厚生労働科学研究研究費補助金

基礎研究成果の臨床応用推進研究事業

自己骨髄細胞を用いた肝臓再生療法の開発に関する研究

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厚生労働科学研究費補助金（基礎研究成果の臨床応用推進研究事業）
総括研究報告書
自己骨髄細胞を用いた肝臓再生療法の基礎と臨床
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研究要旨　我々はより多くの肝不全患者を救命するために、生体肝移植前に行うブリッジ的な治療法として『自己骨髄細胞を用いた肝臓再生療法』の臨床開発を進めたいと考え研究を行ってきた。（自己骨髄細胞を用いた肝臓再生療法）の臨床応用を進める基盤モデルとして骨髄細胞から肝細胞への分化評価モデル(GFP/CCl4モデル)の開発し、骨髄細胞が持続的肝障害の肝硬変時に肝臓に遊走され肝細胞へ分化・増殖することを明らかにした（JB 2003、特公2003-70377）。さらにこのモデルの解析を通じ、骨髄細胞移植により生存率の回復、また肝線維化の改善を発見した（Hepatology 2004, この発見はWiley社よりHepatology News Alert記事とし世界に発信された）。さらに骨髄細胞を用いた再生療法をより効率的に行うための骨髄細胞の肝細胞への分子制御機構をMicro array-Self Organization Map(SOM)解析法にて解析した（FEBS letters 2004, 生データはhttp://liver-project.med.yamaguchi-u.ac.jp/researchのサイトでホームページにて公開している。）。また我々の開発したLiv8抗体は骨髄中の肝再生に有効な分画の分離に有効であることが明らかになった(BBRC 2004)。今後は抗原同定を進め人抗体を作ることで、効率的な再生療法の臨床開発が期待できる。
これらの基礎研究成果を基盤に約２年の月日をかけ臨床研究の準備を進め平成15年11月14日に国内初の（自己骨髄細胞を用いた肝臓再生療法）のPhaseI臨床研究を開始した。臨床研究の対象は（75歳以下、非代償性肝硬変症，ビリルビンの値は3mg/dl以下、血小板5万以上、肝癌はコントロールされていること、また心肺機能正常）が基本的な適応である。実際のプロトコールは，全身麻酔下にて、自己骨髄細胞を400ml分離し末梢静脈より投与する。採取した骨髄液は濃縮器セルプロセッサーCytomateを使用し骨髄細胞を濃縮・洗浄し、患者の静脈より投与し、その後血液検査、画像検査等により肝臓機能の改善について評価する。この臨床研究の開始はNHK等の報道機関にて報道され、多くの肝不全患者から期待されている。現在までに8例の患者に施行し副作用の発生はない。また肝機能について長期に経過観察しえた6症例についての解析をしたところ、(術前、1ヶ月、6ヶ月のエンドポイントでの評価では、平均値で血清アルブミン値は15.2%(1ヶ月後)、および8.7%(6ヶ月後)上昇、血小板値は19.8%、および18.9%上昇、また肝線維化の評価として血清プロコラーゲンⅢ型ペプタイド値は9%および12.3%減少と肝線維化の改善傾向を確認した。また骨髄細胞投与１ヶ月後の肝生検組織において肝再生マーカー蛋白の発現により肝再生も誘導されたと考えられた。この結果は、肝移植以外に有効な治療法がない肝不全患者に対し、自己骨髄細胞を用いた肝臓再生療法の有効性を示し、また安全性が確認された。

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A. 研究目的
C型肝炎の蔓延とともに近年肝疾患が増加している。それとともに肝不全（肝硬変、肝癌、劇症肝炎）患者が増加している。現在肝不全患者に対しては日本においては生体肝移植が行われているが、手術侵襲の問題、ドナーの問題などまだまだ障害が多い。また今後高齢者を対象とした医療を行うには、より侵襲の少ない移植にかわる次世代の再生医療技術の開発が急務である。最近になり、人剖検例にて骨髄中に存在する細胞が肝細胞へ分化転換したことが報告された。また肝臓は胎児期において２次造血の場であるなど、その骨髄細胞から肝細胞への分化の可塑性は存在すると考えられる。我々はその機序を解明し、さらに実際に人の治療に応用するために、新たに骨髄細胞から肝細胞への分化転換についてその分化転換の機序を解明にするために、GFP/CCl4 modelを開発し骨髄細胞から肝細胞への分化転換について評価した。またトランスレーショナル研究の一環として、基礎研究を基盤にし、臨床研究『自己骨髄細胞を用いた肝臓再生療法』のPhaseI臨床研究を行うべく準備を行い実際の臨床研究を開始した。さらに将来の効率的な再生療法の開発のため胎児期に存在する増殖能と分化能を有する肝幹細胞である肝芽細胞の生死を制御する細胞内シグナル伝達系の解明と肝臓の発生や再生に関わる分子の同定を目的とし研究を行った。

B. 研究方法
基礎研究
※GFP/CCl4モデル（骨髄細胞の肝細胞への分化評価モデル）を開発しこのモデルを用いて基礎的検討を行った。
肝硬変マウスに対する骨髄細胞投与による肝機能の回復、肝線維化の改善、生存率の改善効果について検討した。
※骨髄細胞の肝細胞への分化に関与する遺伝子群の解析
GFP/CCl4モデルの解析を通じて、骨髄由来細胞の肝細胞への分化過程に関与する遺伝子群を，Micro array-Self Organization Map(SOM)解析を用い骨髄細胞の投与により誘導された遺伝子群の抽出を目指した。
※骨髄細胞の肝細胞への分化に関与する細胞外マトリックスの解析と肝線維化改善効果に対する解析
骨髄細胞の投与により、肝線維化の改善が認められたことより、この機序についてさらに解析した。実際にMMP9等のコラゲナーゼの発現が増加していたことより In situ zymographyを行いMMP9の活性を評価し、肝線維化改善のメカニズムについて評価した。
※胎児肝特異抗体、また骨髄中の肝幹細胞分画を精製する抗体の開発
胎生期11.5日のマウス肝を抗原にして複数のモノクローナル抗体を作製した。既に作製済みの１０種類以上の胎児肝を認識するモノクローナル抗体のうち、肝芽細胞を特異的に認識する抗Liv2抗体と血液幹細胞を含む細胞を認識する抗Liv8抗体を開発した。特にLiv8抗体については、細胞表面蛋白を認識する抗体で、細胞のsortingに使えることより骨髄中の肝幹細胞の同定に使用できる抗体になりえるかを評価した。
※マウス肝芽細胞特異抗体の作製を行ない、肝形成不全ノックアウトマウスの解析を行なった。また、ヒト疾患モデル生物として期待されているメダカを用いて、肝形成および肝機能不全メダカを単離した。

臨床研究
臨床研究の対象は（75歳以下、非代償性肝硬変症，ビリルビンの値は3mg/dl以下、肝癌はコントロールされていること、また心肺機能正常）が基本的な適応である。実際のプロトコールは，全身麻酔下にて、自己骨髄細胞を400ml

分離し末梢静脈より投与する。採取した骨髄液は濃縮器セルプロセッサーCytomateを使用し骨髄細胞を濃縮・洗浄し、患者の静脈より投与する。その後、経時的に血液検査を施行し，肝再生についてモニターしていく。また画像的にも腹部エコー、ＣＴ検査等にて，肝再生の促進の有無について評価する。First Endpointは１ヶ月後，Second Endpointは６ヶ月後の肝機能に対する改善について評価した。腹水の状況次第であるが、可能であれば，患者の同意の上，エコー下肝生検を行い肝臓の状態について，組織学的検討をしていく。その際にAFP，c-kit，HNF4，CD34，CD45，HHM抗体を用い免疫染色を行い，実際に骨髄細胞投与により肝再生が誘導されるかを評価する。肝線維化の改善の有無については，血清ヒアルロン酸、４型コラーゲン、３型プロコラーゲンペプタイドを測定し評価した。

（倫理面への配慮）
山口大学動物委員会の承認の下、実験動物取り扱いプロトコールに従い実験は行われた。また分担者の仁科の研究については東京医科歯科大学および東京大学動物委員会の承認の下、実験動物取り扱いプロトコールに従い実験は行われた。また実際の自己骨髄細胞を用いた肝臓再生療法は、ドナー細胞として自己骨髄細胞を使用する。また分離した骨髄細胞には分離後遺伝子操作、サイトカイン等は加えない。このため自己血輸血とほぼ同様の安全性で行えると考える。すでに我々は自己骨髄細胞を用いた肝臓再生療法の臨床研究について、山口大学医学部生命倫理委員会にプロトコールを申請し臨床研究の承認を受け、また臨床研究に参加希望の患者さんに対しては、基本的にはPhaseIの安全性試験であるということを十分に本人、患者家族に説明の上インフォームドコンセントを取得ののちに行った。

C. 研究結果
I:基礎研究の結果
自己骨髄細胞を用いた肝臓再生療法の臨床応用を進める基盤モデルとして骨髄細胞から肝細胞への分化評価モデル(GFP/CCl4モデル)を開発し、骨髄細胞が持続的肝障害の肝硬変時に肝臓に遊走され肝細胞へ分化・増殖することを明らかにした（JB2003、特公2003-70377）。さらにこのモデルの解析を通じ、骨髄細胞移植により生存率の回復、また肝線維化の改善を発見した（Hepatology 2004，この発見はWiley社よりHepatology News Alert記事とし世界に発信された）。さらに骨髄細胞を用いた再生療法をより効率的に行うための骨髄細胞の肝細胞への分子制御機構をMicro array-Self Organization Map(SOM)解析法にて解析した（FEBS letters 2004，生データはhttp://liver-project.med.yamaguchi-u.ac.jp/researchのサイトでホームページにて公開している。）。その結果、骨髄細部の肝細胞への分化は初期には形態形成に関与するHOX，HLH型転写制御分子が関与し、後期においては肝細胞の代謝に関与する分子が誘導されていた。また我々の開発したLiv8抗体は骨髄中の肝再生に有効な分画の分離に有効であることが明らかになった(BBRC 2004)。Liv8抗体については、今後は抗原同定を進め人抗体を作ることで、効率的な再生療法の臨床開発が期待できる。また肝芽細胞の増殖には、ストレス応答性SAPK/JNKシグナル伝達系が必須の役割を果たしていることが明らかになり、“肝芽細胞の生死の制御は、増殖シグナルであるSAPK/JNK系と生存シグナルであるNF・B系，細胞死誘導シグナルであるカスパーゼ系の３者のバランスによって制御される”という概念を提示した。また、メダカを用いた大規模スクリーニングを行ない、興味深い変異メダカ（内胚葉形成に影響のある遺伝子変異４種類、肝形成不全７種類、肝機能不全６種類）の発生を国際誌Mechanisms of Developmentのメダカ特集号に報告した。この中には脂肪肝の表現型を呈する変異メダカも存在し、ヒト疾患モデルとして、また薬剤のスクリーニングの対象としての活用が期待されている。これらの成果から、肝臓の発生や再生を考える重要な分子レベルの知見が得られると考えられる。

II:臨床研究の結果
平成15年11月14日に国内初の（自己骨髄細胞を用いた肝臓再生療法）のPhaseI臨床研究を開始した。臨床研究の対象は（75歳以下、非代償性肝硬変症，ビリルビンの値は３mg/dl以下、肝癌はコントロールされていること、また心肺機能正常）が基本的な適応である。実際のプロトコールは，全身麻酔下にて、自己骨髄細胞を400ml分離し末梢静脈より投与する。採取した骨髄液は濃縮器セルプロセッサーCytomateを使用し骨髄細胞を濃縮・洗浄し（この方法については２年の歳月をかけ開発した）、患者の静脈より投与する。この研究の開始はNHK等の報道機関にて報道され、多くの肝不全患者から期待されている。現在までに８例の患者に施行し、副作用の発生はない。また肝機能について長期に経過観察しえた６症例についての解析をしたところ、(術前、１ヶ月、６ヶ月のEnd Pointでの評価では、平均値で血清アルブミン値は15.2%(1ヶ月後)、および8.7%(6ヶ月後)上昇、血小板値は19.8%、および18.9%上昇、また肝線維化の評価として血清プロコラーゲンIII型ペプタイド値は9%および12.3%減少と肝線維化の改善傾向を確認した。また骨髄細胞投与１ヶ月後の肝生検組織において肝再生マーカー蛋白の発現の増加より肝再生も誘導されたと考えられた。

D. 考察
１）達成度について
当初の目的は概ね達成されたと考えられる。実際に基礎研究成果を基盤にし、本助成の目的である、基礎研究成果の臨床応用推進事業として、世界初の臨床研究：自己骨髄細胞を用いた肝臓再生療法研究が開始することができた。また過去の経験では肝硬変患者から十分量の骨髄細胞がとれるかどうかも不明であったが、400ml の骨髄液から約8.5±1.x10^9個の有核細胞がとれることが新たに明らかになった。
２）研究成果の学術的・国際的・社会的意義について
再生医療の理論的根拠は発生や再生の基盤研究に基づいている。GFP/CCl4モデルを通じた基礎研究については、アメリカ肝臓病学会誌に２回ほど我々の発表論文について特別企画として紹介された。（Hepatology 39,4:1143-1146,2004, Hepatology41,1:16-18,2005, Elsewhere, Editorial、特に骨髄細胞投与により肝線維化改善についてはWiley社よりHepatology News Alert記事とし世界に発信された）また論文発表以前の発表としてアメリカ肝臓病学会年会においてもPlenary session, Presidental Plenaryとして口頭発表を行い、この分野に対して国際的にも、大きな影響を与えたと考える。また基礎研究として、発生期の肝幹細胞である肝芽細胞の生死に関わるシグナル機構の解明でき、この情報は再生時の肝細胞のシグナルの理解に繋がると考えられる。また、単離された変異メダカは肝臓の形成や病態に関わる分子の解明につながることから国内外の肝臓研究者から注目を集めており、再生医療への基盤的知見を通して社会的にも意義あると考えられる。臨床研究については、その開始がNHK等の報道機関で取り上げられ非常に大きな期待の中で臨床研究が開始された。また現在も全国各地から臨床研究参加依頼が山口大学病院にきており、肝不全患者に対する本治療法の開発は今後ますます社会的な意義は大きくなると考える

3）今後の展望について

本グランドで開始した臨床研究より、肝移植以外に有効な治療法がない肝不全患者に対し、自己骨髄細胞を用いた肝臓再生療法の有効性を示唆され、また安全性が確認された。本治療法は移植とは違い自己細胞を使うため倫理的問題がほとんどない。今後は我々は本治療法の臨床における有効性のエビデンスを明確にし、増加している肝不全患者を救命するため山口大学で施行したプロトコール、技術を多施設で検討する必要があると考えている。また治療法の開発普及のためには、今までのPhaseI臨床研究により開発した基盤技術（実際のプロトコール）を、他の施設においても施行可能にするようにすることが、全国の肝不全患者によりよい治療法を提供するために不可欠と考えており、旭川医科大学、山形大学、東京医科歯科大学、山口大学と、日本のほぼ全域を網羅するように拠点を選定し多施設にて臨床研究ができる体制が整った。このグループの名はLiver Regeneration with Cell Transplantation (LRCT) Study Group とする。このLRCTにて、『自己骨髄細胞を用いた肝臓再生療法』の PhaseⅡの臨床研究を開始したいと考えている。この PhaseⅡ臨床研究は多施設研究であり、まず初年度は山口大学で開発したプロトコールを各施設で行えるように技術移転を行い各施設ごとに3症例の施行を目標に臨床研究を進める。一方で、我々が行ってきた基礎研究において、骨髄細胞の間歇投与を行うことがより生存率をあげる結果がでた。このため、山口大学では計画1年目において間歇投与の臨床研究の準備を行い、計画の2年目以降は、山口大学で適応症例をランダム化し、現在のプロトコール通り骨髄細胞を採取し1回投与する群と、頻回投与群（6ヶ月間で2ヶ月ごとの3回投与群）とに割付け有効性の比較研究を行う。この臨床研究により本治療法の臨床上のエビデンスが明確になり、肝移植前に行う次世代のスタンダードな治療法の開発につながると考える。また骨髄細胞による肝臓再生療法は、山口大学での実際の費用は平均一人あたり約200万の治療費で入院期間も約1ヶ月であり、生体肝移植がドナー、レシピエントを入れ2000万円程度かかるのに比べ低額で侵襲も少ない。また自己骨髄細胞を使うため拒絶の問題がなく安全に施行でき、増加する肝不全患者を、低額でより多く患者を救命する方法の開発につながる。また我々が開発した骨髄中の肝幹細胞の分離に使える胎児肝モノクローナル抗体 Liv8 抗体の抗原同定を推進し人用抗体の開発できればより効率的な治療法の開発につながる。また基礎研究として行ってきた肝再生時のシグナル系の解明と肝疾患に関わる分子の同定が行なわれ、現在展開中の自己骨髄を用いた再生療法の改良が期待される。

E. 結論

肝再生療法の開発を念頭においた本基盤研究は着実に進展し、肝発生や肝疾患に関わる分子機構が明らかにされつつある。また実際に、我々は基礎研究を基盤とし、PhaseI 臨床研究『自己骨髄細胞を用いた肝臓再生療法』推進してきた。現在までに特に問題となる adverse effect の発生はなく、この治療法は将来、次世代の生体肝移植までのブリッジ的に使用可能な治療法になる可能性がある。

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G．知的財産権の出願・登録状況

１．特許取得
寺井　崇二、高見　太郎、坂井田　功、沖田　極
特許出願 2004-267065 新規肝細胞癌の腫瘍マーカー抗HHMIgG の発見

寺井　崇二、三浦　泉、坂井田　功、沖田　極
特許出願 2005- 79248 生体液中のイオン濃度の測定方法

２．実用新案登録
特になし

３．その他

厚生労働科学研究費補助金（基礎研究成果の臨床応用促進研究事業）
分担研究報告書
自己骨髄細胞を用いた肝臓再生療法の開発
分担研究者　仁科　博史（東京医科歯科大学 難治疾患研究所 発生再生生物学分野・教授）

研究要旨　我々は細胞移植による肝臓再生療法開発のための基盤研究を展開している。発生期の肝臓幹細胞である肝芽細胞の同定法の開発と，肝芽細胞の生存維持および分化誘導機構の解明を行ってきた。まず胎児肝特異的なモノクローナル抗体の作製とスクリーニングにより、肝芽細胞を特異的に認識するLiv2抗体を得ることに成功した。本抗体をツールとして、我々が作出した肝形成不全となるストレスキナーゼSEK1およびMKK7ノックアウトマウスを解析し、“肝芽細胞の生死の制御は、増殖シグナルであるSAPK/JNK系と生存シグナルであるNFκB系，細胞死誘導シグナルであるカスパーゼ系の３者のバランスによって制御される”という概念を提示した。さらに肝臓の発生や肝臓の機能に関わる遺伝子を明らかにする目的で、メダカを用いた大規模スクリーニングを行なった。その結果、肝形成および肝機能に異常のある興味深い変異メダカ１３種類を得ることに成功した。これらの中にはヒト疾患モデルとして利用可能なものも存在した。これら得られた発生期の肝臓幹細胞の特性の解明は、既に進行中の成体に存在する骨髄細胞の肝再生への応用の理論的根拠に貢献している。

A. 研究目的
胎児期に存在する増殖能と分化能を有する肝幹細胞である肝芽細胞の生死を制御する細胞内シグナル伝達系の解明と肝臓の発生や再生に関わる分子の同定を目的とする。

B. 研究方法
マウス肝芽細胞特異抗体の作製を行ない、肝形成不全ノックアウトマウスの解析を行なった。また、ヒト疾患モデル生物として期待されているメダカを用いて、肝形成および肝機能不全メダカを単離した。
（倫理面への配慮）
東京医科歯科大学および東京大学動物委員会の承認の下、実験動物取り扱いプロトコールに従い実験は行われた。

C. 研究結果
肝芽細胞の増殖には、ストレス応答性SAPK/JNKシグナル伝達系が必須の役割を果たしていることを示し、“肝芽細胞の生死の制御は、増殖シグナルであるSAPK/JNK系と生存シグナルであるNFκB系，細胞死誘導シグナルであるカスパーゼ系の３者のバランスによって制御される”という概念を提示した。また、メダカを用いた大規模スクリーニングを行ない、興味深い変異メダカ（内胚葉形成に影響のある遺伝子変異４種類、肝形成不全７種類、肝機能不全６種類）を発生の国際誌Mechanisms of Developmentのメダカ特集号に報告した。この中には脂肪肝の表現型を呈する変異メダカも存在し、ヒト疾患モデルとして、また薬剤のスクリーニングの対象としての活用が期待されている。これらの成果から、肝臓の発生や再生を考える重要な分子レベルの知見が得られると考えられる。

D. 考察
１）達成度について
当初の目的は概ね達成されたと考えられる。
２）研究成果の学術的・国際的・社会的意義について
再生医療の理論的根拠は発生や再生の基盤研究に基づいている。本研究成果は、発生期の肝幹細胞である肝芽細胞の生死に関わるシグナル機構の解明であり、再生時の肝細胞のシグナルの理解に繋がると考えられる。また、単離された変異メダカは肝臓の形成や病態に関わる分子の解明につながることから国内外の肝臓研究者はもちろん臨床医の注目を浴びており、再生医療への基盤的知見を通して社会的にも意義あると考えられる。
２）今後の展望について
肝再生時のシグナル系の解明と肝疾患に関わる分子の同定が行なわれ、現在展開中の自己骨髄を用いた再生療法の改良が期待される。

E. 結論
肝再生療法の開発を念頭においた本基盤研究は着実に進展し、肝発生や肝疾患に関わる分子機構が明らかにされつつある。

F. 健康危険情報

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H. 知的所有権の出願・取得状況（予定を含む。）

１．特許取得　なし

２．実用新案登録　なし

３．その他

本研究プロジェクトで作製された肝芽細胞特異、抗Liv2抗体やシグナル分子SEK1およびMKK7に対する抗体が、（株）生物医学研究所から発売されている。

研究成果の刊行に関する一覧表

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A subpopulation of bone marrow cells depleted by a novel antibody, anti-Liv8, is useful for cell therapy to repair damaged liver

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A subpopulation of bone marrow cells depleted by a novel antibody, anti-Liv8, is useful for cell therapy to repair damaged liver☆,☆☆

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Abstract

We previously reported a new in vivo model named as "GFP/CCl_4 model" for monitoring the transdifferentiation of green fluorescent protein (GFP) positive bone marrow cell (BMC) into albumin-positive hepatocyte under the specific "niche" made by CCl_4 induced persistent liver damage, but the subpopulation which BMCs transdifferentiate into hepatocytes remains unknown. Here we developed a new monoclonal antibody, anti-Liv8, using mouse E 11.5 fetal liver as an antigen. Anti-Liv8 recognized both hematopoietic progenitor cells in fetal liver at E 11.5 and CD45-positive hematopoietic cells in adult bone marrow. We separated Liv8-positive and Liv8-negative cells and then transplanted these cells into a continuous liver damaged model. At 4 weeks after BMC transplantation, more efficient repopulation and transdifferentiation of BMC into hepatocytes were seen with Liv8-negative cells. These findings suggest that the subpopulation of Liv8-negative cells includes useful cells to perform cell therapy on repair damaged liver.

Keywords: Bone marrow cell; Cell therapy; Regenerative medicine; Hepatic stem cell; Migration; Transdifferentiation; Mesenchymal stem cell; Hematopoietic stem cell; Liver regeneration; Niche

Recently, several groups have reported the possible plasticity of bone marrow cells (BMCs) to transdifferentiate into a variety of non-hematopoietic cell lineages [1–4]. Ever since the transdifferentiation of BMC into hepatocytes was documented following a bone marrow transplant from a man donor to a woman recipient [5,6],

☆ *Abbreviations:* BMC, bone marrow cell; CCl_4, carbon tetrachloride; FAH, fumarylacetoacetate hydrolase; GFP, green fluorescent protein; EGFP, enhanced GFP; GFP-Tg mice, C57BL6/Tg14 (act-EGFP) OsbY01 mice; HSC, hematopoietic stem cell; E, embryonic day; MSC, mesenchymal stem cells; MAPC, multipotent adult progenitor cell.

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BMC has been an attractive cell source in regenerative medicine because getting BMC is easier than obtaining other tissue-specific stem cells [7].

However, the results of recent studies have been mixed in that some studies found that BMC was hardly transdifferentiated while others documented high levels of transdifferentiation [8,9]. Successful transdifferentiation in cell therapy involves various cell and recipient factors, and these factors interact in a complex manner. Therefore, it is difficult to identify the conditions necessary for transdifferentiation, contributing to the varied results among past studies. A past study using a fumarylacetoacetate hydrolase (FAH) knockout mice (metabolic tyrosinemia model) showed that hepatic functions could be compensated by transplanting Lin-Kit+Sca+Thy1low (KTLS) marrow cells [10]. In the FAH model, KTLS cells form foci and transdifferentiate into hepatocytes. Results of recent studies suggest that KTLS cells transdifferentiate into hepatocytes due to fusion with hepatocytes [11,12]. The FAH model is a specialized model of metabolic liver damage, making it possible to analyze the transdifferentiation of BMC into hepatocytes and functional compensation. However, a model with which the transdifferentiation of BMC can be analyzed under conditions of more general liver damage is needed. Using autologous transplantation in GFP transgenic mice [13], we established an isogenic transplantation model to assess the transdifferentiation of BMC into hepatocytes. This model is unique in that uncultured BMCs efficiently migrate into the peri-portal area of the liver and transdifferentiate into immature hepatoblasts and differentiate into mature hepatocytes under the specific "niche" of persistent liver damage induced by persistent intraperitoneal administration of carbon tetrachloride (CCl_4) [14]. In this model, liver cirrhosis was induced by 4 weeks CCl_4 injection, and BMCs isolated from GFP transgenic mice were transplanted through the caudal vein. It is possible to chronologically observe colonization and transdifferentiation of BMC in the liver by continuous administration of CCl_4, and we have named this model as the "GFP/CCl_4 model." Furthermore, in this model, as in the natural development of the liver, BMCs appear to be transdifferentiated into hepatoblasts and then into hepatocytes. In our GFP/CCl_4 model, the timing of cell transplantation and the state of recipients appear to be suitable for the transdifferentiation of BMC into hepatocytes. Cell transplantation and continuous liver damage made efficient transdifferentiation of BMC into hepatocytes. In a system similar to ours, human hematopoietic stem cells (HSCs) were transplanted into the bone marrow of immunologically tolerant NOD/SCID mice before administration of CCl_4, and these cells differentiated into albumin-positive hepatocyte-like cells after the CCl_4 administration [15]. These findings suggest that a special "niche" created by CCl_4-induced liver damage is important for the migration of BMC to the liver and transdifferentiation into hepatocytes. Also, it has been reported recently that CCl_4 administration is effective for improving the colonization of HSC to liver of NOD/SCID [16].

The liver functions as a metabolic organ, but during the fetal period, from embryonic day (E) 12 to 16 (E12–E16), the liver functions as a hematopoietic organ [17]. Several studies have reported that mesenchymal cells affect hepatic hematopoiesis during the fetal period [18,19]. After this hematopoietic period, hepatoblasts are involved in a complex manner to develop the liver as a metabolic organ. However, documentation of the existence of HSC in the adult liver suggests that, even in the adult liver, blood cells and hepatocytes still play some role in the maintenance of hepatic function [20]. To further analyze this aspect, we prepared new rat monoclonal antibodies using the fetal liver on E 11.5 as an antigen. One of these antibodies, anti-Liv2, specifically recognizes hepatoblasts in the fetal liver from E 9.5 to 12.5. The results of past studies using the anti-Liv2 antibody have shown that SEK1, a stress-signaling kinase, plays an important role in the proliferation of hepatoblasts, thus suggesting that inflammatory signals are involved in the proliferation of hepatoblasts [21].

Although various theories explain the existence of pluripotent stem cells in BMC, the exact composition of stem cells in BMC is not clear at this time; the following cell types are known to exist in bone marrow: HSC [4,10], side population cells [22], and mesenchymal stem cells (MSC) [23]. Although past studies used the existing antibodies and techniques, there have not been any studies based on the findings associated with natural liver development. Using fetal liver as an antigen, we prepared a new monoclonal antibody, anti-Liv8 antibody, to analyze which subpopulation of BMC could differentiate into hepatocytes under CCl_4-induced continuous liver damage in the GFP/CCl_4 model [14]. This anti-Liv8 antibody recognizes hematopoietic cells using a specific cell surface marker and it can be used to separate cells. In the present study, we used this new antibody to separate BMC of adult mice and then transplanted the different types into mice under identical conditions of the GFP/CCl_4 model to ascertain which types of BMCs transdifferentiate into hepatocytes.

Materials and methods

Mice. C57BL6/Tg14 (act-EGFP) OsbY01 mice (GFP-Tg mice) showed GFP expression in multiple tissue and cells and were kindly provided by Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan) [13]. C57BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). AML1 knockout mice were generated

as described previously [24]. The genetic background of these mice used in this study was C57BL/6 mice. Male and female mice were mated overnight and female mice were scored based on vaginal plaques taken to represent E 0.5. Mice were anesthetized at the completion of experiments. All processes, including surgical steps, were undertaken with the guidance of the committee for animal and recombinant DNA experimentation at Yamaguchi University.

Production of rat monoclonal antibody, Liv8. Eight-week-old WKY/NCrj female rats were immunized in the hind footpads with 100 μg E 11.5 murine fetal liver lysate in complete Freund's adjuvant (0.2 ml). Anti-Liv8 antibodies were raised according to a previously described protocol [21].

Immunohistochemical staining for fetal liver. Fetal liver at E11.5 was obtained from c57/BL/6 mice and AML1 knockout mice. Tissue preparation and immunohistochemical analysis were performed according to a previously described protocol [21]. We analyzed anti-Liv2- and anti-Liv8-positive cells in fetal liver.

Preparation of GFP-positive BMC. For isolation of BMC, GFP-Tg mice were sacrificed by cervical dislocation and the limbs were removed. GFP-positive BMCs were flushed from the medullary cavities of tibias and femurs with PBS culture solution using a 25 G needle. The cell solution was filtered through a cell strainer (16 μm) to remove particular matter and centrifuged at 500*g* for 5 min. After centrifugation, the supernatant was removed and cells were resuspended to prepare 1.0×10^6 cells/ml GFP-positive BMC solutions. Preparation of BMC takes approximately 1.5 h.

FACS analysis of BMC using Liv8 antibody. Prepared GFP-positive BMCs were reacted with rat biotin anti-Liv8 IgG antibody, R-Phycoerythrin (R-PE)-conjugated rat anti-CD45 (leukocyte common antigen) monoclonal antibody (PharMingen, San Diego, USA) at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. Following the incubation with the first antibody, the cells were washed twice by 0.02 M PBS and centrifuged at 500*g* for 5 min. Labeled cells were then reacted to streptavidin–fluorescein isothiocyanate (FITC) conjugate (PharMingen) at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. After that, these were washed out once with 0.02 M PBS and centrifuged at 500*g* for 5 min. The labeled cells were analyzed using FACS Calibur (Becton–Dickinson).

Sort GFP positive BMC by Liv8 antibody. Prepared BMCs were reacted to rat anti-Liv8 IgG antibody at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. Then cells were washed two times by 0.02 M PBS and centrifuged at 500*g* for 5 min. Cells were labeled with rat anti-Liv8 IgG antibody by reacting with Goat Anti-Rat IgG MicroBeads (Miltenvi Biotec GmbH, Bergisch Gladbach, Germany) at the rate of 20 μl per 10^7 total cells, mixed well, and incubated for 20–30 min at 4 °C. Labeled cells were washed once by 0.02 M PBS and centrifuged at 500*g* for 5 min. These cells were separated into Liv8-positive cells or negative cells by the Auto Magnetic Cell Sorting system (Auto MACS) (Miltenvi Biotec GmbH) for 10 min per tube.

Transplantation of Liv8-positive or negative BMC into persistent liver damaged mice. We developed a new in vivo model "GFP/CCl_4 model" for monitoring differentiation of BMCs into hepatocytes [14]. To generate a liver damage group, 0.5 ml/kg of CCl_4 was injected into the peritoneum of 6-week-old C57BL/6 females twice a week for 4 weeks. Liver cirrhosis resulting from the continuous injections of CCl_4 was confirmed. A control group of C57BL/6 mice that had not been treated with CCl_4 was also used. One day after the eighth injection, sorted Liv8-positive or Liv8-negative BMC (1×10^5 cells) was slowly injected into the caudal tail vein of mice using a 31 G needle and a Hamilton syringe. After transplantation, CCl_4 injections (0.5 ml/kg) were continued twice a week. Mice were sacrificed weekly up to 4 weeks.

Tissue preparation. The livers were thoroughly perfused via the heart with 4% paraformaldehyde (Muto, Tokyo, Japan). This step was crucial for washing out contaminating blood cells. For fixation, the perfused livers were incubated with 4% paraformaldehyde (Muto) overnight and then soaked in 30% sucrose for a few more 3 days. Tissues were frozen in dry ice and then sectioned into 18-μm slices using a cryostat (Moriyasu Kounetsu, Osaka, Japan) in preparation for dyeing.

Immunohistochemistry and double immunofluorescence for GFP. To avoid autofluorescence, we used immunostaining to assess the expression of GFP. Cells expressing GFP were analyzed by both fluorescent microscopy and conventional immunohistochemistry with anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA). Immunohistochemical analysis was performed according to a previously described protocol [14,25]. Sectioned tissues were incubated with anti-GFP antibody (1:5000 FL, sc-8334; Santa Cruz Biotechnology), anti-albumin (1:5000, 55462; ICN Pharmaceuticals, Costa Mesa, CA, USA), and anti-Liv2 antibody (1:5000) [21]. For fluorescence immunohistochemistry, tissues were incubated with Alexa Fluor R 488 and 568 donkey anti-goat IgG(H + L) conjugate, Alexa Fluor R 488 goat anti-rabbit IgG(H + L) conjugate, and Alexa Fluor R 568 goat anti-rat IgG(H + L) conjugate (Molecular Probes, Eugene, OR) as secondary antibodies. Positive cells in the liver were quantified using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge coupled devise (CCD) camera and subjected to computer-assisted image analysis with MetaMorph software (Universal Imaging, Downingtown, PA). A total of 10 different areas per liver section were analyzed independently and the areas of positive cells were calculated using the MetaMorph software.

Serum albumin level analysis. Serum albumin levels during the 4 weeks after Liv8-positive or Liv8-negative BMC transplantation were analyzed using the SPOTCHEM EZ SP-4430 dry chemical system (Arkray, Kyoto, Japan).

Statistical analysis. Values are shown as means ± SE. Data were analyzed by analysis of variance with Fisher's projected least significant difference test.

Results

Anti-Liv8 antibody detected hematopoietic progenitor cell in fetal liver at E 11.5

Previously we had raised a rat monoclonal antibody, anti-Liv2, which recognized hepatoblasts at E 9.5 [21]. As shown in Fig. 1A, Liv2-positive cells were also detected in fetal liver at E 11.5. Using the antibody developed in this study, Liv8-positive cells were seen in the fetal liver on E 11.5 (Fig. 1B). Fetal liver at E 11.5 functions as a secondary hematopoietic organ [17]. We analyzed whether anti-Liv8 positive cell is associated with hepatoblast or hematopoietic cell. We found Liv2-positive cells (Fig. 1C), but no Liv8-positive cells (Fig. 1D), in the fetal liver of $AML1^{-/-}$ embryos which do not undergo definitive hematopoiesis [24]. These results suggested that anti-Liv-8 recognizes hematopoietic progenitor cell in fetal liver.

Liv8-positive cells exist in adult bone marrow and express CD45

Next, we investigated Liv8-positive cells in the BMC of adult GFP Tg mice. Liv8-positive cells were found to be present among adult BMCs in adult bone marrow when analyzed in GFP-Tg mice. We found around 32%

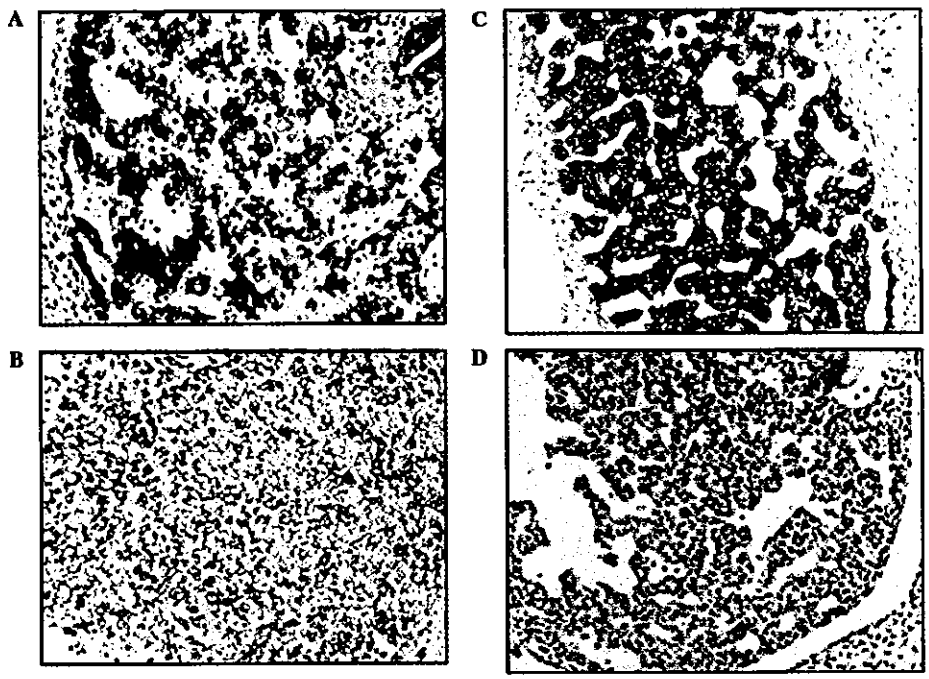

Fig. 1. (A–D) Liv2 and Liv8 expression at E 11.5 in normal and AML1$^{-/-}$ mice. Liv2 (A,C) and Liv8 (B,D) expression at E 11.5 in normal fetal liver (A,B) and AML1$^{-/-}$ mice (C,D). Magnification: (A–D) at 200×.

of Liv8-positive cells in adult GFP-Tg mice (Fig. 2A). We also analyzed the relationship between Liv8 and CD45, and found that 54% of Liv8-positive cells also expressed CD45 (Fig. 2B). These results showed that anti-Liv8 is useful to separate hematopoietic cell and non-hematopoietic cell.

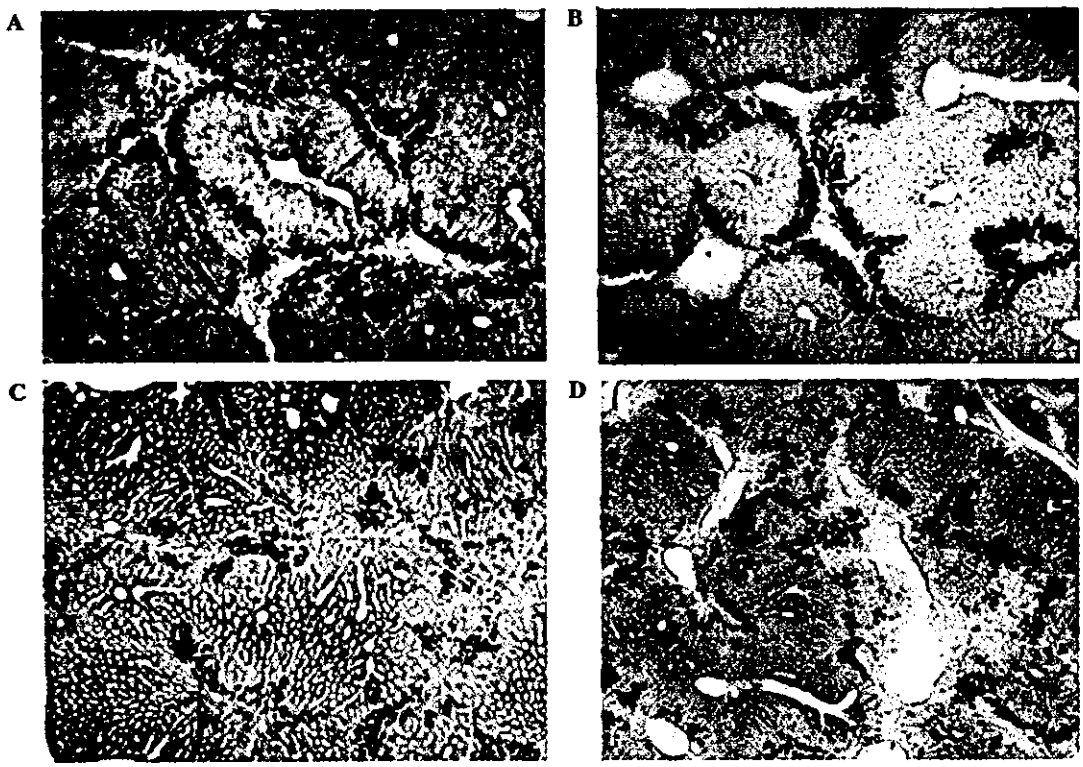

Fig. 3. (A–D) Expression of GFP in liver after transplantation of Liv8-positive and Liv8-negative cells. GFP expression in the liver after transplantation of Liv8-positive BMCs at 1 week (A) and 4 weeks (C), GFP expression at the liver after Liv8-negative BMC transplantation at 1 week (B) and 4 weeks (D) after cell injection. Magnification 200×.

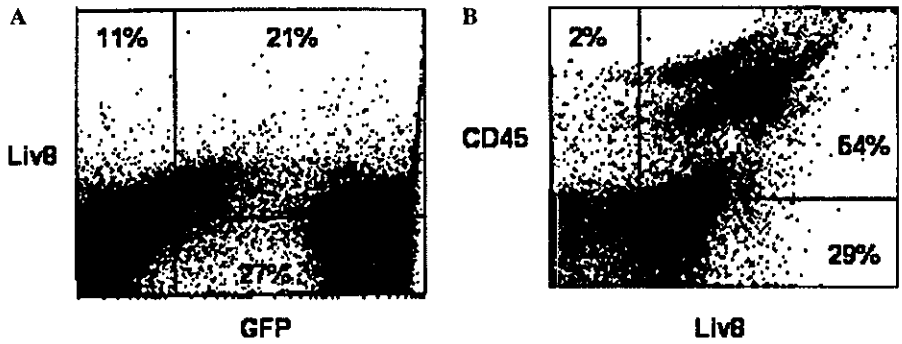

Fig. 2. Expression of CD45, Liv8 in bone marrow cell. FACS analysis of all BMCs of GFP-Tg mice. (A) Staining with Liv8 and GFP. (B) Staining with CD45 and Liv8.

Liv8-negative cells repopulated at the liver more than Liv8-positive cells

After separating Liv8-positive cells from Liv8-negative cells using AutoMACS, these cells were transplanted to recipient mice with CCl_4-induced liver cirrhosis. At one week after transplantation, both Liv8-positive (Fig. 3A) and Liv8-negative cells (Fig. 3B) colonized around the portal vein, with no marked differences in the rate of colonization (Table 1). In the Liv8-positive cell transplanted group, the number of GFP-positive cells in the liver increased transiently, but at four weeks after transplantation, the number of GFP-positive cells was significantly lower in the Liv8-positive cell group (Fig. 3C) than in the Liv8-negative cell group (Fig. 3D). Furthermore, GFP-positive cells were colonized inside the hepatic lobes in the Liv8-negative cell group at four weeks after transplantation. These results showed that Liv8-negative cell repopulated more than Liv8-positive cell.

The Liv8-negative cells transdifferentiate into hepatoblast phenotype

We showed in previous studies that transplanted BMCs transdifferentiate into Liv2-positive hepatoblasts and then further differentiate into hepatocytes [14,21]. In the present study, we also investigated the presence of cells expressing Liv2. Liv2-positive cells were identified by immunostaining, and the results showed that Liv2-positive cells were seen around the portal region one week after transplantation, but that there was no significant difference in the number of Liv2-positive cells between Liv8-positive and Liv8-negative cell groups (Figs. 4A and B, and Table 1). With time, the number of Liv2-positive cells in the liver decreased significantly for the Liv8-positive cell group (Figs. 4C and D and Table 1). The transdifferentiation of myelogenic GFP cells into Liv2 cells was investigated. Cells that expressed both Liv2 and GFP were detected at four weeks after transplantation, and fluorescent staining showed that the expression of Liv2 by myelogenic cells was higher for the Liv8-negative cell group (Figs. 4E and F). These results indicated that Liv8-negative cell could be transdifferentiated into hepatoblast phenotype.

Albumin expression in the liver and serum albumin level following transplantation of Liv8-positive and Liv8-negative BMCs

At one week after cell transplantation, there was no marked change in the expression of albumin for both Liv8-positive and Liv8-negative cell groups (Figs. 5A and B). However, at four weeks after transplantation, the expression of albumin decreased with time for the Liv8-positive cell group (Fig. 5C), but remained the same for the Liv8-negative cell group (Fig. 5D). Furthermore, at four weeks after cell transplantation, the number of yellow cells expressing both albumin and GFP was higher for the Liv8-negative cell group

Table 1
Percent of area for each differentiation marker after Liv8(+) and Liv8(−) cell transplantation under the persistent liver damage

		1 week ($n = 5$)	2 weeks ($n = 5$)	3 weeks ($n = 5$)	4 weeks ($n = 5$)
GFP	Liv8(+)	11.1 ± 1.7	15.1 ± 2. 1	9.4 ± 0.8	5.1 ± 0.6*
	Liv8(−)	11.7 ± 1.0	13.2 ± 0.8	12.4 ± 2.6	9.5 ± 3.6*
Liv2	Liv8(+)	6.0 ± 1.1	7.3 ± 3.5	8.2 ± 1.8	3.3 ± 0.9
	Liv8(−)	5.5 ± 1.3	5.8 ± 0.8	9.2 ± 0.6	7.7 ± 0.9
Albumin	Liv8(+)	15.0 ± 1.9	14.9 ± 2.5	6.8 ± 2.6*	3.7 ± 1.4*
	Liv8(−)	12.7 ± 3.2	12.5 ± 3.2	14.8 ± 1.3*	10.6 ± 2.1*

Values shown are percent of the area occupied.
*showed significant differences at each sampling point ($n = 5$) at $p < 0.05$ between Liv8(+) and Liv8(−) cell transplantation groups.

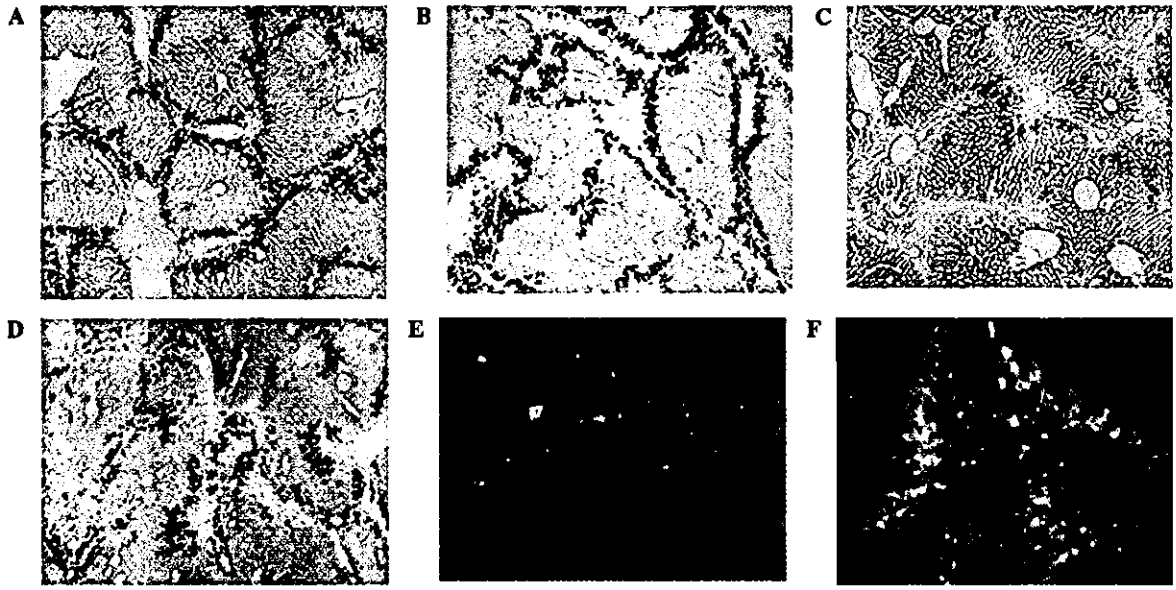

Fig. 4. (A–F) Expression of Liv2 antigen in liver after transplantation of Liv8-positive and Liv8-negative cells. Liv2 antigen expression at 1 week (A) and 4 weeks (C) after Liv8-positive BMC transplantation. Magnification at 200×. Liv2 antigen expression at liver at 1 week (B) and 4 weeks (D) after Liv8-negative BMC transplantation. Double fluorescent staining (red, Liv2; green, GFP; and yellow, Liv2 & GFP) of the liver at 4 weeks after Liv8-positive cell transplantation (E) and Liv8 negative cell transplantation (F) Magnification: (A–D) 200×, (E,F) 400×.

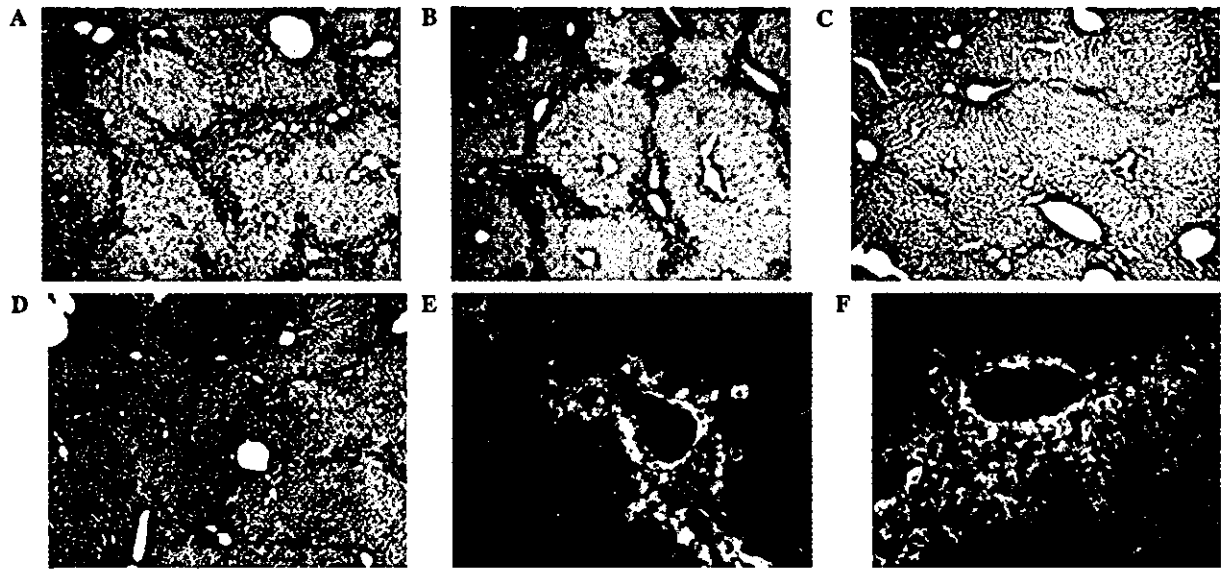

Fig. 5. (A–L) Expression Albumin after transplantation of Liv8-positive and Liv8-negative cells. Albumin expression at 1 week after transplantation of Liv8-positive cells (A) and Liv8-negative cells (B). Albumin expression at 4 weeks after transplantation of Liv8-positive cells (C) and Liv8-negative cells (D). Double fluorescent staining (red, albumin; green, GFP; and yellow, albumin & GFP) of liver at 4 weeks after transplantation of Liv8-positive cells (E) and Liv8-negative cells (F). Magnification: (A–D) 200×, (E,F) 400×.

(Figs. 5E and F). To ascertain whether transplanted cells were functioning as hepatocytes, serum albumin levels were measured. Serum albumin levels increased for both groups and were higher for the Liv8-negative cell group than the Liv8-positive cell group. The serum albumin levels at 4 weeks after Liv8-negative BMC transplantation showed the significantly higher levels for Liv8-negative cell group compared to the Liv8-positive BMC group ($n = 5$, $p < 0.05$) (Fig. 6). These results also showed that Liv8-negative cell could transdifferentiate into albumin-positive hepatocyte.

Discussion

The anti-Liv8 antibody is a useful antibody to separate hematopoietic cells and non-hematopoietic cells in adult bone marrow. We found Liv8-positive cells in fetal

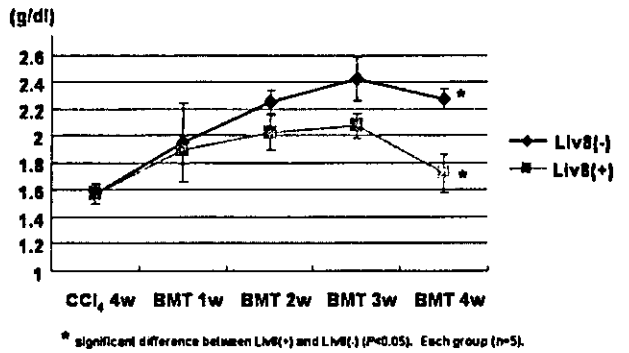

Fig. 6. The level of serum albumin. Serum albumin levels after Liv8-positive or Liv8-negative cell transplantation. CCl_4 4w, 4 weeks CCl_4 injection group. BMT 1w, 1 week after BMC transplantation. BMT 2w, 2 weeks after BMC transplantation. BMT 3w, 3 weeks after BMC transplantation. BMT 4w, 4 weeks after BMC transplantation. * showed significant differences at each sampling point ($n = 5$) at $p < 0.05$.

liver at E11.5, but could not detect no-positive cells in fetal liver of AML1 knockout mice (Fig. 1C) at E 11.5. This result suggested that anti-Liv8-positive cell might be associated with the generation of HSC. We used FACS analysis to understand more about the characterization of Liv8-positive cells in the bone marrow. Around 32% of all BMCs, which were positive for Liv8, also expressed CD45 (Figs. 2A and B). CD45 is the pantrophic marker for hematopoietic cell marker [26,27]. These results suggest that anti-Liv8 recognizes most hematopoietic cells. We separated BMCs into Liv8-positive cells and Liv8-negative cells using AutoMACS, and the repopulation and transdifferentiation of these cells into liver was analyzed in the GFP/CCl_4 model [14].

First we analyzed the colonization of transplanted Liv8-positive or negative cell. There was no change in the ratio of GFP-positive cells one week after transplantation between the Liv8-positive and Liv8-negative cell groups (Figs. 3A and B). In both groups, GFP-positive cells were found around the portal vein. The expression of GFP decreased with time for the Liv8-positive cell group (Fig. 3C), but in the Liv8-negative cell group, GFP-positive cells entered the hepatic lobes (Fig. 3D). At four weeks after transplantation, the rate of colonization for the Liv8-positive cell group was significantly lower than that for the Liv8-negative cell group (Table 1). Previously we found that colonization was not observed when BMCs were transplanted to normal recipients, but colonization was observed when BMCs were transplanted to recipients with liver cirrhosis caused by administration of CCl_4 [14]. Some previous studies also have reported that CCl_4 injection enhances the repopulation of hepatocytes following hepatocyte transplantation via the spleen [28,29]. It has been documented that elevated levels of SDF1 and matrix metalloprotease 9 (MMP9) might have an important role for the migration of BMCs to the liver at liver damage by CCl_4 administration [16,30]. In the GFP/CCl_4, the expression of MMP9 was also increased by the transplantation of BMCs (I. Sakaida, unpublished data). At 1 week after transplantation, there was no marked difference in colonization between the Liv8-positive and negative transplantation groups. These results suggest that the early migration of BMC into liver was determined by the recipient condition. Next we analyzed the transdifferentiation of BMC into functional hepatocyte in the "niche" where transdifferentiation of BMC into hepatocyte is favorable [14]. The results of our past analyses have shown that transplanted BMCs transdifferentiate into Liv2-positive hepatoblasts and then differentiate into hepatocytes only under continuous inflammation. The persistent liver damage made by injection of persistent CCl_4 injection is important for the transdifferentiation of BMC [14]. When human HSCs were transplanted to immunologically tolerant NOD/SCID mice and followed up with administration of CCl_4, it was found that transplanted human HSC was differentiated into albumin express hepatocyte-like cell [15]. Albumin/promoter-Alb-DsRed2 Tg rat was established to monitor the transdifferentiation into albumin positive cell. Albumin-producing DsReds cell was increased by repeated administration of CCl_4 [31]. A study reported recently that the transdifferentiation of BMCs was low when inducing liver damage by CCl_4 administration before or after transplantation [32]. Different results were obtained with these systems because chronic liver damage before and after transplantation was not evident. The persistent liver damage might be the key factor to induce the transdifferentiation of BMC into hepatocyte. We investigated the transdifferentiation of Liv8 positive and negative BMCs into hepatoblast and hepatocytes by Liv2 and albumin expression. Like GFP, Liv2-positive cells were seen around the portal vein one week after transplantation for both Liv8-positive and Liv8-negative cell groups, and there was no marked difference between the two groups (Figs. 4A and B). On the other hand, at four weeks after transplantation, the expression of Liv2 for the Liv8-positive cell group was significantly lower than that for the Liv8-negative cell group (Figs. 4C and D). The results of double staining at four weeks after transplantation also showed that the number of myelogenic Liv2-positive cells was greater for the Liv8-negative cell group (Figs. 4E and F). Figs. 5C and D show the expression of albumin four weeks after transplantation and the expression of albumin for the Liv8-negative cell group was higher (Fig. 5D). The expression of albumin and GFP in myelogenic cells was significantly higher for the Liv8-negative cell group (Fig. 5F). Furthermore, we investigated functional recovery by comparing improvement in hepatic failure between the Liv8-positive and Liv8-negative cell groups.

As shown in Fig. 6, when CCl_4 was administered in the same manner to the Liv8-positive and Liv8-negative cell groups, and the level of serum albumin increased in both groups, but a significant finding in this analysis was significant improvements in the serum albumin levels at four weeks after transplantation in the Liv8-negative cell group compared to the Liv8-positive cell group ($p < 0.05$). These findings support those of immunostaining. These results can be summarized that Liv8-negative cells are more likely to transdifferentiate into hepatocytes with time passed. The subpopulation which was deleted by anti-Liv8 will be useful cells to use cell therapy using BMC to repair damaged liver. The Liv8 negative cell was thought to be non-hematopoietic cells. For example, multi-potent adult progenitor cells (MAPCs) from BMCs differentiate into functional hepatocyte like cells [33,34]. Our results might support that mesenchymal cells may differentiate into pluripotent cells under certain conditions.

Still the precise mechanisms to regulate repopulation and transdifferentiation BMC into hepatocyte are uncertain. To develop a cell therapy using BMC to repair damaged liver, we are planning to further analyze these mechanisms.

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