値 182 mg/dl と軽度の肝機能障害を認める程度で，アンモニア（NH_3）38 μg/dl も正常範囲内であった。高血圧を呈していたために循環器系の精査を施行した。心電図で II，III，AVF で軽度 ST 低下を認めたが，負荷心電図（トレッドミル）では胸痛がなく，心電図でも虚血性変化を認めなかった。さらに心臓超音波検査（UCG）でも異常所見は認めなかった。肝臓の画像検査として超音波検査では肝腎コントラスト(+)で腹部 CT 検査では肝脾 CT 値の比は 1.0 であった。一方，CT 検査で肝臓右葉前区域（S8）から中肝静脈に流入する太い肝静脈（V8）を認めていた。CT volumetry にて予想全肝容積は 1,215 ml，予想右葉肝容積 758 ml，予測残肝容積比 37.6% と概算された。

3. 手術内容

手術時間は 8 時間 40 分，総出血量は 540 ml で，このため輸血を必要とせず，術中ショックもなく順調に肝移植手術は終了した。この際用いたグラフト肝臓は中肝静脈を含む右葉グラフトを用い，その重量は 900 g であった。このことから残肝容積は推定 315 ml と計算され，ドナーの残肝容積は推定 25.9%となった。

グラフト肝を移植し，灌流後の色調は軽度の脂肪肝が示唆された。そして，その術中肝生検所見は macrovesicular steatosis（大型脂肪滴）30%であり，中等度の脂肪肝と組織診断された。

一方，レシピエントの摘出肝臓は小結節型肝硬変の肝表面を呈し，組織学的には自己免疫性肝炎で，同時に門脈血栓が認められた。

4. 術後ドナーの経過

2002 年 8 月 11 日に生体肝移植のドナーとして中肝静脈を含む肝右葉をグラフト肝として切除された。手術直後は当初の予定通り循環障害予防のため ICU 管理とされた。8 月 13 日に黄疸と肝機能障害ならびにアンモニア上昇を認め，肝性脳症 II 度が出現した。同時に呼吸困難も出現したため，気道確保のため挿管した。その後，腹水，黄疸，肝性脳症などを伴う肝障害は継続して観察されていた。そして，8 月から 9 月にかけて大量の腹水が観察された。10 月のドップラーエコー検査では遠肝性門脈血流を認め，腹水はさらに増加した。11 月 14 日に難治性腹水治療のために経静脈的肝内門脈シャント（TIPS）を施行し，腹水は

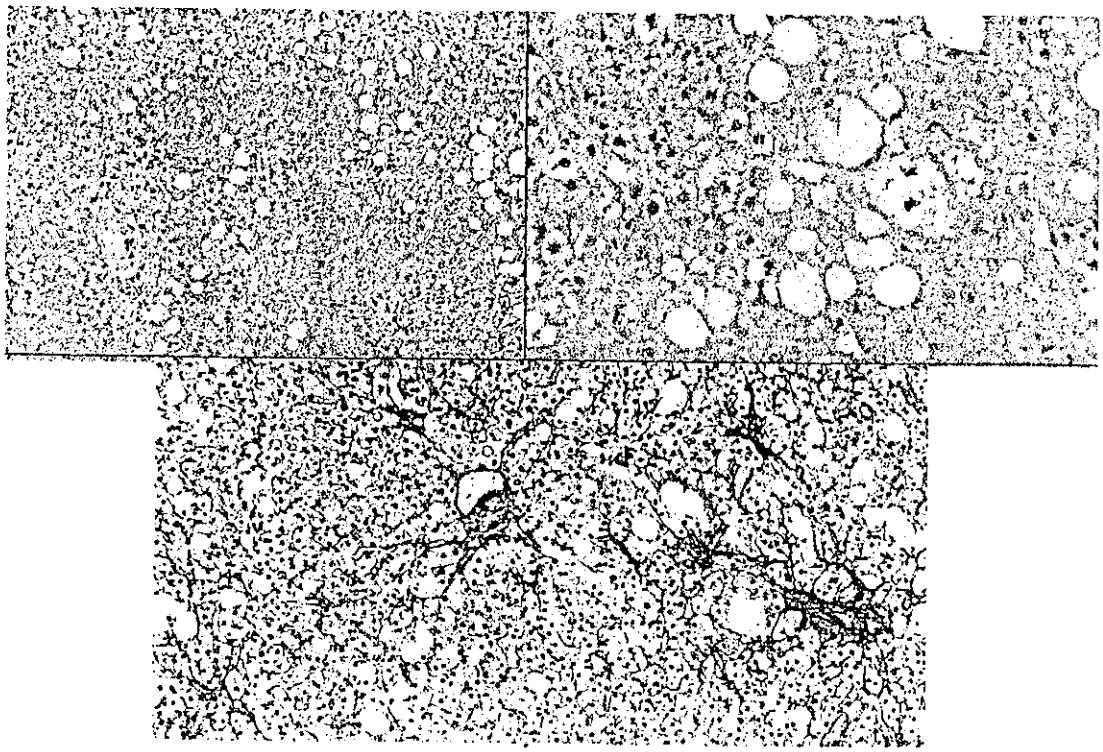

図 1 第 1 回肝生検所見

肝提供手術時 2002 年 8 月 11 日のドナー肝臓の肝生検像で非アルコール性脂肪性肝炎（non-alcoholic steatohepatitis: NASH）の病期 2，活動度 1。上段左：HE 染色中拡大，上段右：HE 染色強拡大，下段：鍍銀染色中拡大

やや減少した。しかしながら，11月頃からは黄疸は遷延し，総ビリルビン値の上昇は続いた。12月に入ると肺炎を合併し，ARDS様となり，腎障害が出現し乏尿となった。12月下旬には意識レベルが低くなり，高アンモニア血症に対する血漿交換を施行した。しかし，意識レベルは低下し，肝性昏睡II度からIII度へと悪化し，血漿交換を続けるも改善傾向を示さなかった。さらに，血液培養から *Stenotrophomonas maltopilia* (MRSA) が検出された。翌2003年1月14日には開眼しているが意思疎通が不可能となり，痛み刺激反応も微弱になり，肝性脳症IV度と判断された。その後，尿量減少が顕著となり，2003年1月27日にドミノ生体肝移植を受けた。

しかし，その後集中治療を積極的に行うも，最終的には改善傾向を示さず，2003年5月4日に死亡された。

IV. ドナー肝組織の病理所見の評価

①第1回肝生検（肝提供手術時2002年8月11日）：脂肪沈着面積は30%くらいだが，肝細胞の風船状腫大，胞体内の好酸性凝集物の出現，肝細胞周囲性線維化が肝小葉中心性にみられ，単なる脂肪肝ではなく，いわゆる脂肪性肝炎と診断される。飲酒歴がないことから非アルコール性脂肪肝炎（non-alcoholic steatohepatitis: NASH）（病期2，活動度1）と考えられた。(図1)

②第2回肝生検（2002年9月26日）：肝小葉中心性にマロリー体を伴う肝細胞の風船状腫大と多核好中球を認めた。肝細胞周囲性の線維化も強く，病期3，活動度3のNASHと考えられた。細胆管内胆汁栓と門脈域の炎症は遷延性胆管炎に由来すると考えられた。その他，類洞内の微小血栓形成を認めた。(図2)

③第3回肝生検（2002年11月14日）：小葉中心性および門脈域，および門脈域周囲性に緻密で高度の線維化を伴う肝硬変でNASH病期4期と診断される。肝細胞の風船状の腫大，マロリー体，好中球浸潤が強く，活動度3と考えられる。高度の細胆管性胆汁うっ滞は遷延性胆管炎に起因するものと考えられた。(図3)

④移植時摘出肝（2003年1月27日）：小結節肝硬変で，NASH（病期4，活動度3）と診断される。そ

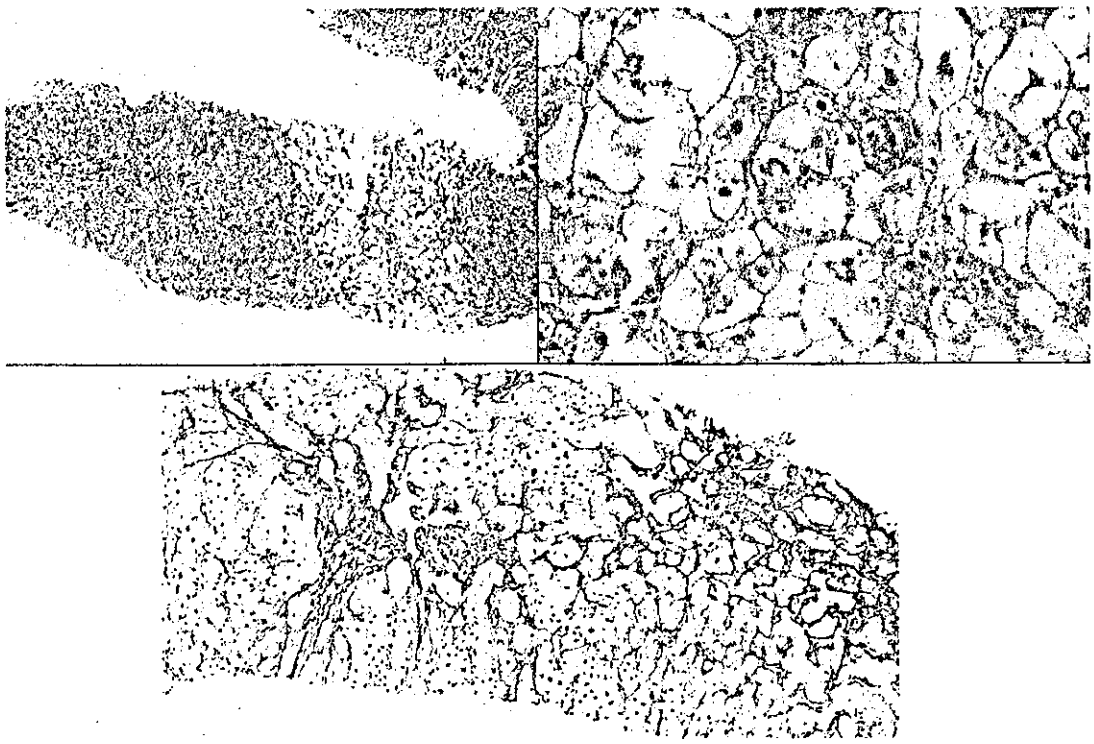

図 2 第2回肝生検所見

ドナー手術後46日目の2002年9月26日の肝生検像。肝小葉中心性にマロリー体を伴う肝細胞の風船状腫大と多核好中球を認めた。NASHの病期3，活動度3。上段左：HE染色弱拡大，上段右：HE染色強拡大，下段：鍍銀染色中拡大

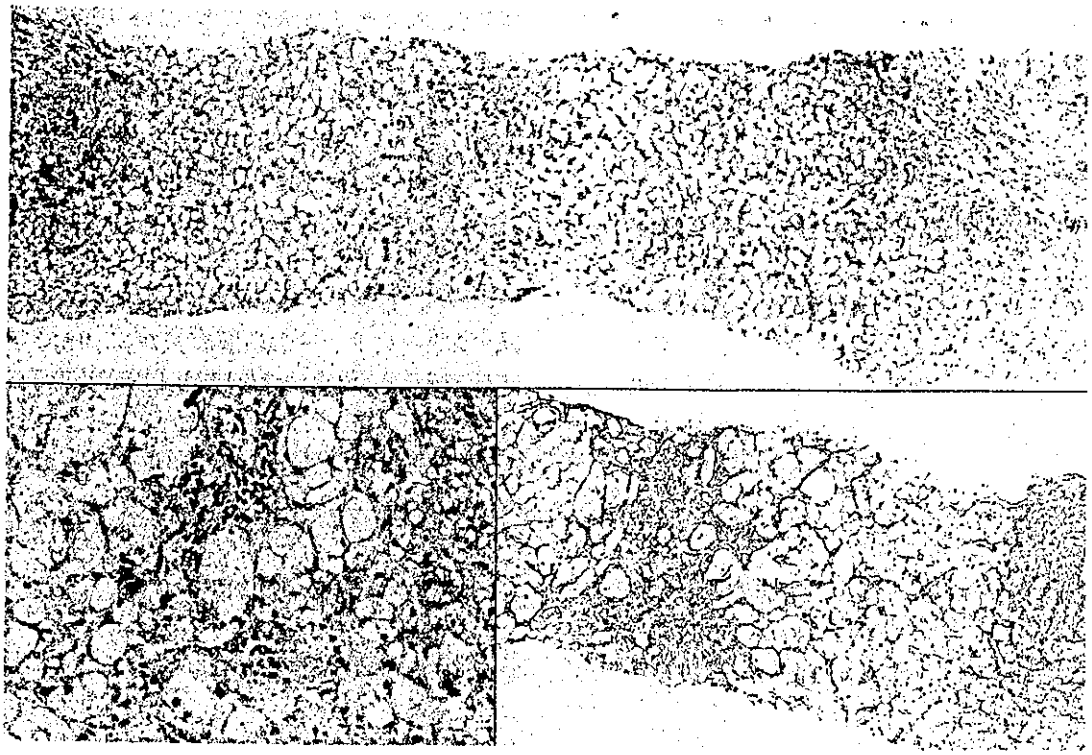

図 3　第 3 回肝生検所見
ドナー手術後 95 日目の 2002 年 11 月 14 日の肝生検像で，緻密で高度の線維化を伴う肝硬変で NASH 病期 4 期。上段：HE 染色弱拡大，下段左：HE 染色強拡大，下段右：鍍銀染色中拡大

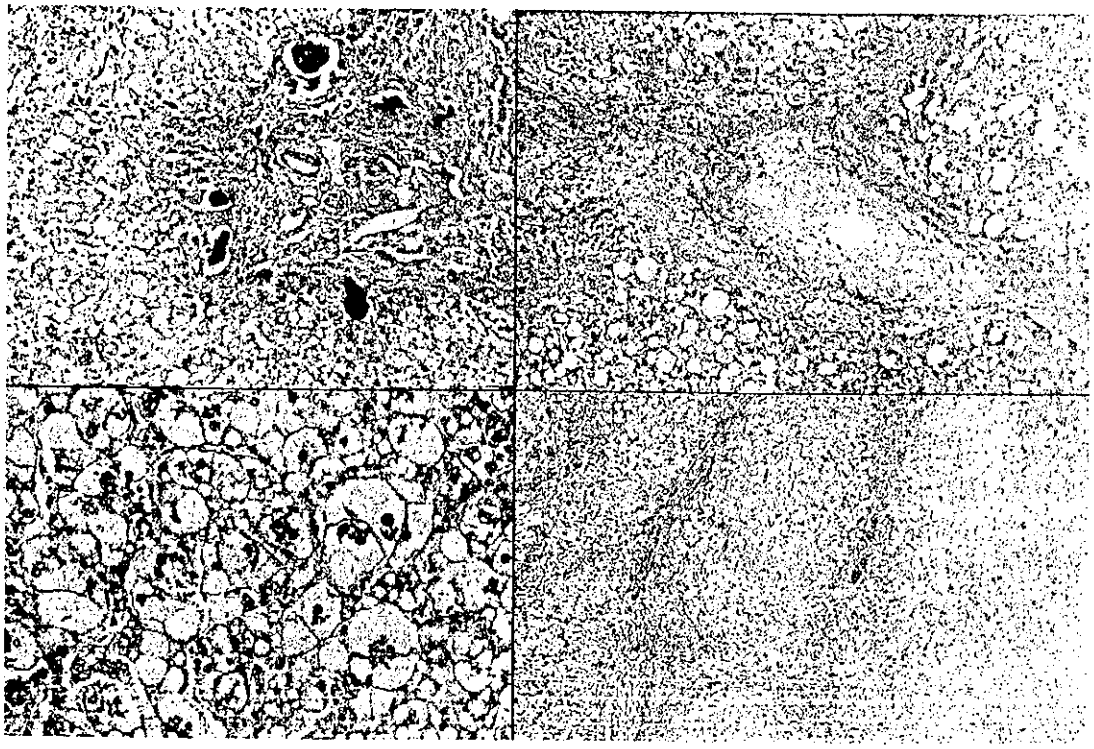

図 4　ドミノ生体肝移植を受けた際の摘出肝組織
ドナー手術後 169 日目の 2003 年 1 月 27 日の肝臓組織像で小結節性肝硬変の所見を呈し，NASH の病期 4，活動度 3。また，小型肝静脈の閉塞性病変，高度の細胆管性胆汁うっ滞，移植直前の循環障害による再生結節の壊死が認められた。上段左：HE 染色中拡大，上段右：HE 染色強拡大，下段左：HE 染色中拡大，下段右：HE 染色中拡大

の他，NASHに続発したと考えられる小型肝静脈の閉塞性病変，高度の細胆管性胆汁うっ滞，移植直前の循環障害による再生結節の壊死を認めた。(図4)

V. 検討した問題点

1. 緊急手術時のインフォームド・コンセントのあり方

ドナーの自発的な臓器提供であることは確認できた。しかし，緊急手術とはいえ，十分なインフォームド・コンセントは必要である。残肝容積が予想より小さめになることはありうるが，右葉グラフトの場合にはそれが術後肝不全につながる可能性があるので，それにさらに詳しく説明すべきであったと思われた。

2. ドナー家族へのサポート

移植コーディネーターがドナーに面談し，精神的に安定していたと判断されたので，精神科医へのコンサルテーションは必要としなかった。しかし，切羽詰まった状況の中でのドナーの心境を思うと，何らかの精神的サポートがあってもよかったと考えられる。家族や家庭生活への心配りなど医療社会福祉士の介入を考慮すべきであるとの提言があった。

3. ドナーの安全を守るための残肝容積の設定

残肝予測の計算式は以下の通りである。

(術前造影CTでの全肝容積－術前造影CTでの予測グラフト肝容積)÷術前造影CTでの全肝容積

本事例では当初，予測残肝容積は(1215－758)÷1215＝0.376の式で計算され37.6%（中肝静脈MHVをつけない右葉グラフト）とした。しかし，レシピエントの状態が重篤なため，グラフト肝に十分な血液灌流を導きうっ血をきたさないようにするため，中肝静脈を含めた右葉グラフトを採取した。この中肝静脈MHVを含めたグラフト採取のために，予測残肝容積値37.6%を下回り，実際に採取したグラフト重量900 gから計算すると残肝容積は(1215－900)÷1215＝0.259という式で計算され，推定容積25.9%となる。ただし，全肝容積の計算はあくまでもCT検査によるものであり，1,215 mlは実測値でないことから，25.9%という値も予測値である。

通常，京都大学の基本方針では正常肝の場合，残肝予測が35%以上で安全域，35～30%で境界域，30%以下で危険域と設定している。

本件とは別に，京都大学肝移植チームの資料によれば，実測残肝容積が30%以下の症例を4例経験し，いずれも合併症はないとしている（残肝容積25.5%，24.6%，26.7%，25.8%）が，この4例の術後の総ビリルビン値が1.9～7.8 mg/dlと異常を示していることは，ドナーに対して残肝容積が少ないと，かなりの負荷がかかっていることが容易に推測される。しかし，脂肪肝があり予測残肝容積が30%未満の症例は3例（このうち実測残肝30%未満は1例の24.6%）であったが，重篤な合併症は発生していないとされている。今回，最終的には推測値で判断するわけであるが，結果的にドナーの残肝容積が25.9%と当初の予想を上回って少なかった。このことが術直後の急性肝不全の要因と考えられる。残肝容積が絶対的に不足した場合に生じる急性肝不全に対する有効な治療法がないことから，予測残肝容積の安全域を少なくとも30%以上とすることを提言したい。

4. 脂肪肝の評価法と肝生検の意義

手術直前のドナーのCT検査で，肝臓と脾臓のCT値の比が1.0と軽度の脂肪肝があることが認識されていた。また，ドナーよりグラフト肝を摘出し，その灌流後に色調が黄色調で軽度の脂肪肝も肉眼的に示唆されていた。さらに，手術時生検の病理所見は9月2日づけ報告で大滴性脂肪沈着macrovesicular steatosisであることが判明していた。今回は，ドナー肝臓の術中迅速診断はなされていない。なお，肝臓表面は平滑であった。

京都大学肝移植チームの方針では脂肪肝の評価は原則として肝生検を行わず，CT検査で判断することとしている。その理由は，①ドナーに肝生検という侵襲的負担をかけたくない，②脂肪沈着には場所により違いがあり，肝生検では全体をみることができない，③CT検査のほうが全体の脂肪沈着を把握できる，の3点である。

さらに，京都大学肝移植チームの基準は肝臓と脾臓のCT値の比0.8以下はドナーとしての適応外と判断し，取り扱わないこととしている。この基準では本例のCT値は1.0と適応範囲内であった。しかし，単純性脂肪肝ではこの考え方で問題はないが，今回の事例のようにNASHによる脂肪肝を疑う場合は，より厳密にかつ正確に診断することが必要と思われた。

5. 肝移植における非アルコール性脂肪性肝炎(NASH)の取り扱い

ドナーの提供時の病理所見から，NASHという診断がなされた。NASHは最近注目されだした代謝性疾患であるが，無症状のものから肝硬変まで病態は広範囲にわたる。原因不明の肝硬変の一因であり，肝不

全に陥り肝移植の適応となることもある。NASHは40～50歳の肥満女性で，糖尿病，高脂血症（特に中性脂肪），高血圧を有する人に発症しやすい。組織学的特徴は大脂肪滴，脂肪嚢（fatty cyst）の形成，肝細胞腫大，グリコーゲン含有核，マロリー体，ミトコンドリアの腫大，多核好中球浸潤，門脈域線維化，肝細胞周囲性線維化などである。

NASH肝を提供した場合のドナーの予後についての報告はない。しかし，肥満患者（おそらくNASHを有する）の胃切除術後急激な肝不全に陥ったとの報告がある。このことは，NASH肝に外科侵襲という負荷が加わった場合肝病変が進行することを示唆する[12]。本ドナーの場合，肝切除という直接的な侵襲が加わり，かつ残肝容積が小さいという二重のリスクを受けている。実際，本ドナーの臨床経過はきわめて急速に悪化の一途をたどっている。

また，NASH肝を受けたレシピエントも肝障害を呈し，組織学的に胆汁うっ滞を含むNASHが発生している。特に脂肪沈着の程度はきわめて高度で移植後4カ月目ですでに前肝硬変まで進展している。その後，レシピエントの肝機能は改善し，退院の運びとなっている。

以上，本ドナーには肝提供時にNASHが存在した。それに残存肝容積のきわめて少ないことを含め，外科的侵襲，循環障害，感染症，DIC，胆管炎そして肝臓の再生など種々の要因が加わり，急激に肝硬変に進展したと推定される。その中でも，最も重要なことは前述の残肝容積が十分でないことである。この残肝容積の少ないことがNASH肝臓の病態悪化を促進したものと推測される。一方，レシピエントにも上述のように術後一時的にNASH病変が肝生検で認められた。このことは，NASHに罹患している肝臓は通常，ドナー肝には慎重でならなければならないことを示唆している。

6. 急激に生じた門脈圧亢進の原因

ドナーに術後すぐに腹水が貯留した原因としては，残肝容積が小さかったために門脈と肝動脈から流入する血液を受け入れることができず，急激に門脈圧が上昇したことが考えられる。著明な腹水が持続した原因は，US検査やCT検査などの画像診断および肝静脈造影から肝静脈の閉塞や狭窄はなく，門脈血栓もなかったことから，ドップラーエコーで確認されたような遠肝性門脈流を認めたことなどが考えられ，このことが急激な肝硬変への進行に伴う門脈圧亢進と中心静脈-肝静脈枝の肥厚・血栓化(いわゆるveno-occlusive disease）を誘発したと考えられる。

7. 遷延性胆管炎の存在

胆汁培養では意義のある細菌培養陽性結果は一度もなかったことから，化膿性細菌性胆管炎は存在しなかったといえる。移植時摘出肝でも胆道系で細菌感染や閉塞を示唆する変化はみられなかった。ドナーの生検肝および摘出肝に認められた細胆管性胆汁うっ滞（好中球浸潤を伴う）や高度の胆汁うっ滞は，遷延性胆管炎に起因すると考えられ，通常の化膿性胆管炎ではみられないもので，敗血症，エンドトキシン血症，あるいはDICに関連して発生したものと思われる。なお，類似の胆汁うっ滞性の病変はレシピエントのグラフト肝（移植後15日，24日，33日）にもみられ，今回の生体肝移植に関連して発生した可能性があるが，この所見は移植後125日では消退していた。

VII. 考　案

今回の事例は，一度生体肝移植を受けたレシピエントが8年間の経過を経て自己免疫性肝炎を発症し，急激に病態が悪化したため緊急の肝移植を余儀なくされた。レシピエント，ドナーを含めた家族に対する説明は十分な時間がなかったが，夫との面談から，ドナーの自発的な臓器提供であることが確認された。今回の手術は2回目ということから了解が得られやすかったと思われる。しかし，緊急手術の場合は緊急がゆえに患者，家族全体をサポートすることがおざなりになる可能性が強いことから，医療福祉士を含めたサポート体制の構築が今後望まれる。

ドナー手術における残存肝容積の緻密な計算を行い，安全域は残肝率35%以上が望まれるが，諸般の事情を考慮した場合でも，少なくとも30%の容積を確保すべきと考える。特に，肝右葉をグラフトに用いる場合は切除が広範囲にならないように注意を要する。京都大学では実測残存肝容積が30%以下を4例経験し，いずれも合併症はないとしている。しかし，この4例の術後の総ビリルビン値が上昇していることは，手術後にドナーに‘肝障害’が発生していると考えるのが妥当である。この黄疸出現の原因は不明であり，今後原因究明が必要であるが，本ドナーでみられた細胆管内胆汁栓を伴った高度の胆汁うっ滞（遷延性胆管炎）は，これら4例でも発生している可能性がある。遷延性胆管炎は病理学的には予後不良の指標とされている。

本事例の残肝容積が25.9%と少ないことと，遷延性胆管炎の存在を考え併せると，推定残肝容積30%以下のドナー手術はドナーの適応条件から除外すべきである。なお，30～35%の残肝容積の場合は慎重に考えなければならないが，少なくとも脂肪沈着があればより慎重に対処すべきと考える。

本事例は，ドナーの肝臓の状態を通常の生化学検査値や画像診断から単純脂肪肝として取り扱ったが，実際はNASHであり，さらにドナー手術で予想以上に残存肝容積が少なくなり（30%以下），それに加えて外科的侵襲，DIC，敗血症，エンドトキシン血症などの様々な術後要因が加わり，NASHの病態が短期間に進行性となり，脂肪性肝硬変を経て非代償性肝硬変に進展したものと推定される。一方，移植されたレシピエントの肝臓にもNASHがみられ，しかも一過性であるが急速に病状が進展したことは，NASH肝がたとえ脂肪沈着率30%以下であっても，ドナー肝臓として適切でないことを示唆しているものと考えられるが，このことに関しては未だに医学的エビデンスは得られていない。京都大学肝移植チームは多くの経験から本グラフトを単純脂肪肝として取り扱ったが，今後は本症例のように肥満，高血圧，高脂血症（疑い），糖尿病（疑い）などを伴う脂肪肝はNASHを鑑別し慎重に対応する必要がある。現時点では，単純脂肪肝とNASHと他の脂肪肝を鑑別すべき血清マーカーがなく，肝組織検査が唯一の診断法である。したがって，NASHが少しでも疑われる場合は肝生検による組織学的診断がきわめて重要であり積極的に肝生検を行い正確な診断をすべきである[13]。

本委員会としては生体肝移植におけるドナーの安全のために，ドナー手術における残存肝容積の緻密な計算とドナー肝臓における脂肪浸潤の原因を，術前に確実に評価することがきわめて重要であることを強調したい。なお，手術手技や術後管理には特に問題となる事象はないと判断された。

VIII. 再発防止の提言

①緊急時の手術であればこそ，十分なインフォームド・コンセントがなされ，患者・家族への全人的サポートが必要である。

②ドナーに脂肪肝がある場合，その正確な原因診断を術前早めに行う必要がある。併発する病態や画像診断から臨床的にNASHが疑われた場合は，積極的に肝生検を行い，確実な診断をすべきである。

③NASHと診断された肝臓は，程度によるがドナー肝臓として不適当である。これはドナーにとってもレシピエントにとっても重症肝障害へ早期に進展する可能性があるからである。したがって，当面はNASHという病態を呈した肝臓は，ウイルス肝炎と同様にドナー肝臓の適応外とすべきである。しかしながら，治療によりNASHが改善した場合は肝生検で再評価し，再びドナー肝臓として適応を考えることは可能である。

④予測残肝容積が少なくとも計算上30%以上を適応とすることが望ましい。

⑤今後，NASHにつき肝移植医療関係者に啓発する必要がある。

IX. ドナーを守るため今後の課題

生体肝移植は，日本においても世界においても標準的な医療として定着しようとしている。特に，脳死ドナーが不足している日本の現況を鑑みると，生体肝移植は重要な選択肢として社会に定着させていくことが必要である。そのためには，何をおいてもドナーの安全と人権を守る生体肝移植医療の確立が必要である。本報告では主に医学的レビューに力を注いだが，今後の課題と医学的原則に加え，倫理的社会的諸課題を包括的に再検討し，安定した基本ルールを明確化するよう努力すべきである。そのためには医学界自らがこの作業に着手すべきであり，急速に変わりつつある諸外国における実情の調査と，その上での国際的な比較も必要である。

X. おわりに

生体肝移植ドナーが自ら肝移植を受けざるをえなくなった事態になったことは，誠に残念である。二度と同じことが繰り返されないよう，ドナーの安全確保に万全を尽くすことは医療者の義務である。本委員会は今回の事例を厳密に検証した上で，今後への提言をまとめた。脳死肝移植が遅々として発展しないわが国においては生体肝移植の必然性を認めざるをえない。しかし，健常者からの臓器提供はたとえ家族といえども安易に考えてはならない。ドナーの安全と人権を守ることは絶対である。そのためには医療者側が厳格かつ透明性あるルールをつくり，それを実行することが求められる[14]。

本委員会としては，このような事例を踏まえて，脳死肝移植がわが国において成熟していくことを強く希

望するものである。

今回の調査にあたり，京都大学附属病院田中紘一病院長，江川裕人助教授をはじめスタッフの皆さんからは，資料の提供，聞き取り調査など全面的協力をいただいた。田中紘一病院長からは徹底的な検証をお願いしたいという言葉を何度もいただいた。ここに厚く感謝申し上げる。同じ医療者として厳正に検証できたと考える。

文責：市田隆文（新潟大学医歯学総合病院・生命科学医療センター）

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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%

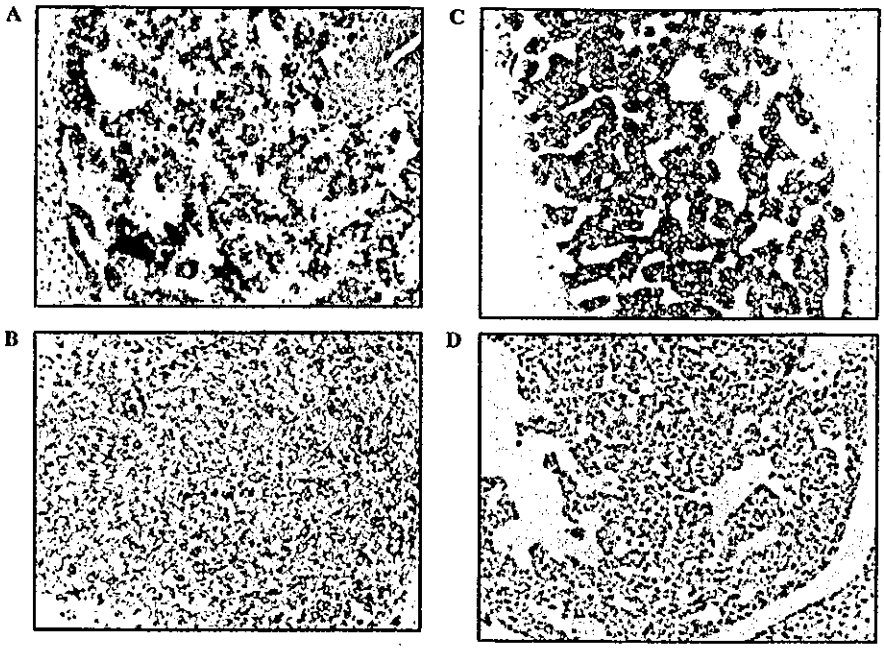

Fig. 1. (A–D) Liv2 and Liv8 expression at E 11.5 in normal and AML1$^{-/-}$ mice. Liv2 (A,C) and Liv8 (B,D) expression at E 11.5 in normal fetal liver (A,B) and AML1$^{-/-}$ mice (C,D). Magnification: (A–D) at 200×.

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.

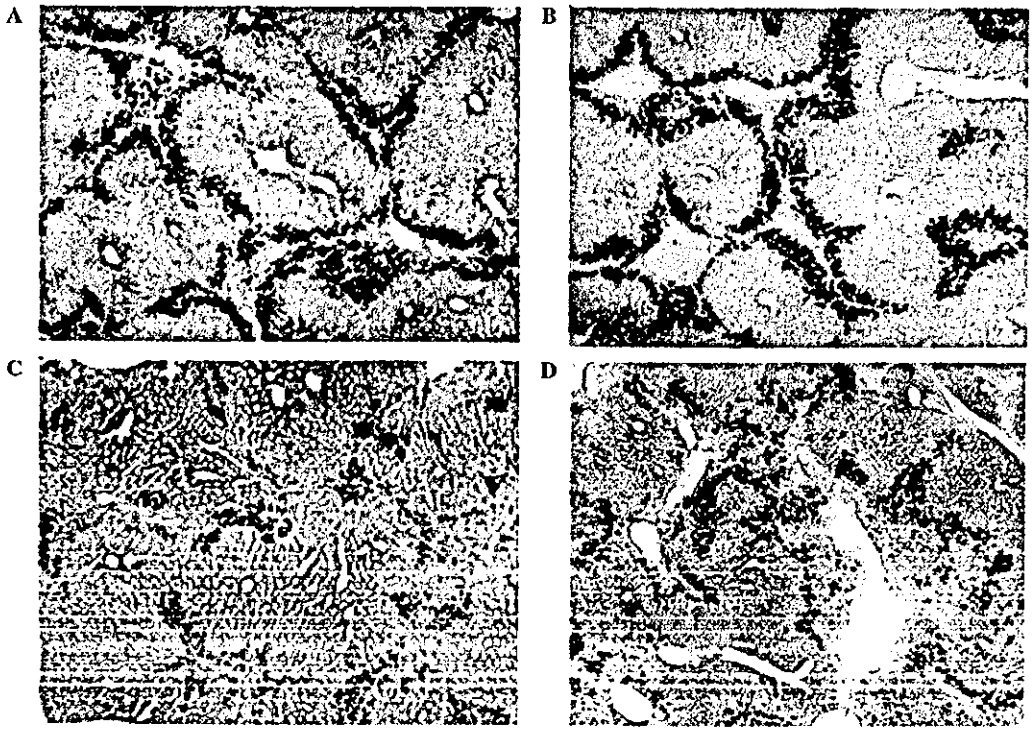

Fig. 3. (A–D) Expression of GFP in liver after transplantation of Liv8-positive and Liv8-negative cells. GFP expression in the liver after transplantation of Liv8-positive BMCs at 1 week (A) and 4 weeks (C), GFP expression at the liver after Liv8-negative BMC transplantation at 1 week (B) and 4 weeks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

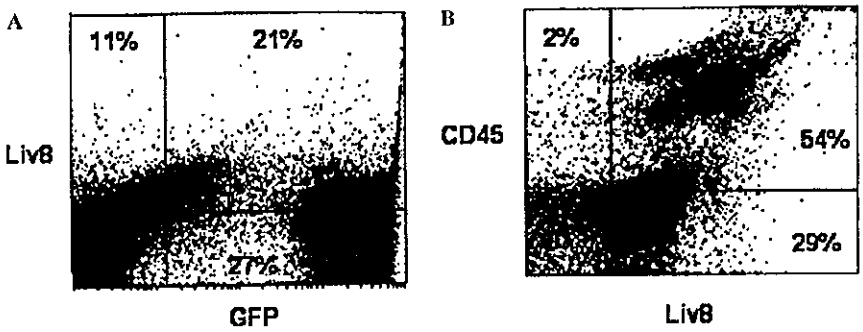

Fig. 2. Expression of CD45, Liv8 in bone marrow cell. FACS analysis of all BMCs of GFP-Tg mice. (A) Staining with Liv8 and GFP. (B) Staining with CD45 and Liv8.

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.

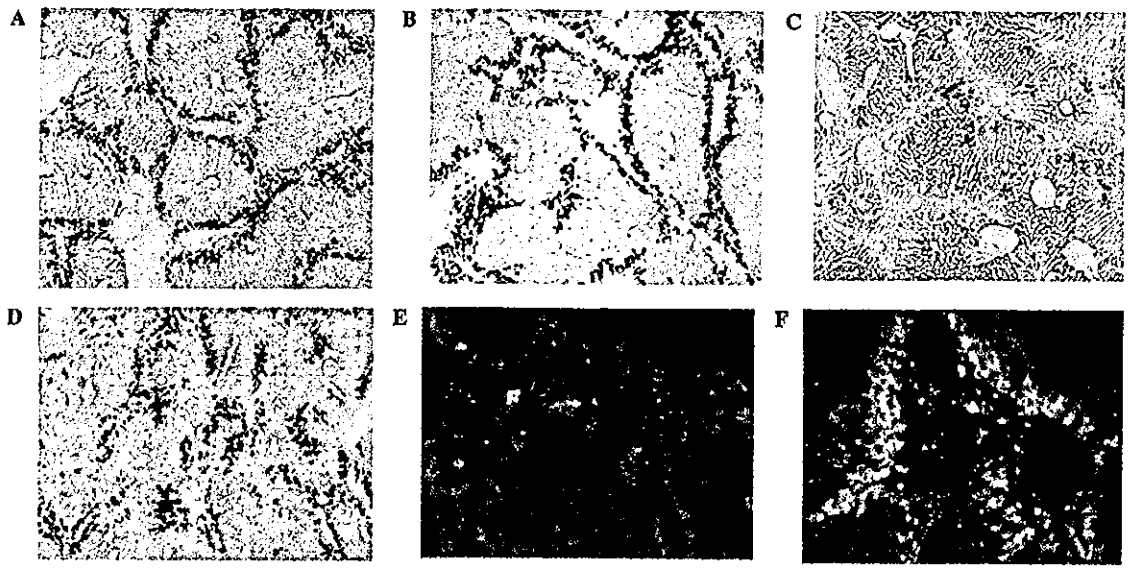

Fig. 4. (A–F) Expression of Liv2 antigen in liver after transplantation of Liv8-positive and Liv8-negative cells. Liv2 antigen expression at 1 week (A) and 4 weeks (C) after Liv8-positive BMC transplantation. Magnification at 200×. Liv2 antigen expression at liver at 1 week (B) and 4 weeks (D) after 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×.

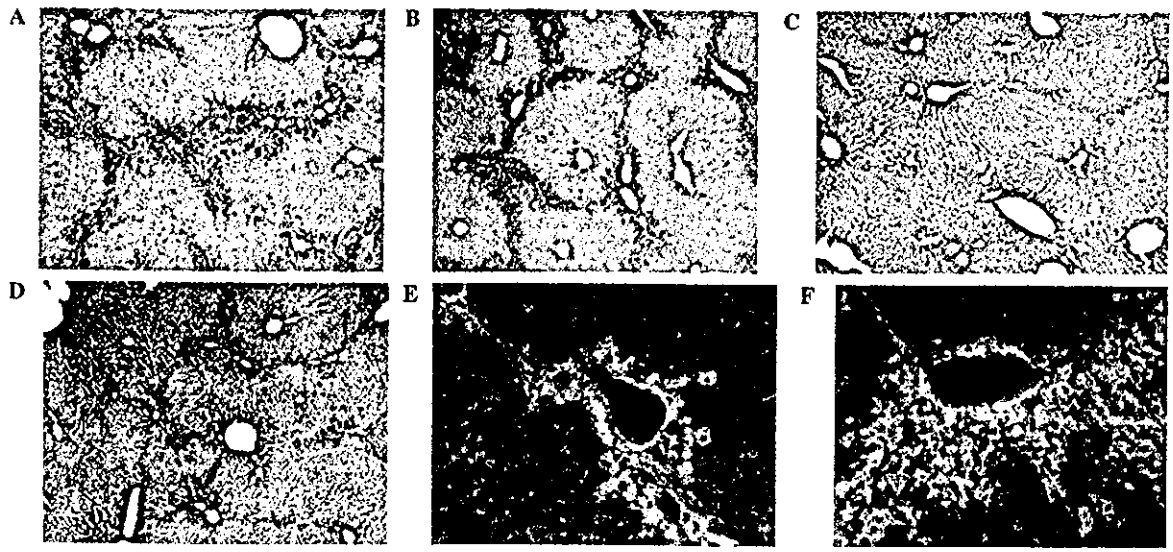

Fig. 5. (A–L) Expression Albumin after transplantation of Liv8-positive and Liv8-negative cells. Albumin expression at 1 week after transplantation of Liv8-positive cells (A) and Liv8-negative cells (B). Albumin expression at 4 weeks after transplantation of Liv8-positive cells (C) and Liv8-negative 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

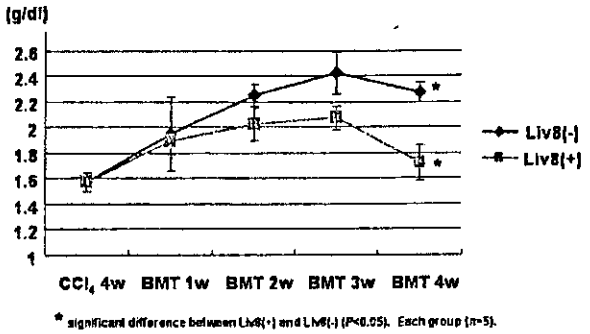

Fig. 6. The level of serum albumin. Serum albumin levels after Liv8-positive or Liv8-negative cell transplantation. CCl_4 4w, 4 weeks CCl_4 injection group. BMT 1w, 1 week after BMC transplantation. BMT 2w, 2 weeks after BMC transplantation. BMT 3w, 3 weeks after BMC transplantation. BMT 4w, 4 weeks 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 Auto-MACS, 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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Herbal medicine Sho-saiko-to (TJ-9) increases expression matrix metalloproteinases (MMPs) with reduced expression of tissue inhibitor of metalloproteinases (TIMPs) in rat stellate cell

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Abstract

We have reported that Sho-saiko-to (TJ-9) prevents liver fibrosis in vivo. To gain further insights into the effect of TJ-9, the matrix metalloproteinases (MMPs)/tissue inhibitors of metalloproteinases (TIMPs) balance was examined. Hepatic stellate cells (HSCs) were isolated from male Wistar rats and cultured with TJ-9 (0-1000 μg/ml) on uncoated plastic dishes for 4 days. To elucidate the effects on the MMPs/TIMPs balance by TJ-9, quantitative analysis of type IV collagen-degrading activity, gelatin zymography and reverse zymography were carried out. Northern blot analysis was performed to determine the expression of MMP-2, 13 and TIMP-1 mRNAs. TJ-9 treatment resulted in dose-dependent upregulation of MMP-2, 13 mRNA and downregulation of TIMP-1 mRNA up to 500 μg/ml. Gelatin zymography, reverse zymography and quantitative analysis of type IV collagen-degrading activity confirmed that TJ-9 increased MMP-2 activity and prevented TIMP-1, 2 activities in a dose-dependent manner. SB203580 diminished the reduction of mRNA as well as the activity of TIMP-1 by TJ-9 and induction of mRNA as well as the activity of MMP-2. These results show that TJ-9 increased MMP-2, 13 activity with reduced TIMP-1, 2 activities on HSCs possibly via P38 pathway.

Keywords: Herbal medicine; Fibrosis; Signal transduction; Metalloproteinase; Tissue inhibitor of metalloproteinase

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Introduction

Hepatic stellate cells have now been clearly identified as the primary cellular source involved in the pathogenesis of liver fibrosis (Friedman, 1991). During the development of liver fibrosis, stellate cells undergo activation, a process characterized by increased cell proliferation, morphological transformation into myofibroblast-like cells and synthesis of excessive extracellular matrix components (Friedman, 1991). These cells are also able to synthesize enzymes, called matrix metalloproteinases (MMPs), which can degrade matrix proteins in the extracellular space. The activity of MMPs is carefully regulated by controlling their conversion from pro-enzymes to the catalytic form and by a family of specific inhibitors, called tissue inhibitors of metalloproteinases (TIMPs).

On the other hand, many studies of signal transduction are now underway. The MAPK family of protein kinases includes the extracellular signal-regulated kinases (ERKs) (Boulton et al., 1991) the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) (Derijard et al., 1994; Kyriakis et al., 1994), and p38 (Han et al., 1994) The MAPK signaling pathway is a multistep phosphorylation cascade that transmits signals from the cell's surface to cytosolic and nuclear targets (Cohen, 1997). It has been suggested that not only do MAPKs serve as the signaling pathways production of MMPs (Esparza et al., 1999; Zeigler et al., 1999), but that they also participate in production of TIMPs (Li and Zafarullah, 1998; Eberhardt et al., 2000). However, the role of ERK, SAPK and p38 pathways in regulating production of MMPs and TIMPs by HSCs is not well known at present.

Many agents have been proposed for the prevention and treatment of fibrosis but no specific inhibitor of stellate cell activation has yet been developed.

TJ-9 has been reported, including by us, to prevent fibrosis via the inhibition of HSCs in different animal models of fibrosis due to choline-deficiency (Sakaida et al., 1998a,b), and pig serum (Shimizu et al., 1999), as well as in isolated stellate cells (Kayano et al., 1998). TJ-9, commonly prescribed in Japan as Sho-saiko-to, is the most popular herbal medicine in Japan and has been widely used in the treatment of chronic liver diseases, especially chronic viral hepatitis. TJ-9 consists of an aqueous extract from the roots of scutellaria, glycyrrhiza, bupleurum, and ginseng; the pinella tuber; the jujube fruit; and the thew ginger rhizome. A number of studies have indicated its cytoprotective effects in experimental liver injuries (Yamamoto et al., 1985; Araki et al., 1988), cancer-preventive effects (Okita et al., 1993, 1994; Tatsuta et al., 1991; Oka et al., 1995), anti-tumor, and apoptotic effects (Matsuzaki et al., 1996; Yano et al., 1994).

From the clinical point of view, treatment for liver fibrosis has been mainly focused on prevention of stellate cell activation instead of acceleration of degradation.

To gain further insights into the effect of TJ-9 on suppression of hepatic fibrosis, we examined changes of the MMPs/TIMPs balance and MAPK pathways of TJ-9 treated HSCs.

Materials and methods

Animals

Male Wistar rats weighting 400 to 450 g (Nippon SLC Co., Ltd., Shizuoka, Japan) were obtained, quarantined for 1 week, and housed in a room under controlled temperature (25 °C), humidity, and

lighting (12 hours light, 12 hours dark). Access to food and tap water was ad libitum. After a 1-week acclimation period on a basal diet (Oriental MF Diet; Oriental Yeast Company, Japan), the rats were used for the experiments.

Isolation and culture of rat hepatic stellate cells

Rat hepatic stellate cells (HSCs) were isolated with Nycodenz as described previously (Kayano et al., 1998).

Yields were 1.0-1.5 $\times$ 10^7 cells/rat. Cell viability was always over 95% as determined by the trypan blue exclusion test. Cell purity was more than 90% as assessed by the presence of yellow droplets and desmin immunoreactivity. Then isolated hepatic stellate cells were cultured at a density of 5.0 $\times$ 10^5 cells/ml in monolayer culture on uncoated 60-mm plastic dishes (Iwaki Glass Co., Ltd., Tokyo, Japan). All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After incubation for 4 h, non-adherent cells were removed with a pipette and the culture medium was replaced with medium containing various concentrations of TJ-9 or medium alone (control). In some experiments, SB203580 or PD98059 (Calbiochem, La Jolla, CA) was added in addition to TJ-9. SB20380 and PD980597 were dissolved in DMSO at the final concentration of 0.1%. SB20238 is known as a highly specific inhibitor of p38 kinase (IC_{50} = 34nM in vitro, 600 nM in cells) and even 100 μM does not significantly inhibit JNK and p42 MAP kinase, and PD98059 is known as specific inhibitor of ERK kinase (IC_{50} = 2 μM), according to the manufacturer's instructions.

The medium was changed every 24 h and cell culture was continued up to 4 days (serum was depleted 24 h before the assessment).

The treated HSCs were scraped after being washed with PBS twice and suspended at 4 °C in 0.5 ml of PBS with 0.1% Triton X. An equal amount of total protein (100 μg) was applied for the quantitative analysis of type IV collagen-degrading activity, zymography and reverse zymography.

Preparation of culture medium with Sho-saiko-to (TJ-9)

Sho-saiko-to (TJ-9, powder) was kindly provided by Tsumura Co., (Tokyo, Japan). Water-soluble ingredients of TJ-9 were obtained as described previously (Kayano et al., 1998). The final concentrations of TJ-9 used were 10, 100, 500 and 1000 μg/ml. The PH values of all culture media with or without TJ-9 were adjusted to within the physiological range.

Hybridization probes

The following probes were used in this study. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) (Sakaida et al., 1996, 1998a,b) was purchased from American Type Culture Collection (Rockville, MD, USA). TIMP-1 cDNAs was used as described (Sakaida et al., 1999). MMP-2 cDNA was a generous gift of Dr. Hiroshi Sato (Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, Ishikawa-Pref., Japan) (Sato et al., 1994). MMP-13 cDNA was the generous gift of Dr Cheryl O. Quinn (Pediatric Research Institute, Department of Pediatrics, Health Sciences Center, Saint Louis University, MO, USA) (Hironaka et al., 2000).

Northern blot analysis

Northen blot analysis was performed after the isolation of 10 μg of total RNA from isolated hepatic stellate cells as described previously (Hironaka et al., 2000). Each signal strength of mRNA was determined after normalization by relevant G3PDH mRNA levels.

Quantitative analysis of type IV collagen-degrading activity

Type IV collagen-degrading activity in conditioned media was determined as described previously (Hironaka et al., 2000) using a Type IV collagenase activity assay kit (YU-18001, Yagai Co., Kamagata, Japan). The principle of the type IV collagenase activity assay kit is based on fluorescent measurement of collagen fragments upon cleavage by gelatinases. It is known that collagen decomposition fragments differ from whole collagen in their temperature of denaturation and ethanol solubility. Upon gelatinase cleavage of fluorescently-labeled collagen type IV, decomposition fragments are produced. These fragments are selectively denatured and extracted with ethanol. The fluorescence intensity of the extracted product is measured and correlates with type IV collagen-degrading activity.

Zymography

As described by Durko et al. (Durko et al., 1997), SDS-polyacrylamide (10%) gels were co-polymerized with gelatin at a final concentration of 1 mg/ml. The concentrated conditioned media were mixed 3:1 (v/v) with sample buffer (0.3 M Tris-HCl, pH 6.8, containing 8% SDS, 0.4% bromophenol blue and 40% glycerol), loaded onto the gels without boiling and separated by electrophoresis. The gels were washed for 1 h in a solution of 2.5% Triton X-100 in 40 mM Tris-HCl, pH 7.6, and for 15 min in 10 mM Tris-HCl, pH 8. For the enzymatic reaction to take place, the gels were incubated for 18 h at 37 °C in a solution of 50 mM Tris-HCl, pH 8, containing 10 mM $CaCl_2$. The gels were stained for 2 h in a 0.5% Coomassie blue R250 solution, then destained in 20% methanol with 10% acetic acid until clear bands (indicating lysis) were apparent on the blue background. Prestained molecular weight markers were resolved on the same gels, separated from other samples after electrophoresis and fixed in 5% acetic acid. To characterize the protease bands, some of the gels were incubated in a buffer (50 mM Tris-HCl, pH 8, with 10 mM CaCl2) containing 20 mM EDTA (a metalloproteinase inhibitor).

Reverse zymography

Reverse zymography was performed as previously described (Herron et al., 1986). Gelatinase inhibitory activity was detected by incubating standard gelatin zymograms for 1 h at 37 °C in a preparation of purified collagenase (2 unit/ml) before incubation at 37 °C for 18 h in a solution of 50 mM Tris-HCl, pH 8, containing 10 mM $CaCl_2$. Gels were stained and destained as described above, after which stained bands manifested the presence of gelatinase inhibitory activity corresponding to TIMPs in the polyacrylamide gels.