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5 急性肝不全に対する骨髄細胞を用いた肝再生療法の現状

要点

- 臨床研究では，急性肝不全（正確には acute on chronic liver failure）に対する G-CSF 投与のランダム化比較試験による有効性を Grag らが論文報告している．
- 基礎研究でも，急性肝障害（肝不全）モデル系に対する G-CSF，全骨髄細胞や骨髄 MSC 投与の有効性を示した論文報告があり，今後の臨床研究における安全性および有効性評価が待たれる．

はじめに

慢性肝疾患の終末像である肝硬変症に対する骨髄細胞を用いた肝再生療法としては，筆者らの「肝硬変症に対する自己骨髄細胞投与（autologous bone marrow cell infusion：ABM*i*）療法」や，国外からも骨髄間葉系幹細胞（mesenchymal stem cell：MSC）投与等の有効性が論文報告されている[1]．その一方，急性肝不全に対する臨床研究論文はこれまでのところ Grag らの acute on chronic liver failure（ACLF）に対する顆粒球コロニー刺激因子（granulocyte colony-stimulating factor：G-CSF）投与が生命予後を改善したという報告のみである[2]．

本項ではおもに骨髄細胞を用いた急性肝不全に対する肝再生療法の基礎研究成果を中心に概説する．

Ⅰ G-CSF を用いた研究

肝硬変症に対する臨床研究論文としては，すでに Gordon らの G-CSF で誘導した自己末梢血 CD34 陽性細胞を門脈または肝動脈から投与したところ血清アルブミン値が上昇したとの報告，アルコール性肝硬変症に対する G-CSF 投与が肝前駆細胞の増殖を促進させたとの Spahr らの報告等がある．一方，**急性肝不全（正確には ACLF）に対するランダム化比較臨床研究**としては，5 μg/kg G-CSF 皮下投与（n＝23）により末梢血好中球や肝内 CD34 陽性細胞数が増加し，肝腎症候群，肝性脳症といった合併症が減少することで投与 2ヵ月後の生存率は有意に高かったとの成績を，Garg らは報告している[2]．基礎研究では，D-ガラクトサミン誘導急性肝不全ラットモデルへの G-CSF 投与が肝内 CD34 陽性細胞を増加させ肝細胞増殖を促進（肝内 Ki-67 陽性細胞が増加）により，生存率と生存期間を有意に延長するとの報告がある[3]．しかし一方で，四塩化炭素急性肝障害ラットモデルに対する G-CSF 腹腔内投与には効果がなかったとの報告もある等[4]，今後の研究の蓄積が待たれる．

Ⅱ 全骨髄細胞を用いた研究

筆者らは四塩化炭素誘導マウス肝硬変モデルへの同種同系全骨髄細胞投与が肝線維化および肝機

肝線維化研究の進歩と治療の展望

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はじめに

肝線維症は、肝炎ウイルス感染、アルコール多飲、非アルコール性脂肪肝炎（non-alcoholic steatohepatitis, NASH）、自己免疫学的機序、薬物性、金属代謝異常など、本章の各稿で取り上げられているおよそ全ての肝細胞傷害によってもたらされる共通の病態である。また、これ以外にも肝外胆管閉塞や肝うっ血により肝線維化は引き起こされる。線維化の進行に伴って肝組織中にはコラーゲンをはじめとする細胞外マトリックスが過剰に沈着し、その終末像である肝硬変では肝細胞機能不全や門脈圧亢進症が問題となる。さらに、高頻度に合併する肝細胞癌の発生を抑止する上でも、肝線維化研究は重要なテーマである。近年、肝線維症の非侵襲的診断と治療法の開発が従来にも増して注目されており、その背景を理解することで肝線維化研究の必要性と重要性とが見えてくる。

肝線維化過程において中心的役割を担う肝星細胞は、かつては伊東細胞（Ito cells）と呼ばれたように、1951年に伊東俊夫教授によって発見された[1]。また、ビタミンＡを貯蔵し[2]、コラーゲンを産生する[3]本細胞の特徴は和氣健二郎教授らにより実証されるなど、発見当初より日本人との関わりが深い細胞である。肝線維化過程において星細胞が果たす役割や、線維肝組織中に増加するⅠ型コラーゲンとこれを分解するmatrix metalloproteinase（MMP）の産生調節機構など、肝線維化研究は大きな進歩を遂げた。しかしながら、臨床の現場に目を向けると、今もって肝線維症に対する特異的かつ効果的な治療薬は存在しない。肝線維化研究の成果はいつになったら臨床に還元できるのかという批判に、その研究に携わる者は真摯に耳を傾ける必要がある。本稿では、近年の肝線維化研究の進歩を概説するとともに、新規治療法の開発に向けて克服すべき問題点と今後の展望に関する私見を述べて、ご批判を仰ぎたい。

肝星細胞のバイオロジーと線維化病態

１）肝星細胞の活性化機構

肝星細胞は肝類洞周囲のDisse腔に存在し、生理的条件下では前述したようにビタミンＡの貯蔵細胞であるとともに、その長い突起と収縮機能を介して類洞内微小循環の調節に寄与している。しかしながら、肝細胞壊死や炎症に伴って放出された液性因子の刺激を受けると次第にビタミンＡの脂肪滴を失い、α-smooth muscle actin（αSMA）陽性の筋線維芽細胞様の細胞（myofibroblast-like cells）に形質転換して、活発にコラーゲン産生を行うようになる。この星細胞の活性化やコラーゲン産生を促進する代表的因子としてplatelet-derived growth factor（PDGF）や後述するtransforming growth factor-β（TGF-β）がよく知られ、最近ではケモカインの関与にも注目が集まっている[4]。その発生学的起源についてはこれまで多くの議論があったが、近年になって中胚葉に由来することが証明された[5]。

正常肝組織から分離した星細胞をプラスティック・ディッシュ上で培養すると、脂肪滴の減少とともにαSMAを発現し、コラーゲン産生を活発に行うようになる。この現象は、生体における星

細胞の活性化を擬するモデルとして、肝線維化機序の解明や抗線維化薬剤の効果判定などに頻用されてきた。しかしながら、線維化刺激により生体内で活性化した星細胞と培養により活性化した星細胞における遺伝子発現プロフィールを網羅的に解析すると、両者は完全には一致しなかった[6]。培養細胞を用いた*in vitro*試験で得られた研究成果を*in vivo*で検証する際に、とりわけ留意すべき点である。

後述するように、肝線維症は可逆的な病態である。四塩化炭素の反復投与を中止して肝線維化所見が改善する際に活性化星細胞数は急激に減少することから、アポトーシスに陥ると考えられてきた[7]。ところが最近、実験的肝線維症の改善過程において、活性化した星細胞の約半数は非活性型に戻るという報告がなされた（図１）[8]。これまでも、培養星細胞の活性型と非活性型との相互移行については、脂肪細胞の分化・脱分化過程と類似してperoxisome proliferator-activated receptor γ（PPAR-γ）などの転写因子による制御が報告されていた[9]が、この可逆性が*in vivo*においても証明された意義は大きい。しかしながら、線維化の改善に伴って非活性型に移行した星細胞は、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。

ビタミンAの活性体であるレチノイン酸に対する受容体のdominant negative formを肝細胞特異的に発現させたマウスでは、脂肪肝炎とともに肝細胞癌が自然発生した[10]。また、活性化星細胞において発現が増加するcytoglobinの遺伝子を欠失させたマウスでは、diethylnitrosamineによる化学発癌が増強された[11]。いずれも、肝線維化と発癌の病態連繋を理解する上で重要な知見である。

２）コラーゲン産生細胞のheterogeneity

活性化した肝星細胞がコラーゲン産生を通じて肝線維化過程において中心的役割を担っていることは前述した通りであるが、星細胞以外にもコラーゲン産生細胞の存在が指摘されるようになった（図１）。その中でも、門脈域に存在するportal fibroblastsは、胆管結紮モデルに代表される胆汁うっ滞に伴う肝線維症の発症と進展において重要な役割を演じている[12]。コラーゲン産生細胞のheterogeneityに関連して、Ⅰ型コラーゲンα1鎖遺伝子（COL1A1）プロモーターとαSMA遺伝子プロモーターのデュアル・レポーターマウスを用いた研究では、正常肝および胆管結紮による線維肝のいずれにおいてもCOL1A1発現細胞とαSMA発現細胞は完全に一致せず、それぞれ単独陽性の細胞と両者陽性細胞が混在していた[13]。星細胞や筋線維芽細胞のheterogeneityは、その起源とも関連して重要な問題を孕んでいる。

近年、骨髄由来細胞がコラーゲンを産生して肝線維化の進展に関わる[14]、あるいは星細胞や筋線維芽細胞自体が骨髄に由来する[15]という報告が相次いだ。しかしながら、COL1A1遺伝子あるいはこれと協調発現するⅠ型コラーゲンα2鎖遺伝子（COL1A2）のエンハンサー・プロモーターをルシフェラーゼやEGFP遺伝子に連結したレポーターマウスを用いて感度ならびに特異度に優れた解析を行うと、骨髄由来細胞のコラーゲン産生は否定的[16]あるいはきわめて限局的であった[17]。全ての実験方法には限界があるが、現在では線維肝組織に浸潤した骨髄由来細胞は炎症性サイトカインやケモカインを産生することで間接的に肝線維化の進展に関わるとする意見が多い。

同様の問題は、上皮間葉移行（endothelial-to-mesenchymal transition, EMT）についても指摘できる。当初は、四塩化炭素投与や総胆管結紮後のマウス、あるいは移植後に再発した原発性胆汁性肝硬変症例において肝細胞や胆管上皮細胞のEMTの関与が報告された。しかしながら、アルブミンやkeratin-19、あるいはα-フェトプロテインといった肝臓における上皮系細胞に特異的な遺伝子のプロモーターを用いたfate mapping studyにより、少なくともマウスを用いた実験的肝線維化過程においてはEMTのコラーゲン産生への直接的関与は否定された[18]。

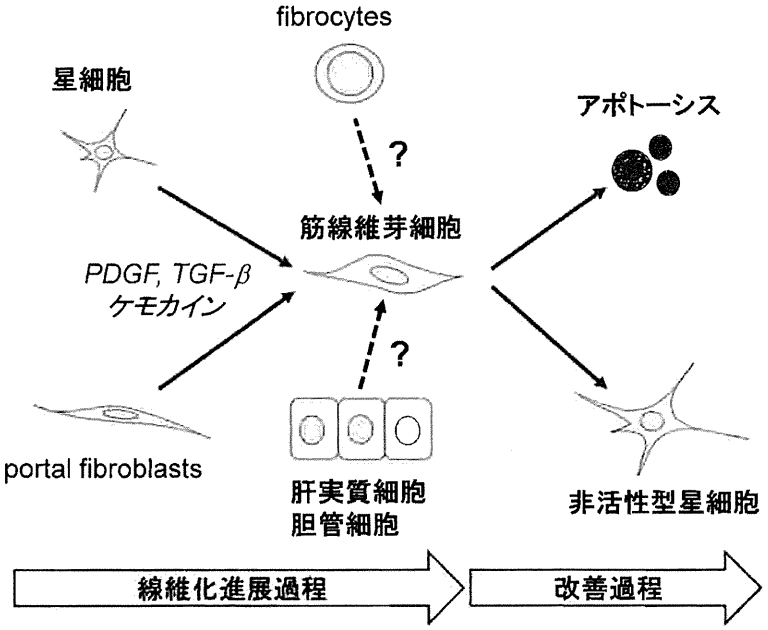

図１　コラーゲン産生細胞のheterogeneityと活性化・脱活性化過程
肝におけるコラーゲン産生細胞である筋線維芽細胞（様細胞）の起源としては、肝実質内の星細胞や門脈域に存在するportal fibroblastsの他、近年では造血細胞由来のfibrocytesや肝実質細胞ないし胆管細胞のEMTの関与が注目された。しかしながら、その後の研究ではfibrocytesやEMTの関与は限局的ないし否定的とする意見が多い。肝線維化の改善過程においては、活性化した星細胞はアポトーシスに陥ることで排除されると考えられていたが、最近ではその一部は非活性型に移行することが報告された。

コラーゲン産生と分解の分子機構

1）TGF-β/Smad3によるコラーゲン遺伝子転写の促進とその制御

TGF-βは肝線維化の進展過程において中心的役割を担っており、その作用は星細胞の活性化、コラーゲン遺伝子転写の促進、MMP発現の抑制、MMP阻害因子tissue inhibitor of metalloproteinase（TIMP）の発現促進など、多岐にわたる[19]。近年、星細胞のToll-like receptor 4（TLR4）を介するTGF-βシグナルの増強機序が明らかにされた[20]。これは自然免疫と肝線維化を繋ぐ接点であると同時に、コレステロールの過剰摂取による肝線維化進展にも関わることで注目されている[21]。

著者らは、COL1A2遺伝子のプロモーター上にTGF-βによる転写促進を伝達する領域を世界で初めて同定し、これをTGF-β-responsive element（TbRE）と命名した[22]。TbREにはSp1とSmad3、さらにCBP/p300 coactivatorが結合し、これらタンパク間の相互作用によりCOL1A2転写を促進させる[23]。また、活性化星細胞では恒常的にリン酸化されたSmad3が核内に蓄積することでCOL1A2転写が亢進しており[24]、核内Smad3が線維化治療の標的になり得ると考えた。例えば、IFN-γの細胞内シグナル伝達物質であるYB-1は、TbREに結合するSmad3とCBP/p300 coactivatorとの相互作用を阻害することで、TGF-βによるCOL1A2転写の促進に対して拮抗的に抑制した[25]。YB-1過剰発現アデノウイルスを投与すると肝線維化の進展が抑制され[26]、これらの知見に基づいてYB-1の核内移行を促進する新規低分子化合物HSc025が開発された。これを四塩化炭素の反復投与により作製した肝線維症マウスに投与すると、血清トランスアミナーゼ値の有意な低下と

ともに、肝細胞の脂肪変性と線維の蓄積が有意に改善された[27]。

コラーゲンは組織や臓器の形態保持のみならず、創傷治癒や組織の修復においても重要な役割を担っていることから、他臓器へ及ぼす副作用を軽減するには線維化組織あるいはコラーゲンの産生細胞特異的に薬剤を運搬・作用させる工夫が必要となる。ビタミンA封入リポソームを用いることで、コラーゲン特異的シャペロンHSP47 siRNAの星細胞選択的な導入と肝線維化の改善が動物実験で示された[28]。

２）骨髄由来細胞の肝線維化改善への関与と細胞治療への応用

コラーゲン合成に関する研究の進歩に比して、分解系の研究は未だに立ち遅れている。問題を複雑にする１つの要因は、齧歯類においてコラーゲン線維を分解する主要な間質性コラゲナーゼであるMMP-13が線維化の改善過程では既存線維を分解する[29]一方で、線維化初期においてはむしろ促進的に働く[30]など、病期によって相反する作用を示すことにある。

著者らは、実験的肝線維症の回復期において骨髄から線維肝組織へと動員された細胞が、MMP-13、MMP-9を順次発現することで線維化改善に寄与することを明らかにした[31]。この際G-CSFを投与すると、骨髄由来細胞の線維肝組織内への生着が増強し、MMP-9の発現亢進とともに線維化の改善が促進された。線維化改善と再生の促進は、肝線維症マウスに自家骨髄細胞を投与した際にも認められ[32]、これら動物実験のデータに基づいて肝硬変症例に対する自家骨髄細胞の注入療法が国内外の複数の施設で試みられている[33][34]。また、骨髄間葉系幹細胞[35]や血管内皮前駆細胞[36]を投与した場合にも同様の効果が認められており、どのような細胞分画を投与するのが臨床的に最も効果的かについてはさらに検証が必要である。

肝線維症治療の問題点と将来展望

１）今、なぜ線維化研究が注目されているのか？

近年、肝線維化研究と線維症治療薬開発の試みが産学をあげて活発になってきたのには、いくつかの理由がある。第１に、C型ならびにB型慢性肝炎に対する治療法の進歩により、近い将来にNASHが慢性肝疾患の主因となることが予想されるなど、わが国における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、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改善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２に、ウイルス性肝炎に対する治療法の進歩は、肝線維症が可逆的な病態であることを証明した[37]。これまで、アルコール性肝硬変の禁酒症例などにおいて日常臨床で感じていた肝線維症の可逆性が実証された意義は大きい。組織におけるコラーゲン含量は合成と分解のバランスの上に成り立っており、コラーゲン合成を促進させる原因を取り除くことによって、進行した肝線維症であっても組織学的に改善する。また、原因治療が困難な場合であっても、コラーゲンの合成を抑制する、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。

第３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。血中線維化マーカーに加えて、超音波装置やMRを用いた肝の弾性度診断が可能になった[38][39]。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、全症例に対して治療前後で実施することは現実的でない。弾性度診断もまだ完全とは言えないが、肝線維化治療薬の開発やその臨床治験において、多数例の中から比較的均一な対象集団を設定し、比較対照試験により治療効果を判定する上で、大きな力となることが期待されている。また、組織学的な線維化のスコア化が、コラーゲン合成と分解のダイナミッ

表１　肝線維症に対する抗線維化治療の試み

１）抗線維化効果が認められたもの

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)
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)
B型慢性肝炎	ラミブジン	Kweon YO. *J Hepatol* (2001) Liaw YF. *N Engl J Med* (2004)
自己免疫性肝炎	ステロイド、アザチオプリン	Dufour JF. *Ann Intern Med* (1997)
原発性胆汁性肝硬変	ウルソデオキシコール酸	Corpechot C. *Hepatology* (2000)
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)
NASH	ピオグリタゾン	Aithal GP. *Gastroenterology* (2008)

２）抗線維化効果が明らかでないもの

疾患名	薬剤名	文　献
NASH	グリタゾン製剤、ビタミンE	Ratziu V. *Hepatology* (2010) Sanyal AJ. *N Engl J Med* (2010)
C型慢性肝炎	IL-10	Nelson DR. *Hepatology* (2003)
(非著効例	インターフェロンγ	Pockros PJ. *Hepatology* (2007)
ならびに	グリタゾン製剤	McHutchison J. *Gastroenterology* (2010)
再燃例)	アンギオテンシンⅡ受容体拮抗薬	Abu Dayyeh BK. *Dig Dis Sci* (2011)

クな変化をリアルタイムで反映しているとは言いがたい。最近、血清タンパク質の糖鎖構造の変化が線維化の進展と改善を鋭敏に反映することが報告され、新たな血中線維化マーカーとして期待されている[40]。

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、進行した線維肝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症治療が模索されており、前述した自家骨髄細胞を用いた肝硬変治療法の開発はその好例である。

２）肝線維症治療薬の現状と問題点

これまで種々の慢性肝疾患に対して用いられてきた治療法の中で、C型慢性肝炎のインターフェロン療法やB型慢性肝炎のラミブジン治療など、原因療法が奏功した症例における肝線維化の改善効果は顕著である（表１）。また、自己免疫性肝炎に対する免疫抑制療法や、原発性胆汁性肝硬変症に対するウルソデオキシコール酸治療でも、線維化の改善が認められた。一方、現在最も注目を浴びているNASHについては、その病態形成に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関わらず、ピオグリタゾン投与の有効性を示した一部の報告を除くと、グリタゾン製剤やビタミンE投与の肝線維化抑制効果は概して否定的である。また、ウイルス学的著効が得られなかったC型慢性肝炎症例に対してインターフェロンγやグリタゾン製剤の投与が試みられたが、いずれも無効であった。さらに、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、長期投与では充分な線維化抑制効果は得られなかった（表１）。

このように、いわゆる「線維化治療薬」が奏功しない理由としては、薬効自体の問題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、様々な進行速度を有す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年前後という短期の比較対照試験で評価することの困難さである。感度ならびに特異度に優れた肝線維化の進展と改善の診断方法、しかも線維化の程度（fibrosis）ではなく、ダイナミックな合成系（fibrogenesis）や分解系（fibrolysis）の非侵襲的診断法の開発が期待される。米国では肝臓病学会と食品医薬品局（Food and Drug Administration, FDA）が合同ワークショップを開催し、抗線維化効果を評価する臨床研究デザインの確立とエンドポイントの設定に一丸となって取り組んでいる。わが国においても、この問題に関する強い認識と早急な対応が必要である。

おわりに

紙面の関係で、近年における肝線維化研究の進歩の全てを紹介することはできなかったが、冒頭に述べたように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。培養細胞を用いた*in vitro*試験や動物実験によって数多くの抗線維化作用物質が同定・報告されていながら、なぜ臨床で充分な効果を発揮できないのか。どのような患者を線維化治療の対象に選んで、どのような評価系を構築すべきか。コラーゲン産生細胞特異的に薬剤を到達させて副作用を軽減するには、さらにどのような工夫が必要か。肝線維症治療薬の一刻も早い臨床応用に向けて、肝臓専門医と創薬研究者に託された課題は大きい。

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Original Article

Enhanced survival of mice infused with bone marrow-derived as compared with adipose-derived mesenchymal stem cells

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Aim: Less invasive therapies using mesenchymal stem cells (MSC) are being developed to treat patients with severe liver cirrhosis. MSC constitute a promising cell source for regenerative therapy and are frequently isolated from bone marrow (BMSC) or adipose tissue (ASC). Therefore, this study assessed the characteristics of these two cell types and their safety for cell infusion.

Methods: *In vitro*, exhaustive genetic analysis was performed using human (h)BMSC and hASC. Subsequently, the expression of mRNA and protein was evaluated. *In vivo*, mouse (m)BMSC or mASC was infused into serial mice via the peripheral vein, and 24-h survival rate, prothrombin time and cause of death were analyzed.

Results: On polymerase chain reaction, western blotting, enzyme-linked immunoassay and fluorescence-activated cell sorting, tissue factor was found to be expressed at higher levels in hASC than in hBMSC. Prothrombin time in mice infused with mASC (>120 s) was markedly longer than that of untreated mice (6.5 ± 1.7 s) and that of mice infused with BMSC (6.7 ± 0.8 s) ($P < 0.001$), indicating that pro-coagulation activity was potently enhanced after ASC infusion. The 24-h survival rates in the mASC- and mBMSC-infused groups were 46.4% (13/28) and 95.5% (21/22), respectively; in the former, the rate decreased with increasing number of infused mASC. This cell number-dependent effect was not observed with mBMSC. A histopathological analysis of mice that died immediately following mASC infusion revealed multiple thrombi in the blood vessels of the lungs.

Conclusion: These results indicate that BMSC are a superior and safer cell source for regenerative therapy.

Key words: matrix metalloproteinase, mesenchymal stem cell, pro-coagulation, tissue factor

INTRODUCTION

REGENERATIVE THERAPY IS a potential cure for organ failure patients. We have previously reported on the efficacy and safety of autologous bone marrow cell infusion (ABMi) therapy,[1] which has shown promise as a therapeutic strategy for treating liver cirrhosis.[2,3] However, current ABMi therapy requires the collection of bone marrow by aspiration under general anesthesia, so there are strict criteria regarding the general health of patients. This prompted us to develop a cell therapy approach using mesenchymal stem cells (MSC) for liver regeneration, which have both healing and immunosuppressive properties.[4] MSC can be derived from bone marrow (BMSC) and adipose tissue (ASC). Although they have similar features, their full characterization can help to determine the optimal source of MSC and the most effective cell type for treating human diseases. The present study compares the safety of infusions using BMSC and ASC. The results showed an enhanced pro-coagulation activity associated with ASC, indicating that BMSC are the more attractive option for cell therapy.

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METHODS

Animals

C57BL/6 WILD-TYPE MICE (SLC Japan, Shizuoka, Japan) were used for experiments. All animal studies were performed in accordance with the institutional

guidelines set forth by the Animal Care Committee of Yamaguchi University.

BMSC and ASC

Human (h)BMSC and hASC derived from six different donors were purchased from Lonza (Basel, Switzerland): BMSC1 (22-year-old man; 1F4287), BMSC2 (21-year-old man; 7F3915), BMSC3 (20-year-old man; 8F3520), ASC1 (26-year-old woman; 1F3651), ASC2 (52-year-old woman; 0F4505), and ASC3 (38-year-old woman; 1F3737). Cells were seeded in 10-cm culture dishes and incubated overnight in a humidified incubator at 37°C and 5% CO_2. The following day, the medium was replaced to remove floating cells. Adherent cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal blood serum (FBS) and penicillin/streptomycin (both from Gibco, Grand Island, NY, USA) on non-coated dishes (Becton Dickinson, Franklin Lakes, NJ, USA) until they reached 80% confluence, and were then detached with 0.05% trypsin-ethylenediaminetetraacetic acid (tripsin-EDTA; Life Technologies) and replated. Mouse (m)BMSC derived from C57BL/6 wild-type mice were purchased from Cyagen Biosciences (Santa Clara, CA, USA), and mASC were obtained from the inguinal subcutaneous adipose tissue of C57BL/6 wild-type mice. Tissue was placed in DMEM with 0.03% collagenase (Wako Pure Chemical Industries, Osaka, Japan), resuspended and centrifuged at 500 *g*. The pellet was resuspended and cultured, and cells from passage 2 or 3 were used for assays. The potential of hBMSC, hASC, mBMSC and mASC to differentiate into adipogenic, chondrogenic and osteogenic lineages was confirmed by oil red O, alkaline phosphatase and Alcian blue staining, respectively (Figs S1,3).

DNA-chip analysis

We compared DNA expression between hBMSC and hASC using the DNA-chip system (Agilent Technology, Santa Clara, CA, USA) and analyzed the expression pattern using the IPA software system (Ingenuity Systems, Redwood City, CA, USA).

Polymerase chain reaction (PCR) and quantitative real-time (qRT)–PCR

Total RNA was extracted from hBMSC and hASC with ISOGEN reagent (NipponGene, Tokyo, Japan) according to the manufacturer's instructions. Contaminating genomic DNA was removed using a DNase kit and RNA was purified with the RNeasy MinElute Cleanup kit (both from Qiagen, Tokyo, Japan), in accordance with the manufacturer's protocols. First-strand cDNA was synthesized using the ReverTra Ace-α system (Toyobo, Osaka, Japan) from 500 ng of total RNA, in accordance with the manufacturer's instructions. Gene expression was evaluated by qRT–PCR using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and TaKaRa ExTaq DNA polymerase (Takara Bio, Shiga, Japan) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Thermal cycling conditions for PCR were 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; the conditions for qRT–PCR were 95°C for 3 s and 60°C for 30 s, until the fluorescence signal exceeded the threshold. The following primer sequences were used: human tissue factor (TF), 5′-CAG ACA GCC CGG TAG AGT GT-3′ (forward) and 5′-CCA CAG CTC CAA TGA TGT AGA A-3′ (reverse); mouse TF, 5′-TCC AGG AAA ACT AAC CAA AAT AGC-3′ (forward) and 5′-CCC ACA ATG ATG AGT GTT TCT C-3′ (reverse); human β-actin, 5′-CCA ACC GCG AGA AGA TGA-3′ (forward) and 5′-CCA GAG GCG TAC AGG GAT AG-3′ (reverse); and mouse β-actin, 5′-TGA CAG GAT GCA GAA GGA GA-3′ (forward) and 5′-GCT GGA AGG TGG ACA GTG AG-3′ (reverse). The identity of MSC was confirmed by analyzing the expression of cluster of differentiation (CD) cell surface markers using previously described primer sequences.[5]

Western blotting and enzyme-linked immunosorbent assay (ELISA)

The hBMSC, hASC, mBMSC and mASC samples were homogenized in 400 μL of cell lysis buffer consisting of *n*-octyl-β-D-glucopyranoside dissolved in radioimmunoprecipitation buffer to a final concentration of 20 mM. Protein samples were mixed with an equal volume of loading buffer consisting of 5% 2-mercaptoethanol and 95% Laemmli sample buffer, heated at 100°C for 3 min, and separated on a 12% polyacrylamide gel. Proteins were transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA, USA), which was blocked for 30 min in blocking buffer (0.1% I-Block in phosphate-buffered saline with Tween [PBST]) and was then washed with PBST before incubation for 12–16 h at room temperature with primary antibodies against TF (Abcam, Tokyo, Japan) and β-actin (Becton Dickinson) in blocking buffer. Membranes were incubated for 30 min at room temperature with appropriate secondary antibodies, and protein bands were detected by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) and autoradiography according to the manufacturer's instructions. TF protein expression in hBMSC and hASC was quantified using the Tissue Factor Human ELISA Kit (Abcam) according to the manufacturer's protocol. Samples were the same as those used for western blot analysis.

Fluorescence-activated cell sorting analysis

Adherent cells were dissociated with 0.05% trypsin-EDTA and resuspended in DMEM containing 10% FBS. Cells were washed with PBS (Life Technologies) and incubated in PBS containing 10% Fc receptor blocking reagent (MACS Miltenyi Biotec, Cologne, Germany) for 20 min on ice, followed by labeling for 20 min on ice with a monoclonal antibody against TF (R&D Systems, Minneapolis, MN, USA); phycoerythrin-conjugated antibodies against CD11b, CD44, CD45, CD90 (Beckman Coulter, Brea, CA, USA), CD105 (eBioscience, San Diego, CA, USA) or CD73 (R&D Systems); or an fluorescein isothiocyanate-conjugated antibody against CD34 (BD Biosciences, San Jose, CA, USA). Isotype-matched immunoglobulin G was used as controls. Flow cytometry was performed using a Gallios system (Beckman Coulter). Cells were also stained with propidium iodide (Sigma-Aldrich, St Louis, MO, USA) to exclude dead cells from the analyses. Each sample was evaluated at least three times. Data were analyzed using Kaluza (Beckman Coulter) or FloJo (Tree Star, Ashland, OR, USA) software.

Clotting assay

Adherent cells were dissociated with 0.01% trypsin-EDTA diluted in PBS. After resuspension in DMEM, cells were washed with ice-cold PBS and HBSA (20 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 0.02% NaN_3; and 1 mg/mL bovine serum albumin). Cell suspensions were frozen at −80 °C and thawed at 37 °C. This lysis step was repeated over three cycles, and 100 μL of cell lysates were transferred to a coagulometer cuvette and mixed with 100 μL of pooled normal human plasma (George King Bio-Medical, Overland Park, KS, USA), to which 100 μL of 25 mM $CaCl_2$ was added. Clot formation time was measured from the moment of $CaCl_2$ addition using a KCl Delta semiautomatic coagulation analyzer (Tcoag, Bray, Ireland).

Prothrombin time

Blood was obtained from cell-infused mice; 3.2% Na citrate was immediately added and the mixture was centrifuged at 2000 g for 15 min. Serum was stored at −80 °C. The procedure was performed using silicone-coated tubes (Sarstedt, Tokyo, Japan), and prothrombin time was measured (Monolis, Tokyo, Japan) using the manufacturer's protocol.

Cell transplantation

Cultured mBMSC and mASC were dissociated into single cell suspensions by trypsinization. Cells were resuspended in PBS and 200 μL (3.0×10^4–3.0×10^6 cells) were carefully infused into the tail vein of syngenic C57BL/6 mice using a 27-G needle.

Statistical analysis

Significant differences were tested with Student's *t*-test. Differences were considered statistically significant at $P < 0.05$. Data are presented as means ± standard deviation for triplicate experiments.

RESULTS

THERE WERE NO phenotypic differences between hBMSC and hASC, namely, with respect to cell surface marker and differentiation potential (Figs S1–S3), and the similar morphology of the two types of cell can be seen on differential interference contrast microscopy (Fig. 1a).

Expression of TF, which can trigger pro-coagulation and induce thromboembolism,[6] was analyzed by DNA-Chip in hBMSC and hASC. TF was expressed at higher levels in hASC than in hBMSC in three donors (Fig. 1b); when the change in expression over multiple cell passages was evaluated by qRT–PCR, the upregulation of TF transcript levels in hASC persisted in cells from passages 2, 3 and 4 in all donors (Fig. 1c). TF protein levels were also higher in hASC than in hBMSC (Fig. 2a–c). In addition, coagulation time, which varied as a function of the number of cells, was shorter in hASC than in hBMSC (Fig. 3).

The safety of mBMSC versus mASC infusion was compared in a mouse model. We previously reported that not only MSC but also macrophages can be easily expanded from murine bone marrow cells in culture dishes.[7] Here, we found that mASC were able to differentiate into adipocytes and osteocytes (Fig. S3). Consistent with the findings in human cells, mBMSC and mASC had similar expression profiles (Fig. S4), but TF mRNA was expressed at higher levels in mASC than in mBMSC (Fig. S5).

Cell infusion analysis using mBMSC and mASC showed that the survival rate at 24 h after mASC infusion was 46.4% (Table 1), with most of the mice dying immediately (i.e. within the first 10 min). The survival rate decreased with increasing cell concentrations. In contrast, the 24-h survival rate after mBMSC infusion was 95.5%, and there was no effect of cell number on survival in this group (Fig. 4).

The prothrombin time, a measure of blood clotting, was assessed in blood obtained from mice that died immediately after mASC injection, those that survived beyond 10 min after mBMSC injection and untreated mice. There were no differences in the prothrombin times of mBMSC-infused and untreated mice (6.7 ± 0.8 s vs 6.5 ± 1.7 s; $P = 0.79$);

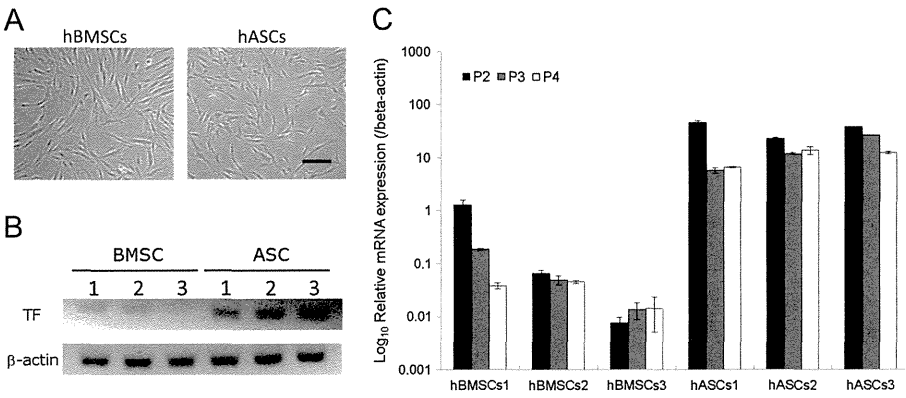

Figure 1 Microscopy of human bone marrow-derived mesenchymal stem cells (hBMSC) and human adipose-derived mesenchymal stem cells (hASC), and tissue factor (TF) mRNA expression in hBMSC and hASC. (a) Similar morphology of the two types of cell can be seen on differential interference contrast microscopy. Scale bar = 200 μm. (b) TF mRNA expression relative to β-actin levels in hBMSC and hASC from three donors for each cell type, as determined by quantitative reverse transcription polymerase chain reaction. TF was expressed at higher levels in hASC than in hBMSC in the three donors. (c) Quantitative analysis of results from (b) (mean ± standard deviation, $n = 3$) in cells from passages 2, 3 and 4 (P2, P3 and P4, respectively). TF transcription in hASC was maintained at high levels even after passage.

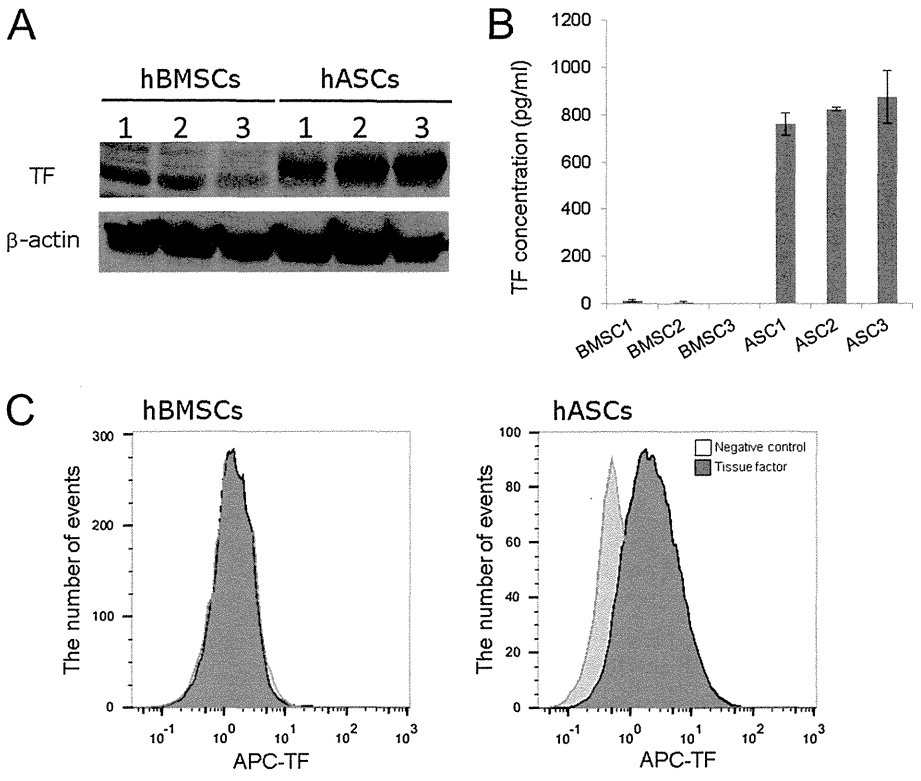

Figure 2 Tissue factor (TF) protein expression in human bone marrow-derived mesenchymal stem cells (hBMSC) and human adipose-derived mesenchymal stem cells (hASC). The 47-kDa TF protein was detected by (a) western blotting and (b) enzyme-linked immunoassay in cells from three donors each. Data represent means ± standard deviation. (c) TF protein expression on the cell surface was analyzed by flow cytometry.

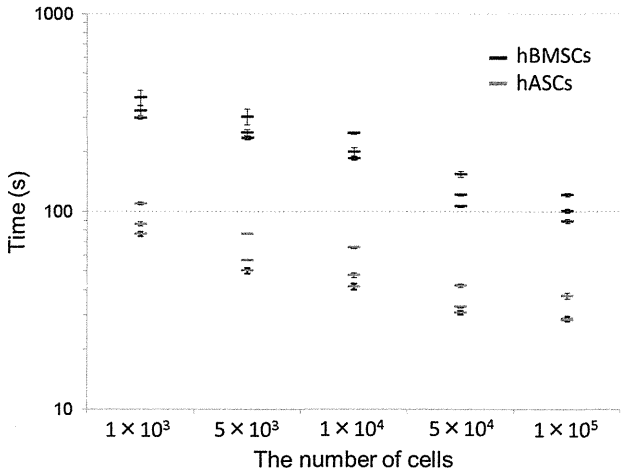

Figure 3 Coagulation time in human bone marrow-derived mesenchymal stem cells (hBMSC) and human adipose-derived mesenchymal stem cells (hASC) using citrated human plasma (pooled). Concentrations ranging 1×10^3–1×10^5 cells were tested. Clotting assay was performed three times for each donor ($n = 3$ for each cell type); data represent means ± standard deviation.

Table 1 Survival rate 24 h after mBMSC or mASC infusion

No. of injected cells	Total mouse number	No. of surviving mice (24 h)	Survival rate (%)
3.0×10^5 mBMSC/mouse	22	21	95.5%
3.0×10^5 mASC/mouse	28	13	46.4%
PBS (same volume)	8	8	100%

ASC, adipose-derived mesenchymal stem cell; BMSC, bone marrow-derived mesenchymal stem cell; PBS, phosphate-buffered saline.

however, the prothrombin time was markedly higher for mASC-infused mice than for untreated mice (>120 s vs 6.5 ± 1.7 s; $P < 0.001$), indicating that pro-coagulation activity was enhanced by mASC infusion (Fig. 5). Histopathological analysis of mice that died immediately following mASC infusion revealed multiple thrombi in the blood vessels of the lungs and liver, which were not observed in the lungs and liver of mBMSC-infused mice (Fig. 6). These results suggest that infusions carry greater risk with mASC than with mBMSC.

DISCUSSION

THIS STUDY COMPARED MSC derived from bone marrow and adipose tissue in terms of their general

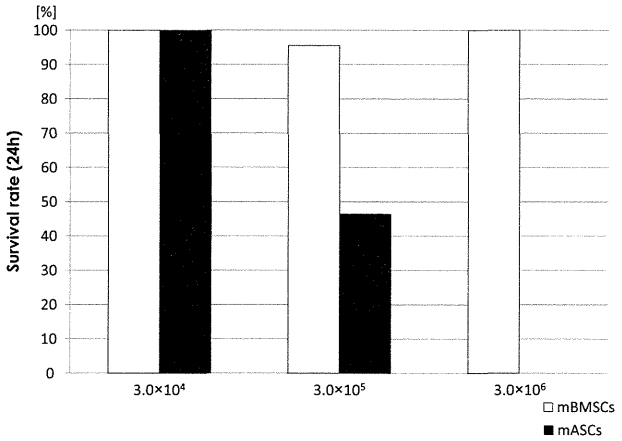

Figure 4 Survival rate in mice infused with mouse bone marrow-derived mesenchymal stem cells (mBMSC) or mouse adipose-derived mesenchymal stem cells (mASC). Survival rate in mASC-infused mice declined markedly as a function of the number of cells infused. On the other hand, mBMSC-infused mice did not show changes in survival rate, regardless of the number of cells infused.

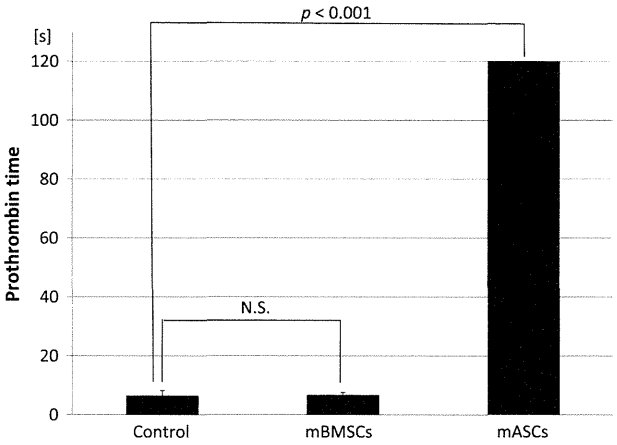

Figure 5 Prothrombin time after mouse bone marrow-derived mesenchymal stem cell (mBMSC) and mouse adipose-derived mesenchymal stem cell (mASC) infusion. Mice were infused through the tail vein and blood clotting was measured in mice that died immediately after mASC injection, those that survived beyond 10 min after mBMSC injection and untreated mice. Prothrombin time in the mASC group was markedly extended, as compared with the other two groups. Data represent means ± standard deviation. N.S., not significant.

characteristics and relative safety for use in infusions. Cell surface marker expression and differentiation capacity were similar in BMSC and ASC; however, TF was differentially expressed and coagulation capacity also varied between the two cell types. Despite the absence of any obvious phenotypic differences between hBMSC and

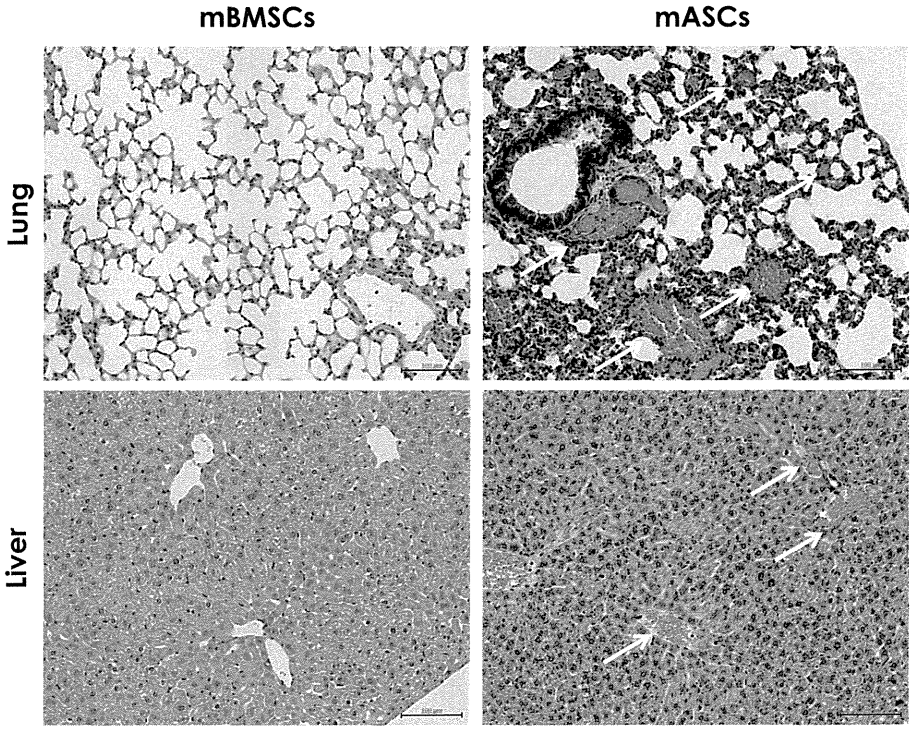

Figure 6 Histopathological analysis of lung and liver tissues after mouse bone marrow-derived mesenchymal stem cell (mBMSC) and mouse adipose-derived mesenchymal stem cell (mASC) infusion. Mice infused with mASC had thrombi in blood vessels (arrows), as seen by hematoxylin–eosin staining of lung and liver tissue sections. In contrast, thrombus was not observed in mice infused with mBMSC. Scale bar = 100 μm.

hASC, TF gene expression was consistently observed in hASC. In addition, TF protein levels were higher in ASC, and were accompanied by a shorter coagulation time than that of BMSC. These results indicate that ASC are distinguished by TF expression, which promotes clotting activity. The survival rate at 24 h was lower and prothrombin time was extended in mASC-infused mice, which also died at a higher rate after infusion. TF is known to stimulate the downstream extrinsic coagulation pathway by forming a compound with factor VII that activates factor VII.[6] Therefore, enhanced pro-coagulation activity by higher expression of TF on the ASC cellular membrane induced multiple thrombi in the peripheral pulmonary and hepatic tissue, inducing deadly multiple organ thromboembolisms. Moreover, prothrombin time is thought to have increased, as extrinsic coagulation factor is consumed in the above process.

We previously demonstrated the safety and effectiveness of ABMi therapy;[2] we also showed the potential for BMSC to improve liver fibrosis and functioning by carbon tetrachloride induction in non-obese diabetic/severe combined immunodeficient mice. These results provide a basis for the development of new types of cell therapy using BMSC.[8,9] Importantly, as the number of transplanted cells may represent an improvement in the efficacy of cell-based therapy, the present data clearly show that BMSC represent a better candidate for this purpose.

In conclusion, the present findings demonstrate that BMSC are a better option than ASC for peripheral vein infusion and provide evidence supporting the use of BMSC for cell therapy in mice.

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Status and Prospects of Liver Cirrhosis Treatment by Using Bone Marrow-Derived Cells and Mesenchymal Cells

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In 2003, we started autologous bone marrow cell infusion (ABM*i*) therapy for treating liver cirrhosis. ABM*i* therapy uses 400 mL of autologous bone marrow obtained under general anesthesia and infused mononuclear cells from the peripheral vein. The clinical study expanded and we treated liver cirrhosis induced by HCV and HBV infection and alcohol consumption. We found that the ABM*i* therapy was effective for cirrhosis patients and now we are treating patients with combined HIV and HCV infection and with metabolic syndrome-induced liver cirrhosis. Currently, to substantiate our findings that liver cirrhosis can be successfully treated by the ABM*i* therapy, we are conducting randomized multicenter clinical studies designated "Advanced medical technology B" for HCV-related liver cirrhosis in Japan. On the basis of our clinical study, we developed a proof-of-concept showing that infusion of bone marrow cells (BMCs) improved liver fibrosis and sequentially activated proliferation of hepatic progenitor cells and hepatocytes, further promoting restoration of liver functions. To treat patients with severe forms of liver cirrhosis, we continued translational research to develop less invasive therapies by using mesenchymal stem cells derived from bone marrow. We obtained a small quantity of BMCs under local anesthesia and expanded them into mesenchymal stem cells that will then be used for treating cirrhosis. In this review, we present our strategy to apply the results of our laboratory research to clinical studies.

Introduction

THE USE OF SOMATIC STEM CELLS in regenerative medicine may help in the development of new treatments for currently intractable diseases. Since November 2003, we have conducted clinical studies in patients with decompensated liver cirrhosis and have supported the development of a liver regeneration therapy using bone marrow cells (BMCs) for autologous BMC infusion (ABM*i*) therapy, a new treatment for liver failure. In addition, aiming to expand the application of the treatment, we are currently conducting research and development studies of a liver regeneration therapy based on cultured mesenchymal cells. In this review, we report on the status of research studies in the field and discuss future challenges in this area.

What is liver cirrhosis?

The liver is composed of a variety of cells such as hepatocytes, cholangiocytes, stellate cells, Kupffer cells, and endothelial cells. Although the liver is an organ with a high regenerative capacity, sustained and chronic inflammation results in the onset of liver fibrosis and the development of cirrhosis when the hepatocytes surrounded by fibrous tissue

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can hardly proliferate, which markedly reduces regeneration and promotes liver dysfunction and cirrhosis. In addition, the risk of liver carcinogenesis increases with the advance of liver cirrhosis. Globally, more than half of the cirrhosis cases are attributable to hepatitis B and C virus (HBV and HBC, respectively) infections; HCV infection is the most common cause in Japan, where approximately 300,000 cirrhosis cases are currently diagnosed. There is, therefore, an urgent need to develop new therapeutic strategies aimed at replacing living donor liver transplantation, which is mostly used for cirrhosis treatment in Japan, contrary to the Western countries where livers from brain-dead donors are used.

Developing improved methods for the administration of BMCs to treat liver fibrosis

A previous study confirmed the presence of Y-chromosomes in the liver tissues of a female leukemia patient who had undergone transplantation of hematopoietic BMCs from a male donor.[1] This groundbreaking finding revealed the existence of cross talk between BMCs and liver tissue, indicating that BMCs were able to fuse with or transdifferentiate into albumin-producing hepatocytes. These results suggested that BMCs might represent a new cell source to repair liver cirrhosis. To investigate the feasibility of the ABM*i* therapy for cirrhosis patients, we developed a mouse green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model where mild cirrhosis induced by administration of CCl_4 was treated by infusion of BMCs fluorescently labeled with the GFP. Our results confirmed that in cirrhotic livers with persistent liver damage, BMCs administered through peripheral blood differentiated into Liv2-positive hepatoblasts and albumin-producing hepatocytes; in addition, the activation of the surrounding A6-positive hepatoblasts was induced.[2,3] Furthermore, we found that liver fibrosis could be improved by the activity of donor-derived BMCs that migrated into the fibrous area of the cirrhotic liver where they expressed matrix metalloproteinase (MMP)-9, an enzyme capable of degrading the fibrotic tissue. As a result, mice with BMC transplants showed liver regeneration and significant improvement in liver function and survival rate.[4] Currently, the beneficial effects of MMPs on hepatic fibrosis and liver function are important in liver repair and regeneration, and there have been several reports indicating that adenoviral delivery of MMPs into the liver ameliorated experimental liver cirrhosis.[5–7] In addition, the analysis of our GFP/CCl_4 model together with the data obtained from human patients showed that the administered BMCs represented a heterogeneous cell population.[2,4] Identification of the cell types that are involved in liver regeneration and repair will be an important step in the development of next-generation treatments for cirrhosis. By now, it is established that administration of these cells clearly improves fibrosis in the cirrhotic liver and may stimulate proliferation of liver progenitor cells and hepatocytes, ultimately leading to the induction of liver regeneration (Fig. 1).

The status of the ABMi therapy

Based on the results of our basic research, we initiated clinical studies involving liver cirrhosis patients ranging from 18 to 70 years (all the participants provided informed consent).

The participants were patients with total bilirubin levels of 3 mg/dL or lower, platelet counts of 50,000/μL or higher, and not showing hepatocellular carcinoma as detected using MRI and CT scans. Bone marrow fluid (400 mL) was collected under general anesthesia; the cells were washed, and the mononuclear cell components were harvested. The end product was administered through a peripheral vein, and follow-up observations were subsequently conducted.[8] This clinical study, initiated at Yamaguchi University in 2003, was, to our knowledge, the first in the world to show that cirrhosis and liver function in patients could be improved without severe adverse effects.[8] Where possible, the biopsy specimens were analyzed, revealing proliferative PCNA-positive hepatocytes in the liver. The results confirmed that the BMC administration induced proliferation of endogenous hepatocytes. Meanwhile, in a joint research project involving Yamaguchi and Yonsei Universities, the ABM*i* therapy was conducted in 10 patients with Child–Pugh B cirrhosis, and significant improvements in the liver function and in the Child–Pugh score were observed. The therapeutic effect was maintained for 12 months. Liver biopsies performed over time confirmed activation of the liver progenitor cell fraction. An additional 20 patients have received the ABM*i* therapy in Yonsei University.[9] In another collaborative effort between Yamagata and Yamaguchi Universities, the ABM*i* therapy was conducted in six patients with alcoholic cirrhosis and a significant improvement in the Child–Pugh score was observed, revealing the benefits of the ABM*i* therapy in treatment of alcoholic cirrhosis. This clinical study also demonstrated the activation of the bone marrow after the ABM*i* therapy confirmed by scintigraphy.[10] Furthermore, a multicenter clinical study on the ABM*i* therapy was conducted in five HIV/HCV coinfected cirrhotic patients, and in three patients with cirrhosis due to nonalcoholic steatohepatitis (Fig. 2). A comparison of laboratory and clinical research data showed that the BMC administration caused rapid changes in the blood levels of granulocyte colony stimulating factor and interleukin-1β.[11] This phenomenon may be attributable to BMCs, which are normally absent in the body, so their rapid administration can induce changes in the host cytokine dynamics. We have previously shown successful treatment based on the administration of autologous BMCs providing a proof-of-concept for the ABM*i* treatment of liver cirrhosis. This treatment aimed at activation of the hepatic progenitor cells and induction of hepatocellular growth through peripheral administration of BMCs, which should result in improvement of fibrosis in cirrhotic livers (Fig. 2).

Our analysis has shown that patients treated with a combination of splenectomy and administration of autologous BMCs showed an improved liver function because splenectomy enhanced the repopulation of peripherally administered BMCs into the cirrhotic liver.[12] Recently, a study on the dynamics of radiolabeled mesenchymal cells in humans showed that immediately after administration, the cells settled primarily in the lungs, then in the spleen, and finally in the liver. This is consistent with our findings that splenectomy promoted establishment of a large number of BMCs in the liver.[13]

A clinical study was conducted in China when 527 patients with liver failure due to HBV received the same medical treatment, and among them, a group of 53 patients received BMCs through the hepatic artery, with 105 designated as the

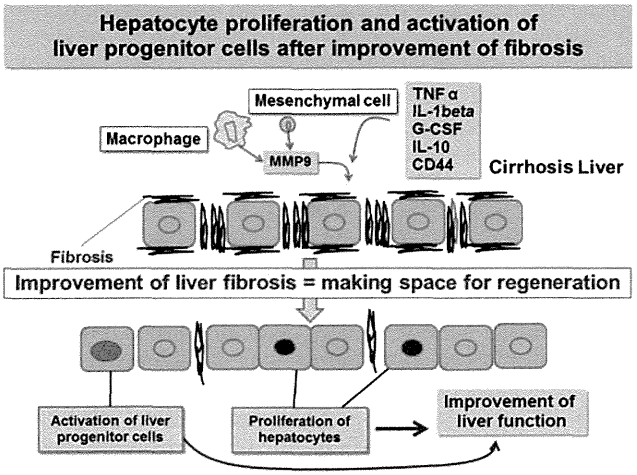

FIG. 1. Mechanism of sequential activation of liver progenitor cells and hepatocytes and improvement of liver fibrosis by BMCs (macrophages and mesenchymal cells).

control group. The analyses showed that the BMC treatment had no side effects. Furthermore, when the patients were divided into early-stage (2–3 weeks) and late-stage (192 weeks) observation groups, improvement of liver function was seen in the early-stage group. In addition, long-term observations suggest that the BMC administration did not increase the incidence of hepatocellular carcinoma.[14] These results are consistent with our findings that frequent administration of BMCs reduces liver carcinogenesis rather than increasing it.[15]

Currently, to substantiate our findings that liver cirrhosis can be successfully treated with transplantation of autologous BMCs, we are conducting randomized multicenter clinical studies designated "Advanced medical technology B" in Japan.

Extending the applications of the ABMi treatment: development of therapeutic methods using the next-generation cultured cells

The current method of the ABM*i*-based therapy consists of administering 400 mL of the bone marrow aspirate under general anesthesia. However, general anesthesia is often inadvisable for individuals with liver failure, which makes the ABM*i* therapy unsuitable for most patients. The problem can be solved when small volumes of bone marrow aspirates are collected under local anesthesia, and fractions of the cells most effective in liver repair and regeneration are expanded and administered to the patients.[16] Figure 3 is a schematic representation of the therapeutic method we are currently developing, which is based on using the cultured BMCs for regenerative treatment of liver cirrhosis. In this method, ~30 mL of bone marrow aspirate is collected under local anesthesia in an outpatient setting and is cultured for about 20 days; the safety is checked before administration to the patient. The development of this therapeutic method and the initiation of the relevant clinical studies are urgently needed, because they will provide treatment options for the patients who thus far have been limited in their choices. A clinical study conducted by Mohamadnejad *et al.* showed that autologous BMCs harvested from four patients

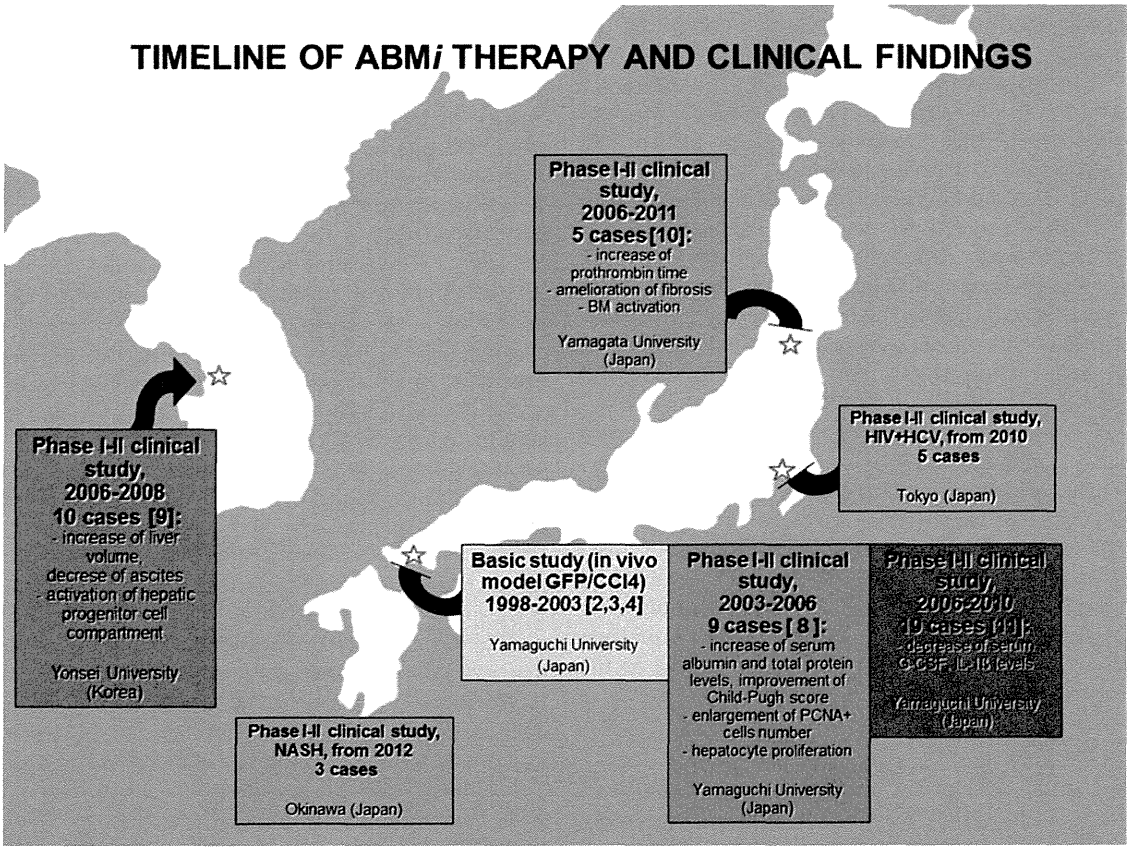

FIG. 2. Timeline of the autologous bone marrow cell infusion (ABM*i*) therapy and clinical findings.