and expressed both hepatocytic (α-fetoprotein and hepatocyte nuclear factor 4α) and cholangiocytic marker genes (*Ck7*), suggesting that CD13$^+$CD133$^+$ cells derived from human iPS cells in culture exhibit characteristics of HPC. Next, we assessed whether human iPS cell-derived HPC had the potential to differentiate into mature hepatocytic and cholangiocytic cells. 3-D biological structures are important for the induction of mature hepatocytic functions and the hanging drop method is often used to self-assemble hepatic stem/progenitor cells into aggregates called spheroids. We found that several hepatic functional genes, such as cytochrome P450, were induced in human iPS cell-derived HPC spheroids. In addition, we tested whether human iPS cell-derived HPC formed cholangiocytic structures during gel culture. Cholangiocytic cells form cysts with epithelial polarity, demonstrating that *in vitro* tubulogenesis occurs in extracellular matrix gel supplemented with cytokines.[47] Many epithelial cysts were formed in gel cultures of human iPS cell-derived HPC. Several of these epithelial cysts expressed cholangiocytic marker CK7, but did not express hepatocytic marker α-fetoprotein. These results indicate that human iPS cell-derived HPC exhibit a bipotent differentiation ability, forming hepatocytes and cholangiocytes.[45]

Efficient methods to establish functional hepatocytes derived from human ES and iPS cells have yet to be identified. One avenue to establish functional livers derived from human iPS cells is to organize hepatic cells *in vitro* in 3-D. Co-culture of human iPS cell-derived hepatocytic cells with human endothelial and mesenchymal cells reportedly forms specific 3-D organoids *in vitro*.[48] These organoids expressed several functional enzymes and proliferated *in vivo* after transplantation. In

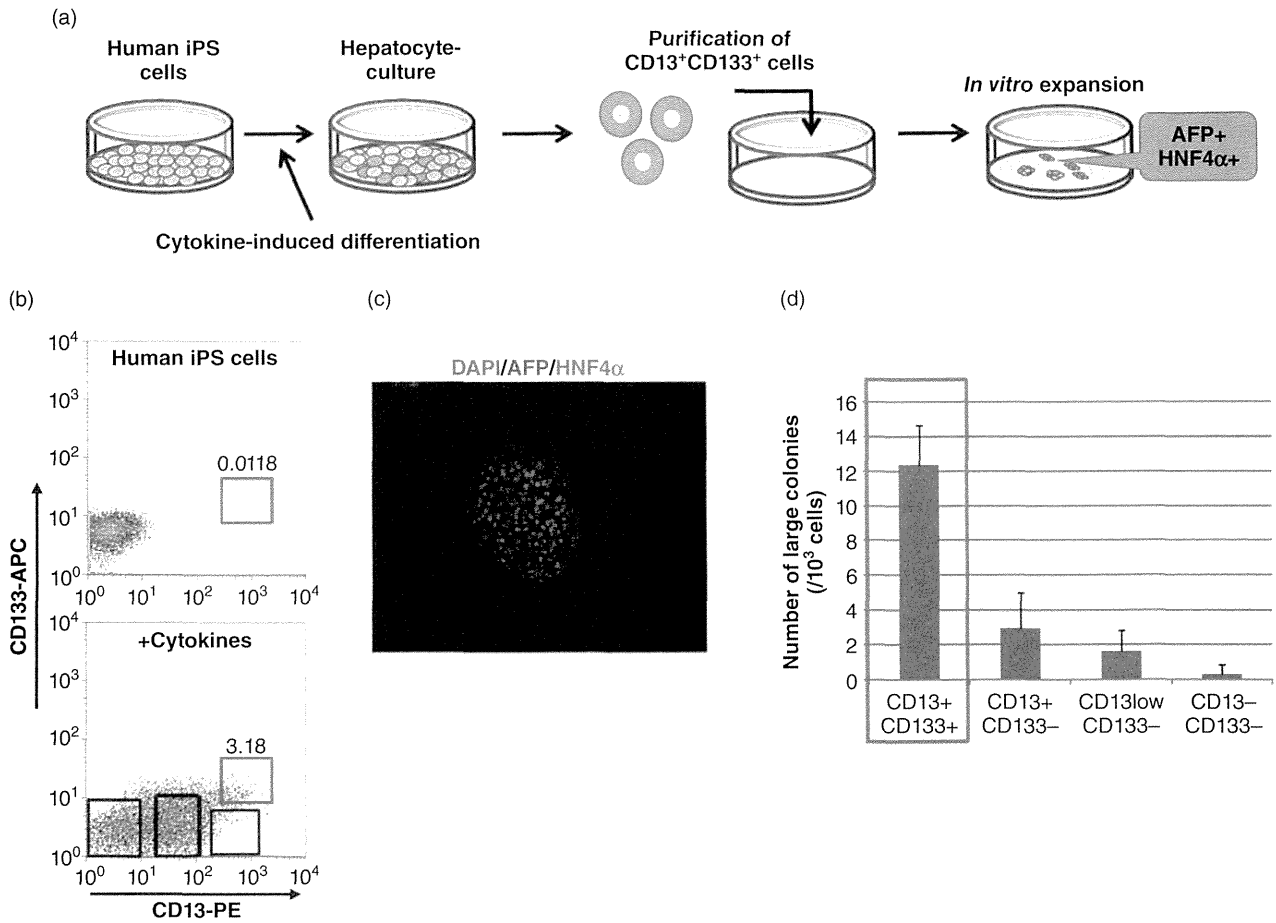

Figure 4 Purification and expansion of hepatic progenitor-like cells derived from human induced pluripotent stem (iPS) cells. (a) Expansion method for hepatic progenitor-like cells. Human iPS cells were differentiated into hepatocytic cells by using cytokines (activin A, fibroblast growth factor, bone morphogenetic protein and hepatocyte growth factor). $CD13^+CD133^+$ cells in the differentiated cell culture were purified and plated onto feeder cells at a low density. (b) Flow cytometry data from human iPS cells and differentiated cells (+Cytokines). (c) Representative colony-derived $CD13^+CD133^+$ hepatic progenitor-like cells. α-Fetoprotein (AFP) and hepatocyte nuclear factor 4α (HNF4α) were detected by immunocytochemistry. (d) The number of large colonies derived from human iPS cells differentiated by cytokines. The $CD13^+CD133^+$ fraction contained many progenitor-like cells. (Reproduced from Yanagida *et al.* with permission.)[45]

addition, organoids were highly vascularized. These results suggested that a suitable environment, such as the presence with non-parenchymal cells, is important for cultured human iPS cells to acquire normal liver functions.

SUMMARY AND FUTURE PERSPECTIVE

IN THIS REVIEW, we present data showing that mesenchymal cells can perform a niche-like function, supporting hepatic stem/progenitor cells in the co-culture system. Recently, we found that small numbers of HPC generated by *in vitro* expansion using the co-culture system could be transplanted into the injured-liver mouse model (unpubl. data). Therefore, stem/progenitor cells in fetal and adult livers are good candidates to be used in regenerative medicine for several liver diseases. Pluripotent stem cells are also potential tools for cell transplantation therapies and drug discovery research. Recently, human ES and iPS cell-derived hepatocytic cells have been established using protocols that mimic the events that occur during normal development. Therefore, culture systems that produce iPS cell-derived hepatic progenitor cells

may also be useful for the study of human hepatic cell development.

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HEPATOLOGY

Efficacy of continuous plasma diafiltration therapy in critical patients with acute liver failure

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Key words
acute kidney disease, acute liver failure, blood purification therapy, plasma exchange.

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Abstract

Background and Aims: Acute liver failure (ALF) is a critical illness with high mortality. Plasma diafiltration (PDF) is a blood purification therapy that is useful for ALF patients, but it is difficult to use when those patients have multiple organ failure or unstable hemodynamics. In these patients, symptoms are also likely to exacerbate immediately after PDF therapy. We developed continuous PDF (CPDF) as a new concept in PDF therapy, and assessed its efficacy and safety in ALF patients.

Methods: Ten ALF patients (gender: M/F 6/4, Age: 47 ± 14) were employed CPDF therapy. The primary outcomes were altered liver function, measured by the model for end-stage liver disease (MELD) score, and total bilirubin and prothrombin time international normalized ratios (PT-INR), 5 days after CPDF therapy. Secondary outcomes included sequential organ failure assessment (SOFA) scores, 5 days after CPDF therapy, and the survival rate 14 days after this therapy.

Results: The MELD score (34.5–28.0; $P = 0.005$), total bilirubin (10.9–7.25 mg/dL; $P = 0.048$), PT-INR (1.89–1.31; $P = 0.084$), and SOFA score (10.0–7.5; $P < 0.039$) were improved 5 days after CPDF therapy. Nine patients were alive, and one patient died because of acute pancreatitis, complicated by ALF. There were no major adverse events related to this therapy under hemodynamic stability.

Conclusion: In the present study, CPDF therapy safely supported liver function and generally improved the condition of critically ill patients with ALF.

Introduction

Acute liver failure (ALF) is a rapidly progressing critical illness associated with a high mortality rate, and characterized by jaundice, ascites, hepatic encephalopathy, and bleeding due to severe impairment of liver function caused by massive liver necrosis.[1,2] Acute, severe hepatic necrosis releases toxic metabolites such as ammonia from the splanchnic circulation.[3]

The therapeutic strategy for ALF differs between Western countries and Japan. In Japan, an artificial liver support system (ALSS) is commonly the first-line therapy because liver transplantation is usually accomplished with living donors.[4–7] In Western countries, deceased donor liver transplantation is the first-line therapy for ALF.[8]

Plasma exchange (PE) is a fundamental and simple ALSS. However, the procedure is accompanied by adverse effects such as hypernatremia, metabolic alkalosis, citrate poisoning, and abrupt changes in colloid osmotic pressure.[9,10] In Japan, PE is often combined with continuous hemodiafiltration (CHDF), a process in which electrolyte imbalance is corrected and fluids are controlled, simultaneously. PE/CHDF therapy, however, can be expensive because it requires 3.2–4.8 L of fresh frozen plasma (FFP), along with the necessary equipment; a risk of infection is also associated with this therapy.

Plasma diafiltration (PDF or selective plasma filtration with dialysis) has been developed as an alternative to PE/CHDF therapy. PDF is a blood purification therapy in which simple PE is performed with a membrane plasma separator, while dialysate flows outside the hollow fibers.[11] In ALF patients, PDF is a useful bridge therapy for liver regeneration or transplantation.[12,13] However, PDF therapy is difficult to use in critically ill ALF patients with complicated multiple organ failure, especially in those with unstable hemodynamics. Symptoms can also be exacerbated immediately after PDF therapy because this conventional PDF therapy is used intermittently throughout an 8-h day.

In this study, we designed a new PDF therapy concept, termed continuous PDF (CPDF), and conducted an observational study to assess the efficacy and safety of this therapy for patients with ALF.

Materials and methods

The study protocols conformed to the ethical guidelines of the 2008 Declaration of Helsinki. This study was approved by the

Table 1 Demographics of ten ALF patients

No.	Age (years)	Gender (M/F)	Etiology	MELD score	PT-INR	Total bilirubin (mg/dL)	Encephalopathy grade	M.V.	SOFA score	Total duration (days)	Outcome
1	33	F	HELLP	29	1.81	5.4	—	+	13	9	Alive
2	37	M	Alcohol	21	1.57	11.3	—	−	5	14	Alive
3	60	F	AIH	40	1.90	34	III	−	11	5	Death
4	38	M	Drug	43	4.83	5.1	III	−	8	8	Alive
5	54	M	HBV	34	1.88	28	II	+	14	12	Alive
6	63	F	AIH	26	2.30	15.2	II	+	8	9	Alive
7	26	M	Unknown	36	1.96	10.5	IV	−	11	9	Alive
8	47	F	HBV	51	9.57	4.5	III	+	9	7	Alive
9	34	M	Drug	35	1.76	10.4	II	+	14	14	Alive
10	38	M	Unknown	27	1.57	65.3	—	−	12	11	Alive

—, not detected; AIH, autoimmune hepatitis; ALF, acute liver failure; HBV, hepatitis B virus; HELLP, hemolysis elevated liver enzymes; MELD, model for end-stage liver disease; M.V., mechanical ventilation; PT-INR, prothrombin time international normalized ratios; SOFA, sequential organ failure assessment.

institutional review board of the Kanazawa University Graduate School of Medicine.

Patients. Ten patients in the intensive care unit (ICU) at Kanazawa University Hospital from January 2011 to April 2013 received CPDF therapy. These patients fulfilled the Japanese diagnostic criteria for ALF,[14] which consists of prothrombin time international normalized ratios (PT-INR) of > 1.5 caused by severe liver damage within 8 weeks of onset of the symptoms when prior liver function was estimated as normal. Informed written consent of the patients or responsible family members was obtained prior to enrollment.

CPDF decision process and implementation. The decision to employ CPDF for each patient included fulfilling the diagnostic criteria of ALF; complicated renal dysfunction that compromised fluid management; and ongoing fluid management concerns including significant ascites, edema, and/or fluid overload. The patient characteristics are listed in Table 1. CPDF therapy was performed using an Evacure EC-2A plasma separator (Kuraray, Tokyo, Japan) at a blood flow rate of 80 mL/min. Filtered replacement fluid for artificial kidneys (Sublood-BS; Fuso Pharmaceutical, Osaka, Japan) was infused at a dialysate flow rate of 400 mL/h and a replacement flow rate of 280 mL/h. FFP was infused intravenously at 120 mL/h, and nafamostat mesilate (Futhan; Torii Pharmaceutical, Tokyo, Japan) was used as an anticoagulant.

The CPDF column was replaced every 24–48 h unless disabled. Patients were monitored closely for signs and symptoms of adverse effects or complications during this therapy. We decided that the criteria for discontinuation of CPDF was a total bilirubin of < 5.0 mg/dL and PT-INR < 1.2, and the point in time at which the survival rate improved.

The primary outcomes were altered liver function measured by the model for end-stage liver disease (MELD) score, total bilirubin, and PT-INR 5 days after CPDF therapy. This time point was chosen because patients with ALF are generally re-assessed for liver transplantation every 5 days. Secondary outcomes included sequential organ failure assessment (SOFA) scores 5 days after CPDF therapy and survival rate 14 days after CPDF therapy. SOFA is a scoring system to determine the extent of a person's multiple organ function or rate of failure based on six different scores, such as respiratory, cardiovascular, hepatic, coagulation, renal, and neurological systems.[15]

Statistical analysis. Data are expressed as medians and interquartile ranges. Differences in variables before and after CPDF therapy were examined by paired Student's *t*-test after a symmetrical distribution was confirmed. $P < 0.05$ indicated statistical significance. We also considered clinical efficacy analyzed by effect size (ES) using Cohen's *d*, which measures the strength of the relationship before and after CPDF therapy due to the low number of patients in this study. We determined that ES > 0.2 = small, ES > 0.5 = moderate, and ES > 0.8 = large efficacy of this therapy based on Cohen's criteria.

Results

Demographics. We assessed 10 patients with ALF. All patients were diagnosed with ALF and received CPDF therapy. The characteristics of the patients are shown in Table 1. The etiology for ALF was variable, and the average age of patients was 47 ± 14 years (range, 26–64). Seven ALF patients had overt hepatic encephalopathy. Five ALF patients received mechanical ventilation therapy, while there was no patient with inotropic and vasopressor support.

Primary outcomes. The MELD score improved significantly from 34.5 to 28.0 ($P = 0.005$; Fig. 1a), resulting in high clinical effectiveness (ES = 0.78; Table 2) after CPDF therapy. Total bilirubin also significantly improved from 10.9 to 7.25 mg/dL ($P = 0.048$; Fig. 1b), resulting in moderate clinical effectiveness (ES = 0.65; Table 2) after CPDF therapy. PT-INR had a tendency to improve from 1.89 to 1.31 ($P = 0.084$; Fig. 1c), resulting in high clinical effectiveness (ES = 0.92; Table 2) after CPDF therapy.

Secondary outcomes. The SOFA score decreased from 10.0 to 7.5 ($P < 0.039$; Fig. 1d), resulting in moderate clinical

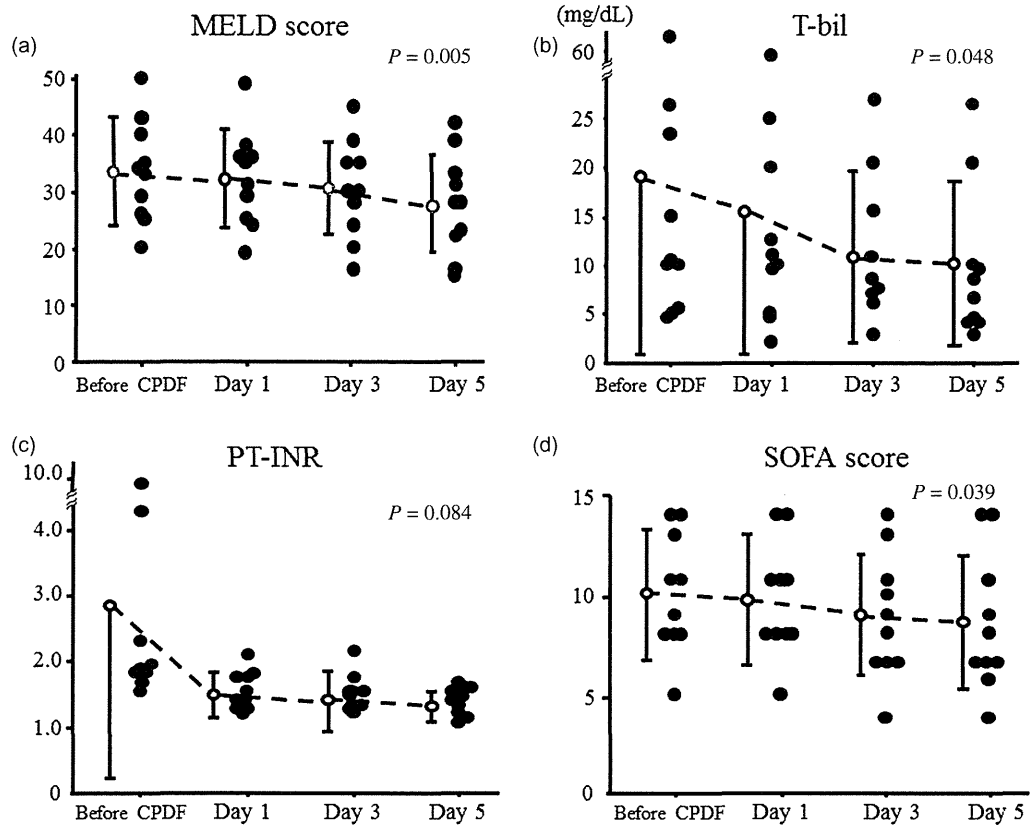

Figure 1 ALF patient parameters. (a–c) Primary study outcomes of this study. MELD score (a), total bilirubin value (b), and PT-INR (c) were improved 5 days after CPDF therapy. (d) Secondary study outcomes of this study. SOFA score (d) were improved after 5 days CPDF therapy. Each value are plotted, and also expressed as means ± SD.

Table 2 Parameter alteration 5 days after CPDF Treatment

Variables	Before CPDF	After CPDF	Effect size	95% CI	*P* value
SOFA score	10 (5–14)	7.5 (4–14)	0.47	0.085–2.715	0.039
MELD score	34.5 (21–51)	28 (14–42)	0.78	2.611–10.789	0.005
Total bilirubin (mg/dL)	10.9 (4.5–65.3)	7.25 (3.4–27.7)	0.65	0.076–17.82	0.048
Ammonia (mg/dL)	119 (60–316)	143 (58–407)	0.46	−115–24.2	0.174
Creatinine (mg/dL)	1.44 (0.47–4.79)	1.36 (0.27–3.12)	0.37	−0.176–1.198	0.127
PT-INR	1.89 (1.57–9.57)	1.31 (1.17)	0.92	−0.258–3.384	0.084
ALT (IU/L)	123 (21–10 892)	39 (17–482)	0.77	−743–495	0.129
AST (IU/L)	109 (60–15 183)	52.5 (28–436)	0.82	−759–6440	0.108
Albumin (g/dL)	3.1 (2.6–3.7)	2.7 (2.3–3.2)	1.08	−0.017–0.777	0.059
MAP (mm Hg)	82 (62–112)	89.5 (72–115)	0.23	−22.4–3.42	0.131
Heart rate (beat/min)	89.5 (76–122)	92 (57–135)	0.34	−5.49–17.49	0.268
PaO_2/FiO_2 ratio	330 (188–583)	383 (240–567)	0.38	−139–59.4	0.195

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; CPDF, continuous plasma diafiltration; MAP, mean arterial pressure; MELD, model for end-stage liver disease; PT-INR, prothrombin time international normalized ratios; SOFA, sequential organ failure assessment.

effectiveness (ES = 0.47; Table 2) after CPDF therapy. Nine patients were alive and discharged from the ICU, and one patient died due to acute pancreatitis complicated by ALF. CPDF therapy had no major adverse effect, including bleeding, and maintained hemodynamic stability.

Other outcomes. Parameters of hepatocyte injury such as aspartate aminotransferase/alanine aminotransferase also decreased (ES = 0.82 and 0.77, respectively) by CPDF therapy. The creatinine value (ES = 0.37) had a small clinical effectiveness (Table 2).

Five ALF patients with overt encephalopathy were controlled by CPDF therapy, while two patients with uncontrolled encephalopathy were treated with CPDF therapy combined with hemodialysis to control overt encephalopathy. Circulation parameters such as mean arterial pressure and heart rate were maintained (Table 2) without inotropic and vasopressor support during CPDF treatment period. Oxygenation index (PaO_2/FiO_2) as a measure of pulmonary function increased after this treatment (Table 2). In this study, mechanical ventilation was indicated not only by hepatic encephalopathy but also by impaired respiratory function because of massive ascites or pleural effusion, or an unstable hemodynamic state. Two patients were withdrawn from mechanical ventilation after pulmonary function improved.

Moreover, we could employ this treatment without any adverse events, such as infections and unstable hemodynamics.

Discussion

In the present study, we observed that CPDF therapy improved liver function in critical ALF patients with beneficial effects on renal, pulmonary, and hemodynamic function that led to an improved SOFA score which reflected the severity of critical illness without any adverse effects.

ALSS is the first-line therapy for ALF patients until liver regeneration or transplantation because only a small proportion of ALF patients can receive deceased donor liver transplants, in Japan.[4,6] Recently, several types of ALSS methodologies such as Prometheus or the Molecular Adsorbent Recirculating System have been developed, primarily to eliminate toxic substances. However, these therapies require complex equipment and are expensive.[16,17]

Conventional PDF therapy is simple, less expensive, and results in fewer adverse events than other therapies. Consequently, this therapy has been demonstrated to be one of the most useful blood purification therapies for ALF patients.[11,13] However, conventional PDF therapy, which is used intermittently throughout an 8-h day, does not usually maintain hemodynamic stability. Because we dealt with critical patients in ALF, a group in whom hemodynamic instability and a high SOFA score implying high mortality in this observational study, we employed CPDF therapy, which can maintain stable hemodynamics in most cases.

In this study, we showed that the efficacy of CPDF liver support. Moreover, CPDF therapy provides some of the characteristics of renal replacement treatment for patients with ALF; these patients frequently have renal functional impairment. CPDF therapy, as well as CHDF, avoid abrupt changes and successively remove toxic substances while managing fluid balance.[18] This reduces pulmonary edema and the exacerbation of impaired respiratory function and satisfactorily supports liver function.[19,20] In fact, CPDF therapy improved a pulmonary function in several patients of this study. Thus, CPDF therapy can improve the function of multiple organs, possibly making this therapy superior to conventional PDF therapy. Moreover, these benefits suggest that CPDF therapy is cost-effective and helps avoid the possibility of infection.

Whether CPDF therapy can effectively remove toxic substances (e.g. ammonia) during rapid disease progression, caused by hepatic encephalopathy, remains to be determined. Similarly, an assessment of how CPDF therapy maintains decreased plasma albumin value also remains to be determined, as we did not observe improvements in ammonia values. This study included two patients with high ammonia values and uncontrolled encephalopathy; these patients employed CPDF, combined with hemodialysis, to control the encephalopathy. The plasma albumin value was decreased during the CPDF therapy because the sieving coefficient of 0.3 for albumin selectively removed low- and intermediate-molecular weight, albumin-bound substances in the plasma separator (Evacure EC-2A). Albumin loss was managed by intravenously administering 12.5–25 g of albumin to maintain the plasma albumin level.

Some of the limitations of this study should also be considered. The first is that the therapeutic strategy for ALF is different, in Japan, from that in Western countries because few deceased donor liver transplants are performed. The second is that although our study was prospective, it was not a randomized controlled study (RCT), and the sample size was small. An RCT should be explored to determine the effects of CPDF in patients with ALF.

In summary, CPDF therapy, a new concept in ALSS, improved liver function in critically ill ALF patients and had beneficial effects on multiple organ functions, suggesting that it may be an alternative or at least one of the useful and desirable forms of ALSS for ALF patients.

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Original Article

Characteristics and prediction of hepatitis B e-antigen negative hepatitis following seroconversion in patients with chronic hepatitis B

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Aim: We analyzed the characteristics of alanine aminotransferase (ALT) abnormality after achieving hepatitis B e-antigen (HBeAg) seroconversion (SC) and other factors associated with the occurrence of HBeAg negative hepatitis.

Methods: We followed 36 patients with chronic hepatitis B from 3 years prior to at least 3 years after SC (mean, 11.6 years) and examined ALT, hepatitis B virus (HBV) DNA, HB surface antigen, HB core-related antigen (HBcrAg) levels and mutations related to HBeAg SC.

Results: ALT normalization (<31 IU/L for at least 1 year) was primarily observed until 2 years following SC, after which it became more infrequent. We next divided patients into abnormal (≥31 IU/L, $n = 20$) and normal (<31 IU/L, $n = 16$) groups based on integrated ALT level after the time point of 2 years from SC, and considered the former group as having HBeAg negative hepatitis in the present study. Although changes in median levels of ALT and HBcrAg differed significantly between the groups, multivariate analysis showed ALT normalization within 2 years after SC to be the only significant determining factor for this disease ($P = 0.001$). We then assessed the 19 patients whose ALT was normal at 2 years following SC, four of whom developed HBeAg negative hepatitis. Increased levels of HBV DNA ($P = 0.037$) and HBcrAg ($P = 0.033$) were significant factors of potential relevance.

Conclusion: ALT abnormality after 2 years of SC may be evaluated as HBeAg-negative hepatitis. ALT, HBV DNA and HBcrAg levels may be useful in predicting the outcome of patients who achieve HBeAg SC.

Key words: hepatitis B core-related antigen, hepatitis B virus, reactivation, seroconversion

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern with an estimated 350–400 million carriers worldwide. Whereas acute infection in adults is generally self-limiting, that during early childhood develops into persistent infection in most individuals, which can lead to chronic hepatitis and eventually liver cirrhosis and hepatocellular carcinoma (HCC).[1–3] The natural history of chronic HBV infection can be classified into several phases based on levels of alanine aminotransferase (ALT) and HBV DNA, hepatitis B e-antigen (HBeAg) status and estimated immunological status.[4] In the immune tolerance phase, HBeAg is positive, ALT level is normal, histological evidence of hepatitis is absent or minimal, and HBV DNA level is elevated. The chronic hepatitis B phase is characterized by raised ALT and HBV DNA levels. In this phase, the host's immune system initiates a response that results in active hepatitis. In patients who are HBeAg positive, active hepatitis can be prolonged and may result in cirrhosis. However, chronic hepatitis B eventually transitions into an inactive phase with a loss of HBeAg positivity in the majority of patients. Seroconversion (SC) of HBeAg to HBe antibodies and the fall of HBV DNA level result in the disappearance of disease activity despite persisting hepatitis B surface antigen (HBsAg) and low HBV DNA level. The SC of

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HBeAg marks the transition from the hepatitis phase to the inactive carrier phase, which is generally thought to be a benign course for the HBV carrier, although hepatitis can sometimes reactivate spontaneously.[5]

Patients experiencing HBV reactivation undergo another transition characterized by increases in HBV DNA and ALT levels and disease activity without the reappearance of HBeAg. This phase is referred to as HBeAg negative chronic hepatitis B. Occasional severe hepatitis B flare-ups with moderate HBV DNA level occur in this phase.[6,7] It is thought that HBeAg negative chronic hepatitis B is caused by mutant strains of HBV that are unable to produce HBeAg[6,8] and tends to develop into cirrhosis and HCC more frequently than does HBeAg positive chronic hepatitis B.[9–13] Therefore, it is important to identify patients who are likely to develop HBeAg negative hepatitis after HBeAg SC from those who can maintain an inactive carrier phase. In the present study, we evaluated 36 patients with HBeAg SC to examine the effects of host factors and viral factors, including serum quantitative HBsAg, hepatitis B core-related antigen (HBcrAg), HBV DNA, PC (A1896) mutation and BCP mutations (T1762 and A1764) before, during and after SC.

METHODS

Patients

A TOTAL OF 36 patients with sustained HBeAg SC (24 men and 12 women; median age, 38 years [range, 23–65]) were enrolled in this study after meeting the following criteria: (i) follow ups for at least 3 years before and after HBeAg SC; and (ii) serum samples at several time points before, during and after SC available for testing. HBeAg SC was defined as seroclearance of HBeAg with the appearance of anti-HBe that was not followed by HBeAg reversion or loss of anti-HBe. All patients were seen at Shinshu University Hospital from 1985 to 2009. The median follow-up period after SC was 11.6 years (range, 3.2–26.0). HBsAg was confirmed to be positive on two or more occasions at least 6 months apart in all patients. No patients had other liver diseases, such as alcoholic or non-alcoholic fatty liver disease, autoimmune liver disease or drug-induced liver injury. Patients who were complicated with HCC or who showed signs of hepatic failure were excluded from the study. HBV genotype was C in all patients, who were also negative for antibodies to hepatitis C virus and HIV. Nucleoside/nucleotide analog (NUC) therapy was introduced in 14 patients after HBeAg SC on physicians' decision, and then follow up was stopped. No patient was treated with interferon during the study period. ALT, albumin, bilirubin, platelet and other relevant biochemical tests were performed using standard methods.[14] The integration value of ALT after SC was calculated using the method described by Kumada *et al.*[15] (median determination frequency, 4.7/year per person [range, 1.6–13.9]) because a previous study showed integration values to be more meaningful than arithmetic mean values in long-term follow-up cohorts.[16] As guidelines released by the Ministry of Health, Labor and Welfare of Japan advise consideration of antiviral therapy for patients with ALT levels of 31 IU/L or more,[17] an ALT integration value of less than 31 IU/L was defined as normal in this report. Serum samples were stored at −20°C until tested. Liver biopsies were performed by percutaneous sampling of the right lobe with a 14-G needle in eight patients with HBeAg negative hepatitis, as reported previously.[14] All biopsies were 1.5 cm or more in length. Liver histological findings were scored by the histology activity index of Knodell *et al.*[18] The protocol of this study was approved by the ethics committee of our university and was in accordance with the Declaration of Helsinki of 1975. Informed consent was obtained from each patient.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg and anti-HBe, were tested using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan).[19] Quantitative measurement of HBsAg was done using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex (Kobe, Japan).[20] The assay had a quantitative range of −1.5 to 3.3 log IU/mL. Serum HBcrAg level was measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio, Tokyo, Japan) as described previously.[21] We expressed HBcrAg level in terms of log U/mL, with a quantitative range set at 3.0–6.8 log U/mL. End titers of HBsAg and HBcrAg were determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range. HBV DNA level was measured using an Amplicor monitor assay with a dynamic range of 2.6–7.6 log copies/mL.[22] Six major genotypes (A–F) of HBV were determined using the method reported by Mizokami *et al.*,[23] in which the surface gene sequence amplified by polymerase chain reaction was analyzed by restriction fragment length polymorphism.