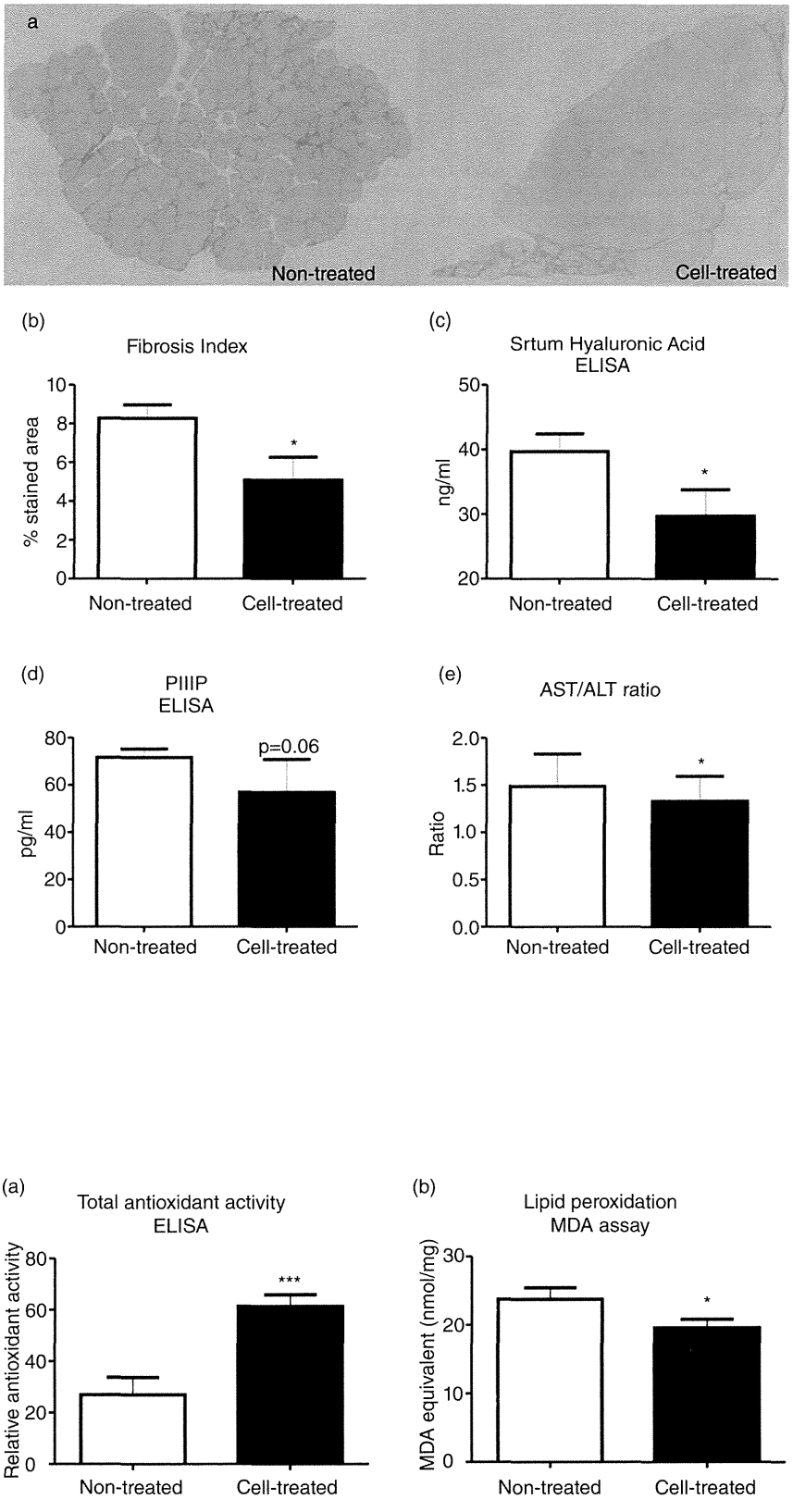

Figure 5 Fibrosis quantification. (a–b) Morphometry analysis of Sirius red-stained liver samples showed that the cell-treated group had lower levels of fibrosis (original magnification ×4). Complementarily, serum collected from cell-treated mice presented lower mean values for (c) hyaluronic acid (HA), (d) procollagen N-terminal peptide (PIIIP) and (e) aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio ($*P < 0.05$).

Figure 6 Antioxidant activity in cell-treated mice. (a) Total antioxidant activity was higher in the cell-treated group compared to the non-treated group ($***P < 0.001$). Additionally, (b) lipid peroxidation in liver tissue was lower in the cell-treated group compared to the non-treated group ($*P < 0.05$).

translocation of redox response elements such as nuclear factor-κB and Nrf2. They suggested that the prosurvival pathways that are activated in MSC *in vitro* could be a part of an adaptive response employed by stromal cells under injury conditions.[65]

A direct and specific effect of ROS in viability was ruled out using H_2O_2 and NAC in cultures. As expected, these molecules increased (H_2O_2) and decreased (NAC) intracellular ROS, but no direct relationship between viability and ROS levels was seen at the time points tested (data not shown). Additionally, to assess whether cMSC could potentially prevent oxidative stress in liver cells, we utilized a co-culture model with murine hepatocytes and cMSC. In this experiment, we found a lower ROS level in co-cultured murine hepatocytes treated with TAA (Fig. 3), suggesting a hepatoprotective effect of cMSC via antioxidant activity. Using a mouse primer for Nrf2 with no cross-reactivity against canine samples *in silico*, we verified the higher amount of mRNA in co-cultured hepatocytes (Fig. S1). However, unexpectedly, monocultured hepatocytes showed higher ROS levels when TAA was absent from the culture medium, suggesting that hepatocytes have a mechanism similar to cMSC in the presence of TAA. The underlying mechanisms are now under investigation.

Our above *in vitro* results motivated us to test cell therapy using cMSC in TAA-induced liver injury in NOD/SCID mice. In chronic TAA-induced injury, the animals that received cMSC infusions by tail vein showed better results for the biochemical parameters. The serum injury markers (ALT, AST and LDH) were reduced with successive cell infusions, suggesting protection of hepatocytes from necrosis and apoptosis (Fig. 4). Because ALT and AST are enzymes that reveal hepatocyte damage, these results strongly support our *in vitro* findings showing that cMSC have hepatoprotective effects against TAA-induced injury. We cannot rule out the possibility that infused cMSC may act systemically to aid the liver in its recovery. Consistent with our results and considering the possibility that Nrf2 may be involved in this process, Xu *et al.*[66] demonstrated a delayed ALT decrease in sera from Nrf2-knockout mice after treatment with hepatotoxin. Because Nrf2 is crucial for induction of expression of a wide range of antioxidant genes, antioxidant activity may be essential for promoting liver regeneration.

As already discussed, oxidative stress plays an important role in liver injury, and some authors have recently demonstrated that cell-based therapy can be an effective treatment. Recently, Cho *et al.* have shown that MSC have an antioxidant potential to ameliorate acute liver injury induced by carbon tetrachloride.[34] In a murine model of carbon tetrachloride-induced acute liver injury, they found increased Nrf2 activity and lower ROS, ALT and AST levels in animals treated with syngeneic MSC.

Okuyama *et al.* reported that transgenic mice with high expression of thioredoxin, a small redox-active protein with antioxidant effects, showed not only ameliorated liver injury but also decreased liver fibrosis.[67,68] Consistent with this result, we showed that the possible antioxidant activity of cMSC reduced necrotic and inflammatory areas (Fig. 4d,e) and fibrosis levels by measuring of different parameters (Fig. 5). We also found higher concentration of matrix metalloproteinase 9 in liver tissues harvested from cell-treated group what can in part explain the results found in fibrosis analyses (Fig. S2).

In this present study, we confirmed that animals in the cell-treated group had better redox homeostasis by showing higher total serum antioxidant activity and lower lipid peroxidation in liver tissues (Fig. 6). The cMSC infusions seemed to sustain normal overall total antioxidant activity in these animals, which may explain the decreased lipid peroxidation (Fig. 6b), serum injury markers (Fig. 4a–c) and histological findings *in vivo* (Figs 4,5). At this juncture, we can clearly see that cMSC can act efficiently in combating oxidative stress in liver.

As far as we know, this study is the first to use a complete approach (*in vitro* + *in vivo*) to evaluate the role of antioxidant activity in ameliorating liver injury using cells from a medium-sized animal. These results reveal potent antioxidant activity and hepatoprotective effects of cMSC *in vitro* and *in vivo* and support more studies examining the antioxidant activity of stem cells to combat liver diseases.

In conclusion, we showed that cMSC can protect hepatocytes by reducing ROS damage induced by TAA both *in vivo* and *in vitro*. These results suggest a potential for MSC treatment in several hepatic diseases.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Figure S1 Relative quantification of NF-E2-related factor 2 (Nrf2) mRNA in hepatocytes in co-culture showed higher values when compared to samples from monoculture under thioacetamide (TAA) condition ($^*P < 0.05$).

Figure S2 Enzyme-linked immunoassay revealed that liver tissues harvested from cell-treated group presented higher concentration of matrix metalloproteinase 9 ($^*P < 0.05$).

14. 肝臓の再生療法

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key words　liver cirrhosis, liver regeneration, stem cell, bone marrow cell, mesenchymal stem cell

動　向

B型またはC型肝炎ウイルスに起因した肝硬変であっても代償期であれば，核酸アナログ製剤やインターフェロンによりウイルスの排除や肝炎鎮静化が可能となり，肝不全や肝癌といった重篤な病態への進展を防ぐことができるようになった．またC型肝炎ウイルス排除を目的としたインターフェロン治療やB型肝炎ウイルスに対する核酸アナログ製剤内服に対する医療費公費助成の整備も進み，C型肝炎ウイルスが経口内服薬のみで排除できる見込みも立ってきた．しかしすでに進行した肝硬変にはインターフェロンの適応はなく，肝炎ウイルス排除後の肝線維化改善にも時間がかかる．また少なくとも現時点でも非代償性肝硬変をはじめとする重症肝疾患の根治療法は肝移植（生体肝移植あるいは脳死肝移植）である．2010年7月以降は改正脳死臓器移植法の施行により「本人が拒否していない限り家族（遺族）の同意で臓器提供ができる」ようになったが，慢性的ドナー不足，手術侵襲，免疫拒絶や医療経済面などの諸問題は解決されておらず，肝硬変に対する有効な再生療法の開発が求められている現状に変わりはない．

1999年にPetersenらがメス骨髄移植ラット肝臓に投与オス骨髄由来細胞が[1]，2000年にはTheiseらが男性ドナーから骨髄移植を受けた女性患者の剖検例において慢性炎症の肝臓および消化管組織内にY染色体陽性細胞が存在していたことを報告したことから，骨髄細胞中には多分化能を有する幹細胞が存在することが示唆された[2]．これ以降，肝臓の再生療法に用いる細胞源として骨髄（幹）細胞が注目され，世界中で基礎・臨床研究が進められている[3-8]．われわれも，骨髄細胞から肝細胞への分化・増殖評価マウスモデル「Green fluorescent protein (GFP) /carbon tetrachloride (CCl_4) モデル；GFP/CCl_4モデル」による基礎研究により自己骨髄細胞投与が肝線維化および肝機能を改善させることを報告し，その成果を基盤に2003年11月から臨床研究「肝硬変症に対する自己骨髄細胞投与療法（ABM*i*療法）」を開始し，2005年度からは多施設臨床研究を推進してきた．これまでに国内外の施設を含めてABM*i*療法の肝機能改善・修復効果を確認し論文報告しており，2013年6月に「C型肝炎ウイルスに起因する肝硬変患者に対するABM*i*療法の有効性と安全性に関する研究（ランダム化比較試験）」が日本初の先進医療Bとして認可された．

一方，その他の肝臓再生療法としては，イギリスではG-CSFで誘導した末梢血CD34陽性細胞を用いた細胞療法，ドイツではCD133陽性単核

球細胞の門脈内投与療法，培養自己骨髄由来細胞を用いた細胞療法などの臨床研究の論文報告がある．本稿では，われわれのABM*i*療法を含む肝硬変症に対する肝臓再生療法の状況や，今後の展望について概説する．

A. 基礎研究；マウスGFP/CCl_4モデル

われわれは，四塩化炭素（CCl_4）による肝細胞直接障害モデルを用いて基礎研究を進めてきた．われわれのマウスGFP/CCl_4モデルの特徴は，1）四塩化炭素の反復投与により慢性肝障害環境下にあること，2）骨髄細胞投与後も四塩化炭素投与を継続しこの炎症環境を維持すること，3）自家骨髄細胞投与を想定して同種同系GFPトランスジェニックマウスをドナーとしたことである[9,10]．また本モデルでは，6週齢のC57BL/6マウスに四塩化炭素（1.0mL/kg）を4週間（計8回）腹腔内反復投与することで慢性肝障害（肝硬変）状態とし，これに同種同系GFPトランスジェニックマウス大腿骨から分離した全骨髄細胞を洗浄後に末梢静脈である尾静脈から投与し，その後も四塩化炭素投与は継続したうえで，経時的に肝機能改善効果を評価している．結果，骨髄細胞を投与することにより，血清アルブミン値の改善，生存率の有意な上昇さらにはシリウスレッド染色で評価した肝線維の減少が認められた[9-11]．投与した骨髄細胞は肝障害がないマウス肝臓には生着しないものの，四塩化炭素による持続肝障害環境下で投与した骨髄細胞は投与後1日目から門脈域周囲の線維に沿って生着し，さらに週を追うごとに既存の線維の中にも観察された[11]．またこの過程で，障害肝に生着した骨髄由来GFP陽性細胞がmatrix metalloproteinase（MMP）9などのコラゲナーゼを産生し，肝線維の溶解に働くことを確認した[11]．これらの基礎研究結果から，慢性肝障害環境下に自己骨髄細胞を末梢静脈から投与することにより，レシピエントの肝合成能・肝線維化さらには生命予後までも改善させたと考えた．さらにその後の検討により，本過程に関与する因子としてFGF2（fibroblast growth factor 2）が促進的に働くことも明らかとなった[12]．さらに高発癌肝硬変マウスに対して骨髄細胞を頻回投与したモデル系において肝発癌は有意に抑制されており，骨髄細胞投与により酸化ストレスが制御されていた可能性が示唆された[13]．

B. 臨床研究

1. 自己骨髄細胞を用いたABM*i*療法

われわれの自己骨髄細胞（非培養）を用いたABM*i*療法の適応条件などの詳細は以下である．

【対象疾患】肝硬変症

【治療適応基準】1）総ビリルビン値3.0mg/dL以下，2）血小板数5.0×10^{10}/L以上，3）食道胃静脈瘤および肝細胞癌コントロール良好，4）心肺機能良好で重篤な併存疾患を認めない，5）CTやMRIなどの画像診断で肝細胞癌が存在しない．

【プロトコール】全身麻酔下に約400mLの自己骨髄細胞を採取し，GMPグレード設備が完備された再生・細胞療法センターでSOP（Standard Operating Procedures）に準じて骨髄単核球細胞を精製濃縮し，平均5.2×10^{9}個の自己骨髄単核球細胞を採取同日に本人の末梢静脈から点滴投与した．細胞投与後は6カ月間経過観察を行い，血液生化学検査，肝組織検査（肝生検），腹部超音波検査や腹部CT検査により安全性および有効性の評価を行った．また経過観察中は，内服薬剤や抗ウイルス剤などの変更は行っていない[14]．

【結果】骨髄細胞投与後6カ月間経過観察可能であった症例において，投与6カ月後の血清アルブミン値，総蛋白値やChild-Pughスコアは有意に改善し[14]，さらに15カ月間経過観察可能であった9例でも同様の改善効果を認めた．なお2013

年7月現在において，特に問題となる有害事象の発生は認めていない[14-16]．さらに2011年には，非代償性B型肝硬変を対象としたABM*i*療法が血清アルブミン値やChild-Pughスコアを改善させたとの報告がKimらによりなされ，そのメカニズムとして経時的な肝生検からhepatic progenitor cell(HPC)を活性化させる可能性が示唆された[17]．さらにアルコール性肝硬変に対するABM*i*療法の有効性と安全性が齊藤らにより報告された[18]．

2. 先進医療B「C型肝炎ウイルスに起因する肝硬変患者に対するABM*i*療法（ランダム化比較試験)」

これまでのABM*i*療法の実績により，「C型肝炎ウイルスに起因する肝硬変患者に対するABM*i*療法の有効性と安全性に関する研究（ランダム化比較試験)」が2011年12月に「ヒト幹細胞を用いる臨床研究に関する指針」の承認を，2013年6月には先進医療Bの認可を受け，今後，実施していく．なお，本ランダム化比較試験の概要は以下のとおりである．

【目的】C型肝炎ウイルスに起因する肝硬変患者に対する自己骨髄細胞投与療法の有効性と安全性の検討

【適格基準】以下の選択基準をすべて満たし，かつ，以下の除外基準のいずれにも該当しない症例を適格症例とする．

【選択基準】

（1）C型肝炎ウイルスに起因する肝硬変症例
（2）90日以上離れた2点において，Child-Pughスコアが7点（Child-Pugh B）以上の状態にあり，現行の内科的な治療法では改善が見込めない症例
（3）20歳以上75歳以下の症例
（4）インフォームドコンセントを取得可能で，研究参加の同意が得られた症例

【除外基準】

（1）C型肝炎ウイルス以外の原因で肝硬変へ至った症例，もしくは肝硬変へ至った原因が不明の症例
（2）悪性新生物を合併する，または既往を有する症例
（3）破裂の危険性を有する食道・胃静脈瘤を有する症例
（4）血清クレアチニン値 2mg/dL以上の腎機能障害を合併する症例
（5）ヘモグロビン値が 8g/dL未満，あるいは血小板数が 50,000/μL未満の症例
（6）血清総ビリルビン値が3.0mg/dL以上の症例
（7）Performance Status 3あるいは4の症例
（8）同種血輸血に関する同意を得られない症例
（9）B型肝炎ウイルス感染症，ヒト免疫不全ウイルス感染症，成人T細胞白血病ウイルス感染症，パルボウイルスB19感染症が否定できない症例
(10) 妊娠中の女性
(11) 全身麻酔を行うことが適切でないと担当医が総合的に判断した症例
(12) 造影剤に対する重篤なアレルギーのある症例もしくは造影剤に対する重篤なアレルギーの既往を有する症例
(13) その他，担当医が不適当と判断した症例

【主要評価項目】細胞投与群は細胞投与後，標準的治療群は登録後24週の時点でChild-Pughスコアの1点以上改善する割合

【副次評価項目】細胞投与群は細胞投与後，標準的治療群は登録後24週の時点の以下の指標を副次エンドポイントとする．

（1）効果維持率の推移（効果維持の定義：Child-Pughスコアが悪化しない）
（2）血清アルブミン値の推移
（3）血清線維化マーカー値の推移

（4）腹水量の推移
（5）下腿浮腫の改善率及び消失率の推移
（6）自覚症状の推移
（7）有害事象の発生頻度
【有害事象・重大な事態の評価】有害事象とは，臨床研究参加期間中（同意取得時～プロトコル治療後観察期終了もしくは中止時）に被験者に生じたあらゆる好ましくない，意図しない徴候（臨床検査値の異常変動を含む），症状又は病気をいい，当該プロトコル治療との因果関係の有無は問わない．

3. ABM*i*療法以外の臨床研究

まずG-CSF（granulocyte-colony stimulating factor）を使った肝硬変に対する臨床研究論文としては，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[19]，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[20]，Child-Pughスコアを改善させたとのPaiらの報告や[21]，HanらによるB型肝硬変症に対するG-CSF投与の報告がある[22]．しかしながら，G-CSF投与により健常人でも脾破裂を起こしたとの報告があることから，脾腫を伴う肝硬変症例へのG-CSF投与には注意が必要である[23]．最近では，Spahrらが非代償性アルコール性肝硬変症を対象にG-CSF投与しその後に採取した自己骨髄単核球細胞を投与したものの，標準治療群であるステロイド投与群と同等の改善であったと報告している[24]．またG-CSFを使用せず200mLの自己骨髄液から分離した濃縮CD34陽性細胞を肝動脈から投与するPhase I臨床研究の報告があるが，本臨床研究では造影剤投与が原因と考えられる腎不全による死亡例の報告がある[25]．このことは，投与細胞の種類，細胞濃度や投与速度を至適化することの重要を示している．

その他にはドイツから，肝悪性腫瘍切除術後の残肝に自己CD133陽性細胞を経門脈投与する細胞療法の有効性も報告されている[26,27]．

一方，ABM*i*療法と同様に自己骨髄細胞を用いる肝臓再生療法には以下のような論文報告がある．PengらはB型肝硬変症を対象に120mLの自己骨髄単核球細胞（間葉系幹細胞を含む）を肝動脈から投与する群（n=53）と，投与しないコントロール群（n=105）で解析した．その結果，骨髄投与により副作用はなく，早期には肝機能の改善を確認した．また長期観察では，骨髄細胞投与後の肝細胞癌の発生率は低い傾向で（p=0.107），われわれの高発癌肝硬変マウスと矛盾しない結果であった[28]．その他，ブラジルのLyraらによる，肝移植待機例10例に対して腸骨から採取した自己骨髄単核球細胞を肝動脈から投与したところ血清アルブミン値の上昇と血清ビリルビン値の低下を認め，投与経路は末梢静脈より肝動脈投与が有効であったとの報告がある[29,30]．

さらには，培養骨髄由来細胞を用いた臨床研究の論文報告もある．少量の骨髄液から肝再生・修復作用を有する細胞を分離培養し再投与することができれば，全身麻酔下に骨髄液を採取する必要がなくなるため適応は拡大し，さらには凍結保存した培養細胞を分割投与することも不可能ではなくなり，患者負担は大きく軽減される．まず2007年にMohamadnejadらはPhase I臨床研究として4例の非代償性肝硬変症に対して，平均3.2×10^7個の培養自己骨髄間葉系幹細胞を末梢静脈から投与し，うち2例でMELD（the model for end-stage liver disease）スコアが改善したと報告している[31]．さらにKharazihaらは8例の肝硬変症に対して，局所麻酔下に腸骨から採取した約20mLの骨髄液から単核球をフィコール法で分離し約2週間通常培養した．この培養細胞はCD44/CD73/CD105（間葉系細胞マーカー）陽性で，3～5×10^7個を末梢静脈または門脈から

表1　論文報告されたおもな肝臓再生療法

投与細胞の種類	投与細胞数	改善効果	対象症例	文献番号
CD34陽性細胞 (G-CSF 誘導末梢血由来)	$1\times10^6\sim2\times10^8$	血清アルブミン・ ビリルビン改善	5 Alcohol	19
5日間G-CSF投与のみ	記載なし	血清HGF上昇 肝Ki67$^+$/CK7$^+$細胞増加	24 Alcohol (うち11コントロール)	20
培養CD34陽性細胞 (G-CSF 誘導末梢血由来)	平均2.3×10^8	血清アルブミン・ Child-Pughスコア改善	9 Alcohol	21
末梢血単核球細胞 (G-CSF 誘導末梢血由来)	$10^7\sim10^8$/kg	血清アルブミン・ Child-Pughスコア改善	40 HBV (うち20コントロール)	22
G-CSF+単核球細胞	4.7 ± 10^7/kg	MELDスコア，肝脂肪化 (標準治療群でも改善)	48 Alcohol (うち30コントロール; ステロイド投与)	24
骨髄単核球細胞; ABM*i*療法	$5.20\pm0.63\times10^9$	血清アルブミン・ Child-Pughスコア改善	5 HCV, 3 HBV, 1成因不明	14
骨髄単核球細胞; ABM*i*療法	$0.48\sim1.48\times10^8$/kg	肝CK7陽性細胞増加 肝容量増加 Child-Pughスコア改善	10 HBV	17
骨髄単核球細胞; ABM*i*療法	$8.0\pm7.3\times10^9$	血清アルブミン・PT% Child-Pughスコア改善	10 Alcohol (うち5コントロール)	18
骨髄単核球細胞(MSC)	記載なし	血清アルブミン・ ビリルビン・PT% MELDスコア改善 HCC増加なし	158 HBV (うち105コントロール)	28
骨髄単核球細胞	$>1\times10^8$	血清アルブミン・ ビリルビン改善	10	29
骨髄単核球細胞	$3.78\pm2.69\times10^8$	血清アルブミン・ ビリルビン・ Child-Pughスコア改善	30 (うち15コントロール)	30
骨髄由来CD133陽性細胞	$2.4\sim12.3\times10^6$	肝切除後の肝容量増加	6肝癌 (うち3コントロール)	26
骨髄由来CD34陽性細胞	平均5.25×10^6 (CD34$^+$, 90.5%)	造影剤による腎不全死亡 (1例)	1 HBV, 1 PBC 1 AIH, 1成因不明	27
培養骨髄由来MSC	平均31.7×10^6	MELDスコア改善 (ただし2例のみ)	3 成因不明, 1 AIH	31
培養骨髄由来MSC	$3\sim5\times10^7$	MELDスコア・ 血清クレアチニン改善	4 HBV, 2成因不明 1 HCV	32
HGF含有培地培養MSC	2×10^8	MELDスコア改善	40 HCV (うち20コントロール)	33

G-CSF, granulocyte colony-stimulating factor; HGF; hepatocytes growth factor; PT, prothrombin time; MSC, mesenchymal stem cell; MELD, Model for End Stage Liver Disease〔Takami T, et al. Curr Opin Gastroenterol. 2012; 28(3): 203-8[7]より改変〕

投与したところMELDスコアが平均17.9から10.7へと改善したと報告している[32]．Amerらは20例のC型肝硬変症から局所麻酔下に腸骨から約120mLの骨髄液を採取し，HGF (hepatocyte growth factor) 含有培地で培養した間葉系幹細胞をエコーガイド下に肝臓または脾臓内へ直接注入し，コントロール20例に比べて有意にMELDスコアおよびChildスコアが投与後2週から6カ月の間は改善したと報告している[33]．今後は，エビデンスレベルの高い有効性を示すためにランダム化比較試験の実施などが求められよう．

なお，以上のおもな肝臓再生療法の概要を表1にまとめているので参照いただきたい[7]．

むすび

これまでの基礎研究や臨床研究成績から，肝硬変症に対する（自己）骨髄細胞を用いた肝臓再生療法の有効性は強く示唆されている．今後は，エビデンスレベルの高い有効性を示すためにランダム化比較試験を推進することや，低侵襲な骨髄由来培養細胞を用いた治療法の開発が必要となっていくであろう．

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Bone marrow cell-based regenerative therapy for liver cirrhosis

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Abstract

Bone marrow cells are capable of differentiation into liver cells. Therefore, transplantation of bone marrow cells has considerable potential as a future therapy for regeneration of damaged liver tissue. Autologous bone marrow infusion therapy has been applied to patients with liver cirrhosis, and improvement of liver function parameters has been demonstrated. In this review, we summarize clinical trials of regenerative therapy using bone marrow cells for advanced liver diseases including cirrhosis, as well as topics pertaining to basic *in vitro* or *in vivo* approaches in order to outline the essentials of this novel treatment modality.

Key words: Bone marrow; Liver regeneration; Cirrhosis; Stem cell; Transplantation

Core tip: Bone marrow cells, which include multipotent progenitor cells, are capable of differentiation into liver cells. Autologous bone marrow infusion therapy has been applied to cirrhotic patients, and improvement of liver function parameters has been demonstrated. Although the efficacy of this treatment modality needs to be evaluated in more detail in a large number of patients, regenerative therapy using bone marrow cells for advanced liver diseases has considerable potential.

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INTRODUCTION

Bone marrow cells (BMCs) are capable of differentiating into liver cells[1-4] because they include stem cells known as multipotent adult progenitor cells[5,6]. These cells have been shown to produce albumin when cultured with hepatocyte growth factor (HGF)[7] and various liver-specific proteins, including albumin, when cultured with mature hepatocytes[8]. Using cells obtained with a negatively selective magnetic cell separation system for efficient sorting of rat BMCs enriched with stem cells, we have shown that BMCs differentiate into cells expressing liver-specific genes when cultured with mature hepatocytes or HGF[9]. As there is now much evidence indicating that BMCs can differentiate into cells resembling liver cells *in vitro*[6-11], the characteristics of such BMCs are of great interest in the context of liver-regenerative medicine[12-14].

Liver cirrhosis is the end stage of chronic liver disease, and is associated with many serious systemic complications resulting from both liver failure and portal hypertension. This condition has a poor prognosis and is difficult to treat. Therefore, development of an effective liver-regenerative therapy for liver cirrhosis is an urgent priority. Liver transplantation is the only curative remedy for cirrhotic patients, but is associated with many problems such as donor shortage, surgical complications,

rejection and high cost. As an alternative approach, regenerative cell therapy using stem cells is now attracting attention. Multipotent stem cells present in bone marrow are a particularly promising candidate for this purpose. In this review, we summarize clinical trials of liver-regenerative therapy using BMCs for advanced liver diseases including cirrhosis, as well as topics pertaining to basic *in vitro* or *in vivo* approaches in order to outline the essentials of this novel treatment modality.

MIGRATION AND ENGRAFTMENT OF TRANSPLANTED BMCs TO THE INJURED LIVER IN STUDIES USING ANIMAL MODELS

Although BMCs can show liver cell lineage differentiation *in vitro*, an understanding of the dynamics of transplanted BMCs *in vivo* is essential for the development of BMC-based regenerative therapy. In this context, two important issues need to be clarified: (1) How do transplanted BMCs migrate to and engraft in the liver? and (2) Is there a relationship between the degree of liver damage and the extent of migration of transplanted cells? A previous study using model rats with carbon tetrachloride (CCl_4)-induced liver injury has demonstrated that transplanted BMCs derived from transgenic rats expressing green fluorescent protein[15] in the spleen migrated to and remained in the periportal area of the recipient's damaged liver[16]. These transplanted cells expressed liver cell markers such as alpha-fetoprotein as well as Notch signaling markers for stem cells, suggesting that the BMCs retained in the recipient liver possess the potential to differentiate into liver cells.

Migration of transplanted BMCs to the liver after injection into the spleen has been compared in two models of liver injury induced by administration of CCl_4 and 2-acetylaminofluorene (2-AAF)[17], respectively, focusing particularly on differences in levels of liver mRNA for growth factors such as HGF and fibroblast growth factor (FGF), which have been shown to be responsible for efficient liver cell lineage differentiation of BMCs[9,18,19]. Interestingly, transplanted BMCs were found to engraft into CCl_4-induced injured liver characterized by submassive hepatic necrosis and induction of high levels of HGF and FGF, but not into liver damaged by 2-AAF[20]. A higher degree of HGF induction is characteristic of more severe liver damage[21,22]. These findings suggest that transplanted BMCs migrate more effectively to a liver with greater damage, and that this transplantation approach would be clinically promising for treatment of advanced liver diseases. However, further studies are needed to clarify the factors produced by both BMCs and hepatocytes that contribute to better differentiation of BMCs into liver cells *in vivo*, thus improving the effectiveness of BMC transplantation.

HUMORAL FACTORS BENEFICIAL FOR LIVER REGENERATION AFTER BMC TRANSPLANTATION

The degree of liver function and fibrosis, as well as survival rate, have been shown to improve significantly after BMC transplantation in animal models of severe liver injury[23,24]. With regard to the mechanisms of liver regeneration resulting from BMC transplantation, many of the physiological and regenerative roles of transplanted BMCs remain unclear. However, it can be said with certainty that humoral factors produced in the liver during the regenerative process after BMC transplantation have a crucial role in both improvement of liver fibrosis and liver cell lineage differentiation of stem cells originating from BMCs and hepatic epithelial stem cells.

Improvement of liver fibrosis results from fibrolysis through the proteolytic action of BMC-induced factors. In this context, matrix metalloproteinase (MMP) activity is particularly noteworthy[25]. Sakaida *et al*[23] showed that BMC transplantation ameliorated liver fibrosis in the CCl_4-induced liver-injury model, and that the fibrolytic change was attributable to MMP-9 secreted by BMCs that had migrated to fibrotic areas of the liver.

The liver cell lineage differentiation of BMCs occurs through the cooperative action of a variety of growth factors such as HGF or FGF induced in the injured liver[11,20,26]. Such differentiation may be accompanied by early elevation of the apolipoprotein A1 level in serum and liver[27]. Administration of FGF2 in combination with BMC transplantation synergistically ameliorates liver fibrosis in models of liver injury induced by CCl_4[28]. In addition, in severe liver injury where hepatocyte proliferation is strongly inhibited, hepatic stem cells such as oval cells are induced and show differentiation toward a liver cell lineage, thus leading to liver regeneration[29,30].

As BMC transplantation is successfully adaptable to cases of severe liver injury, it has been hypothesized that transplanted BMCs interact with hepatic epithelial stem cells and influence the subsequent proliferation and differentiation of stem cells. Studies of the interaction between BMCs and hepatic stem cells can provide new insight into the mechanisms of recovery from severe liver damage through liver regeneration after BMC transplantation. In this context, *in vitro* analysis using a system for co-culture of BMCs and an established epithelial hepatic stem cell line has been conducted. Haga *et al*[31] demonstrated that the expression of *FGF2* mRNA was upregulated in BMCs co-cultured with hepatic stem cells, and that expression of mRNAs for both albumin and tyrosine aminotransferase, representative of mature hepatic cells, became detectable in hepatic stem cells after culture with FGF2 protein. Thus, BMCs stimulate both proliferation and differentiation of hepatic stem cells into the hepatocyte lineage, and FGF2 is one of the factors produced by interaction with BMCs, which stimulates

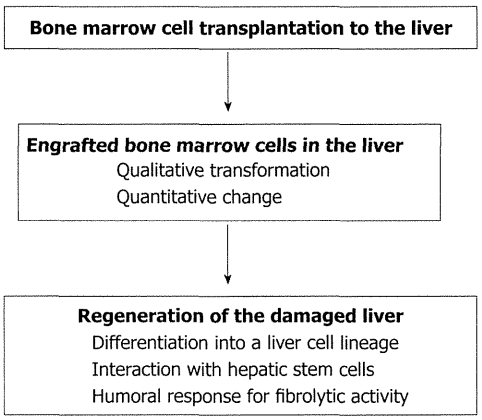

Figure 1 Putative action of transplanted bone marrow cells that include multipotent stem cells for regeneration of damaged liver.

such differentiation. Cross-talk between bone marrow stem cells and hepatic epithelial stem cells may underlie the process of liver regeneration, and this is an area of interest for future investigation. Figure 1 shows an overall representation of the putative action of transplanted BMCs in the regeneration of damaged liver.

CLINICAL TRIALS OF BMC TRANSPLANTATION FOR ADVANCED LIVER DISEASES

BMC transplantation has received increasing attention as a promising therapy for advanced and severe liver diseases such as cirrhosis. Clinical trials of BMC administration to patients with advanced liver diseases have been performed, and improvement of liver function parameters such as the serum level of albumin, Child-Pugh score or Model for Endstage Liver Disease score have been reported[32-40]. Another study has shown that intraportal administration of autologous $CD133^+$ BMCs and subsequent portal venous embolization of right liver segments resulted in a 2.5-fold increase in the mean proliferation rate of the left lateral segment, in comparison with controls not receiving BM transfusion[41]. These findings suggest that transplanted BMCs have a potential role in liver regeneration and proliferate in the recipient liver. Recently, autologous BMC transplantation - a technique named autologous BMC infusion (ABMi) therapy - has been applied to multi-center patients with liver cirrhosis due to hepatitis C[42], hepatitis B[43] and excess alcohol intake[44] using almost the same protocol, and a series of studies have demonstrated improvement of the serum albumin level, leading to improvement of the Child-Pugh score.

Although BMC administration for advanced liver diseases including cirrhosis is an attractive strategy in the field of cell therapy for liver regeneration, many concerns need to be addressed[45-47]. As *in vitro* and *in vivo* experiments have clearly shown, BMCs induce fibrolysis and show hepatocyte differentiation, and they may interact with hepatic epithelial stem cells to aid their differentiation into the hepatocyte lineage. However, it is still unclear how infused BMCs work to improve liver function in humans. A clinical trial of ABMi for patients with cirrhosis demonstrated that the number of AFP-positive cells increased significantly in the liver relative to the situation before ABMi[42]. In addition, ABMi appeared to induce hepatocyte proliferation in the liver, as expression of proliferating cell nuclear antigen, a marker of hepatocyte proliferation, was significantly increased after ABMi in comparison with the pretreatment situation. Although these findings suggest that transplanted BMCs have a potential role in liver regeneration and proliferate in the recipient liver, it remains unknown whether fully functional hepatocytes are induced by ABMi. The characteristics of stem cells present among BMCs that show hepatocyte differentiation require further elucidation.

The factors that determine the difference between effectiveness and non-effectiveness of ABMi are unclear. Collateral circulation resulting from the portal vein disorganization that characterizes liver cirrhosis may affect the flow and effective migration of infused BMCs to the liver, and thus migration of infused cells to the liver may partly depend on the portal venous pressure. In addition, the expression levels of cellular adhesion molecules associated with the attachment of infused cells to liver tissue may vary a great deal among patients. The long-term effectiveness of this therapy in terms of survival rate has not been demonstrated. These issues should be evaluated by a randomized controlled trial involving a large number of patients. Additionally, other issues that impact the efficacy of this therapy, *i.e.*, the long-term culture conditions optimal for stocking BMCs for repeated infusion, the optimal cell population to employ, the optimal number of cells to infuse, the effectiveness of repeated infusion and the optimal route for cell delivery need to be investigated further.

In conclusion, regenerative therapy using BMCs for advanced liver diseases including cirrhosis has considerable potential. Further studies are needed to develop a better method of BMC transplantation that can contribute to improvement of liver function and to clarify the long-term effectiveness of this therapy.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Transmission of hepatitis C virus: Self-limiting hepatitis or chronic hepatitis?

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Abstract

It has been suggested that hepatitis C virus (HCV) is selectively transmitted to a new host as an infectious clone from multiple HCV variants (quasispecies) in the donor. Most individuals with HCV infection develop chronic hepatitis, but approximately 15%-40% of them clear the virus spontaneously and the hepatitis is resolved in a self-limiting manner in the acute phase of infection. This difference in the outcome of acute hepatitis C is attributable to both viral characteristics and genetic regulation of infection. In particular, the evolutionary dynamics of the infecting virus and host genetic polymorphisms pertaining mainly to the immune system, including polymorphisms in the region of the Interleukin 28B gene encoding interferon-λ-3, are associated with susceptibility to HCV infection.

Key words: Hepatitis C; Spontaneous clearance; Interleukin 28B; Single nucleotide polymorphism; Interferon-λ

Core tip: Most individuals with hepatitis C virus (HCV) infection develop chronic hepatitis, but in some the hepatitis is resolved in a self-limiting manner in the acute phase of infection. What factors are responsible for this difference in the outcome of hepatitis C? The evolutionary dynamics of the infecting virus and host genetic polymorphisms pertaining mainly to the immune system, including the Interleukin 28B gene, as well as susceptibility to HCV infection, are important in determining the outcome of infection.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major threat to public health, and about 170 million people are estimated to be infected worldwide with a potential risk of progression to cirrhosis and hepatocellular carcinoma[1,2]. This review summarizes the two current topics of HCV study: the transmission mode of HCV with multiple variants (quasispecies) and the factors associated with susceptibility to HCV infection, with special reference to viral characteristics and host genetic variation.

MODE OF HCV TRANSMISSION: HOW IS HCV WITH MULTIPLE VARIANTS TRANSMITTED?

HCV shows significant genetic heterogeneity among isolates, and the degree of variability is unevenly distributed throughout the viral genome: some regions are conserved and some are highly variable[3]. In particular, the hyper-

variable region 1 (HVR1) of the *HCV E2* gene encoding a putative envelope glycoprotein mutates at a high rate, resulting in a wide spectrum of mutants referred to as "quasispecies" during infection[4,5]. Some virions may contain defective RNA genomes, which also affect the infectivity and replicability of the virus[6]. The mixture of clones present determines the biological and immunological properties of the virus.

How is HCV with multiple variants (quasispecies) transmitted to the new host? Does the status of transmitted HCV consist of multiple clones or a selected single clone? The transmission mode of HCV has been investigated by sequencing of the recovered viral genome from both donor and recipient[7,8]. HCV infection in human communities has occurred sporadically because no effective neutralizing vaccine against HCV has been developed. In particular, HCV infection in health-care workers through exposure to patient's blood due to a needle stick accident or accidental droplet transmission is a serious problem[9-12]. We previously reported a case of HCV infection resulting from a needle stick accident, and had an opportunity to investigate how HCV variants from the donor are transmitted to the recipient by comparing the HCV HVR1 genome encoding the envelope E2 protein recovered from the serum of both the donor and recipient[7]. In this case, we had observed the recipient before the onset of hepatitis and collected serum samples after obtaining informed consent. Thus, we were able to compare the HCV HVR1 genome between the donor's HCV at inoculation and the recipient's HCV just after onset of viremia. Interestingly, a minor subset of the donor's HCV clones was selectively transmitted to the recipient, and this selection determined the predominant clone in the new host. Several clones that appeared to stem from the recipient's predominant clone had one amino acid change within the HVR1 region during this short period. This particular case progressed to chronic hepatitis, and the same phenomenon has been demonstrated in the case of acute, self-limiting hepatitis[8]. These data suggest that a minor clone of the donor's HCV is transmitted and adapts to the new host. The precise mechanism of this viral selection in the initial phase of transmission has not been elucidated.

The simplicity of the transmitted viral strain in the initial phase of infection may explain some of the important clinical manifestations. Anti-viral therapy using interferon elicits a favorable response in the acute phase of HCV infection[13-16]. In addition, if a single strain is transmitted selectively in the initial phase of infection, this specific strain may be one of the factors determining disease activity. In fact, a study using a model of HCV transmission has demonstrated that a specific HCV strain recovered from a patient with fulminant hepatitis caused unusually severe hepatitis in a chimpanzee to which it was transmitted[17]. At present, the specific strain of HCV responsible for progressive liver disease cannot be discriminated from viral quasispecies in contaminated blood. Further investigation would be useful for clarifying the specific viral strain responsible for the disease, and such efforts would be important for planning future strategies for the development of an effective therapeutic vaccine.

SELF-LIMITING HEPATITIS OR CHRONIC HEPATITIS? HOW IS SUSCEPTIBILITY TO HCV DETERMINED?

The spontaneous clearance rate of HCV in the acute phase of infection

Most individuals with HCV infection fail to clear the virus and develop chronic hepatitis with a risk of progression to cirrhosis and hepatocellular carcinoma. However, a small proportion of individuals are known to show resolution of the infection in a self-limiting manner. The rate of spontaneous viral clearance in acute HCV infection is reported to be approximately 15%-40% of all HCV-infected individuals[18-20]. Although differences in study populations such as race may influence the clearance rate in each cohort, a systematic review of 31 studies has estimated this rate to be 26%[20]. We have previously reported a Japanese population-based cohort study of the natural history of HCV infection in an area where community-acquired acute hepatitis C is endemic; here, the spontaneous viral clearance rate was estimated to be approximately 20%[21,22]. What is the difference between self-limiting resolution of hepatitis and progression to chronic hepatitis? Comparative studies of this issue have focused on both viral characteristics and genetic regulation.

Viral characteristics influencing the outcome of acute hepatitis C

After the establishment of HCV infection, the viral genome mutates at a high rate, especially in the HVR1 of the HCV E2 region. The evolutionary dynamics of the infected virus are associated with the outcome of acute hepatitis C; genetic stasis and a high rate of evolution of HCV HVR1 are associated with resolution of infection in self-limiting hepatitis and progression to chronic infection, respectively[23]. The case we experienced progressed to chronic infection and 8 of 30 homogeneously predominant HCV HVR1 clones recovered from the recipient developed one amino acid mutation within this region during a short period of only 6 wk after infection[7]. As for the relationship between the viral load at the time of infection and the outcome of acute HCV infection, a recent study has shown that a high viral load in the initial phase of infection is associated with spontaneous viral clearance, leading to self-limiting resolution of hepatitis[24]. A high viral load may trigger strong innate immunity in the acute phase. However, it has also been reported that viral clearance may occur after a low infectious dose of HCV has been transmitted[25]. In addition, spontaneous viral clearance rarely occurs in the chronic phase of HCV infection where a low viral load is associated with spontaneous clearance[26]. The spontaneous clearance of HCV may thus depend on the immune system of indi-

viduals rather than the viral load. Further studies using a greater number of cohorts are needed to clarify the relationship between spontaneous viral clearance and the initial viral load, as well as the degree of induction of the innate immune response.

Genetic regulation of HCV infection

HCV-specific humoral and cellular immune responses are detectable in infected individuals, and a strong immune response against HCV favors viral clearance[18,27]. Genetic variation in host genes involved in immune response is likely to account for the difference in outcome. In particular, induction of natural killer (NK) cells in the innate immune response during the acute phase of infection plays a crucial role in resolving HCV infection. We have previously reported differences in genetic variations between HCV-infected individuals with and without viremia in the Japanese population[22], where a single nucleotide polymorphism (SNP) of transforming growth factor (TGF)-β1, which suppresses the proliferation and cytotoxicity of NK cells (the -509CC genotype or -509C allele), was associated with high HCV clearance rates and low transcriptional activity of TGF-β1[28]. The killer cell immunoglobulin-like receptor (KIR) and its human leukocyte antigen (HLA) have been reported to influence the outcome of HCV infection. Combinations of genotypes involving genes encoding the inhibitory NK cell receptor KIR2DL3 and HLA-C1 ligand directly influence HCV clearance in Caucasians and African Americans with an expected low infectious dose of HCV[25]. These data suggest that a diminished inhibitory effect of NK cells resulting from such gene regulation confers protection against HCV.

In a recent genome-wide association study, SNPs in the region of the Interleukin 28B (*IL28B*) gene encoding interferon-λ-3 were shown to be closely associated with the virologic response of HCV to antiviral therapy[29-31]. Patients carrying an *IL28B* homozygote for the major alleles of rs12979860 (CC genotype)[29] or rs8099917 (TT genotype)[30] show a greater propensity to achieve a sustained virologic response to pegylated interferon-α and ribavirin therapy than those carrying an *IL28B* heterozygote or homozygote for its minor allele. This SNP (rs12979860) also influences the outcome of HCV infection in the context of natural history; the CC genotype enhances resolution of HCV infection with spontaneous clearance among individuals of European and African ancestry[32]. This CC genotype has also been reported to be associated with a higher rate of spontaneous clearance in Asian populations[33]. In addition, a recent study has demonstrated that SNPs in the region of *IL28B* (rs12979860) and HLA class II (rs4273729) are independently associated with spontaneous resolution of HCV infection in individuals of European and African ancestry[34]. A prospective follow-up study of patients who developed acute hepatitis C also revealed a strong correlation between the *IL28B* C allele at rs12979860 and clearance[24]. Taken together, the SNP of *IL28B* (rs12979860) can be a marker

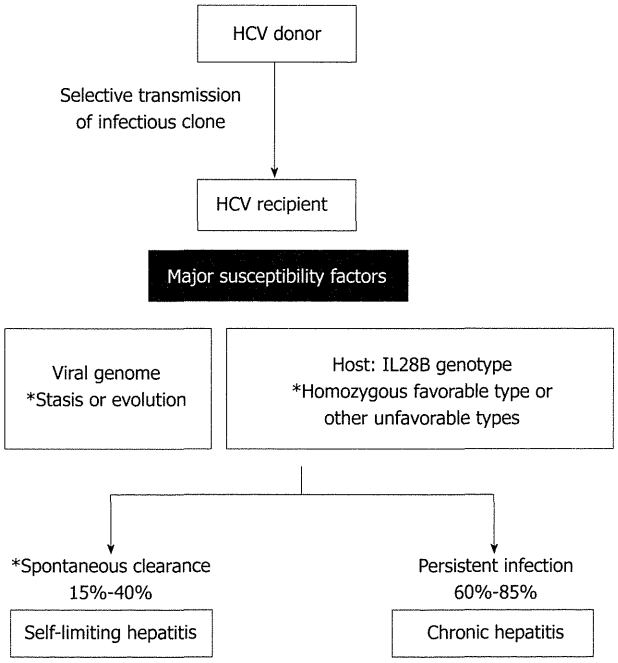

Figure 1 Transmission of hepatitis C virus, and the significance of viral and host factors for predicting the outcome of infection. HCV: Hepatitis C virus; IL28B: Interleukin 28B.

for indicating whether immediate antiviral treatment needs to be started in patients with acute hepatitis C[35]. Recently, upstream of the *IL28B* gene, a dinucleotide variant ss469415590 (TT or ΔG), in which ss469415590 (ΔG) activates the *IFNL4* gene encoding interferon-λ-4 protein through a genome frameshift, has been reported to be more strongly associated with HCV clearance in individuals of African ancestry than the SNP of *IL28B* (rs12979860), but comparable to that in Europeans and Asians[36]. This variant is in high linkage disequilibrium with rs12979860, and further investigations are expected to elucidate the functional role of ss469415590 (ΔG) that activates the *IFNL4* gene in association with the innate immune response to HCV.

CONCLUSION

Both the viral characteristics of an infecting clone and genetic regulation of infection by the host determine differences in the outcome of acute HCV infection (Figure 1). The evolutionary dynamics of the virus and genetic polymorphisms in the host pertaining mainly to the immune system influence susceptibility to HCV. In particular, the discovery of SNPs in the region of the *IL28B* gene has led to the characterization of a novel genetic marker of hepatitis C that is able to predict self-limiting viral clearance in the acute phase of infection as well as the response to antiviral therapy.

REFERENCES

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