cells) and, after the cells had been processed, we administered an average of 5.2×10^9 cells (Terai et al. 2006). In the BM-MNCs, there were 0.001–0.01 % MSC present (Pittenger et al. 1999). Therefore, the number of MSCs administered in ABM*i* therapy converts to a range of 0.5 to 5×10^5 cells. In this research, approximately 4×10^6 BM-MNCs were cultured through two passages; assuming 94 % of the approximately 3×10^6 recovered cells were MSCs, we obtained approximately 2.8×10^6 MSCs. For example, in 10 mL of bone marrow aspirate, which can be collected under local anesthesia, the number of BM-MNCs is approximately 2×10^8 cells, which converts mathematically to approximately 0.2 to 2×10^4 MSCs; but after these cells undergo two passages, the recovered number of cells is 1.5×10^8 and multiplying that by 94 % means that approximately 1.4×10^8 MSCs can be collected. Even when the loss accompanying the various processing steps is taken into account, the number of recovered MSCs is considered adequate. In other words, this research has demonstrated that ABM*i* therapy will be possible using cultured cells that can be collected under local anesthesia.

With regard to the carcinogenicity of human bone marrow cells, a group at Sun Yat-Sen University in China has shown that, when a bone marrow aspirate was administered via the hepatic artery to patients with HBV-induced liver failure, there was no change in the onset of hepatocarcinoma due to the bone marrow cell infusion for up to 192 weeks (Peng et al. 2011). A safety evaluation by oncogenicity tests involving nude mice will be necessary before clinical application but we have already shown that the administration of bone marrow cells does not promote the onset of liver cancer in mice with hepatocarcinogenic liver cirrhosis (N-nitrosodietylamine/GFP-CCl_4 model) and, conversely, it significantly inhibits hepatocarcinogenesis (Maeda et al. 2012).

No reports on either the safety or danger of the administration of cultured human bone marrow-derived cells have been published concerning the intravenous infusion of autologous cultured BM-MSCs but a death resulting from contrast nephropathy did occur when CD34-positive hematopoietic stem cells were administered via the hepatic artery (Pai et al. 2008). The majority of cultured human bone marrow-derived cells (P2) that we have studied are MSC and they contain almost no CD34-positive cells (0.06 %). Moreover, we are considering infusion via a peripheral vein in the same manner as ABM*i* therapy, so, in that respect, the administration of P2 cells should be safe. In addition, it has been reported that no malignant transformation of bone marrow cells has occurred in the past, even with long-term culturing. In this case, the duration of the P2 cell culture is 20–22 days and, from a quality standpoint, we believe that malignant transformation is unlikely to occur (Bernardo et al. 2007).

Finally, in this study, we have revealed the liver fibrosis-improving effect of MSC originating in cultured human bone marrow-derived cells (P2). In the future, further analysis of the liver fibrosis-improving mechanism will be needed but we have now shown that cultured human bone marrow-derived cells (P2) can improve liver fibrosis by regulating the expression of MMP-9 and HSC activation that is mediated by the humoral factors TGFβ and TNFα. This analysis has also revealed that ABM*i* using cultured cells, which can be collected on an outpatient basis under local anesthesia, is a method with sufficient clinical applicability.

Acknowledgment This study was supported by Grants-in-Aid for scientific research from the Japan Society for the Promotion of Science (JSPS); Ministry of Health, Labour and Welfare, health and labour sciences research grants and Japan Science and Technology Agency (JST), the project of realization of regenerative medicine and highway. Ms. Mariko Yamada, Ms. Isako Fujimoto and Ms. Yoko Fukusumi helped us with several analyses.

References

Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M et al (2000) Hepatocytes from non-hepatic adult stem cells. Nature 406:257

Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D et al (2007) Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res 67:9142–9149

Hisanaga T, Terai S, Iwamoto T, Takami T, Yamamoto N, Murata T, Matsuyama T et al (2011) TNFR1-mediated signaling is important to induce the improvement of liver fibrosis by bone marrow cell infusion. Cell Tissue Res 346:79–88

Huang CK, Lee SO, Lai KP, Ma WL, Lin TH, Tsai MY, Luo J et al (2013) Targeting androgen receptor in bone marrow mesenchymal stem cells leads to better transplantation therapy efficacy in liver cirrhosis. Hepatology 57:1550–1563

Iwamoto T, Terai S, Hisanaga T, Takami T, Yamamoto N, Watanabe S, Sakaida I (2013) Bone-marrow-derived cells cultured in serum-free medium reduce liver fibrosis and improve liver function in carbon-tetrachloride-treated cirrhotic mice. Cell Tissue Res 351:487–495

Kharaziha P, Hellstrom PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, Telkabadi M et al (2009) Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I–II clinical trial. Eur J Gastroenterol Hepatol 21:1199–1205

Kim JK, Park YN, Kim JS, Park MS, Paik YH, Seok JY, Chung YE et al (2010) Autologous bone marrow infusion activates the progenitor cell compartment in patients with advanced liver cirrhosis. Cell Transplant 19:1237–1246

Maeda M, Takami T, Terai S, Sakaida I (2012) Autologous bone marrow cell infusions suppress tumor initiation in hepatocarcinogenic mice with liver cirrhosis. J Gastroenterol Hepatol 27(Suppl 2):104–111

Mizunaga Y, Terai S, Yamamoto N, Uchida K, Yamasaki T, Nishina H, Fujita Y, et al. (2012) Granulocyte colony-stimulating factor and interleukin-1beta are important cytokine in repair of the cirrhotic liver after bone marrow cell infusion -comparison of humans and model mice. Cell Transplant (in press)

Mohamadnejad M, Alimoghaddam K, Mohyeddin-Bonab M, Bagheri M, Bashtar M, Ghanaati H, Baharvand H et al (2007) Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis. Arch Iran Med 10:459–466

Pai M, Zacharoulis D, Milicevic MN, Helmy S, Jiao LR, Levicar N, Tait P et al (2008) Autologous infusion of expanded mobilized adult bone marrow-derived CD34+ cells into patients with alcoholic liver cirrhosis. Am J Gastroenterol 103:1952–1958

Peng L, Xie DY, Lin BL, Liu J, Zhu HP, Xie C, Zheng YB, et al. (2011) Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. Hepatology (in press)

Phinney DG, Kopen G, Isaacson RL, Prockop DJ (1999) Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem 72:570–585

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Saito T, Okumoto K, Haga H, Nishise Y, Ishii R, Sato C, Watanabe H et al (2011) Potential therapeutic application of intravenous autologous bone marrow infusion in patients with alcoholic liver cirrhosis. Stem Cells Dev 20:1503–1510

Sakaida I, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, Okita K (2004) Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. Hepatology 40:1304–1311

Takami T, Terai S, Sakaida I (2012) Advanced therapies using autologous bone marrow cells for chronic liver disease. Discov Med 14: 7–12

Terai S, Sakaida I, Yamamoto N, Omori K, Watanabe T, Ohata S, Katada T et al (2003) An in vivo model for monitoring trans-differentiation of bone marrow cells into functional hepatocytes. J Biochem (Tokyo) 134:551–558

Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, Yokoyama Y et al (2006) Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 24:2292–2298

Terai S, Tanimoto H, Maeda M, Zaitsu J, Hisanaga T, Iwamoto T, Fujisawa K et al (2012) Timeline for development of autologous bone marrow infusion (ABMi) therapy and perspective for future stem cell therapy. J Gastroenterol 47:491–497

Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, Henegariu O et al (2000) Liver from bone marrow in humans. Hepatology 32:11–16

Thomas JA, Pope C, Wojtacha D, Robson AJ, Gordon-Walker TT, Hartland S, Ramachandran P et al (2011) Macrophage therapy for murine liver fibrosis recruits host effector cells improving fibrosis, regeneration, and function. Hepatology 53:2003–2015

Hepatology Research

Hepatology Research 2013 doi: 10.1111/hepr.12204

Original Article

Canine mesenchymal stem cells show antioxidant properties against thioacetamide-induced liver injury *in vitro* and *in vivo*

Luiz Fernando Quintanilha,[1,4] Taro Takami,[1] Yoshikazu Hirose,[1] Koichi Fujisawa,[2] Yasuhiko Murata,[1] Naoki Yamamoto,[1,3] Regina Coeli dos Santos Goldenberg,[4] Shuji Terai[1] and Isao Sakaida[1,2]

[1]*Department of Gastroenterology and Hepatology,* [2]*Center for Reparative Medicine, Yamaguchi University Graduate School of Medicine,* [3]*Yamaguchi University Health Administration Center, Yamaguchi University, Ube, Japan; and* [4]*Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*

Aim: To overcome current limitations of therapy for liver diseases, cell-based therapies using mesenchymal stem cells (MSC) have been attempted through basic and clinical approaches. Oxidative stress is a crucial factor in hepatology, and reactive oxygen species (ROS) are well-established molecules responsible for its deleterious effects. The antioxidant properties of MSC were recently demonstrated, and therefore we examined the antioxidant activity of canine MSC (cMSC), their effects on isolated hepatocytes *in vitro* and their curative potential against thioacetamide (TAA)-induced liver injury *in vivo*.

Methods: To evaluate the ability of cMSC to challenge oxidative stress, cell viability, cytotoxicity and ROS were measured in cultured cMSC treated with TAA. Also, cMSC were co-cultured with hepatocytes in the same injury condition, and the ROS level was measured exclusively in hepatocytes. Finally, to verify the curative potential of cMSC, 2.0×10^6 cells or phosphate-buffered saline were injected systemically in non-obese diabetic/severe combined immunodeficiency mice that received TAA injections twice a week for 13 weeks. We then evaluated histological parameters, serum injury markers and redox homeostasis.

Results: cMSC overcame TAA-induced oxidative stress *in vitro*, as shown by increased viability and lower cytotoxicity and ROS levels. Moreover, hepatocytes co-cultured with cMSC also showed decreased cellular ROS. The *in vivo* study showed that mice treated with cMSC presented with an ameliorated histological pattern, suppressed fibrosis, lower serum injury marker levels and better oxidative parameters.

Conclusion: We concluded that cMSC injection reduce TAA-induced liver injury through antioxidant activities and hepatoprotective effects, showing a curative potential in liver diseases.

Key words: liver, mesenchymal stem cells, NF-E2-related factor 2, oxidative stress, reactive oxygen species

INTRODUCTION

LIVER DISEASES ARE highly prevalent in the population worldwide. Currently, despite different alternatives that have been tested, the standard treatment for end-stage chronic liver disease that is available and effective is whole liver transplantation. However, liver transplantation has serious limitations such as donor scarcity, immunological incompatibilities, high cost, and significant morbidity and mortality associated with the procedure.[1–3] Additionally, considerable long-term side-effects have been reported.[4–7] Given the inherent limitations of this treatment, alternative therapies are urgently needed.

In recent years, cell-based therapy, especially therapy using bone marrow cells (BMC), has emerged as an alternative to improve damaged liver function. An increasing number of studies have been published showing evidence of therapeutic effects of BMC in liver diseases,[8–15] including clinical trials worldwide.[16–20]

Correspondence: Dr Taro Takami, Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Email: t-takami@yamaguchi-u.ac.jp
Received 24 February 2013; revision 19 June 2013; accepted 8 July 2013.

The interest in this particular cell niche comes from previous reports showing the presence of donor-derived cells in the liver of bone marrow transplant recipients.[21,22] This observation, which has been proven in animal models,[23,24] showed potential cross-talk between BMC and the liver under certain conditions. Among the different cell types found in bone marrow, mesenchymal stem cells (MSC) have shown promising results in tissue regeneration.[8,12,14,25] These cells can be easily isolated from the patient, cultured, expanded and used as an autologous cell-based therapy.

Although promising results have been shown, important questions remain. For example, no consensus exists about the mechanisms of liver repair by BMC infusion. This topic constitutes one of the most debated issues in regenerative medicine.

Recently, oxidative stress has been shown to be an important factor in liver diseases such as liver fibrosis, cirrhosis, viral hepatitis, hepatocellular carcinoma and others.[26–30] Oxidative stress is partly generated by reactive oxygen species (ROS), which are produced by different pathways such as NAD(P)H oxidases, xenobiotic metabolism, mitochondrial leakage and cytochrome P450 activity, which lead to hepatocyte damage through lipid peroxidation and alkylation of proteins, nucleic acids and lipids.[31–33] Although the liver itself has an efficient antioxidant defense system, sometimes this system is not sufficient to repair the damage and/or an imbalance exists between oxidative stress elimination and production. MSC were recently reported to have an antioxidant ability that may contribute to oxidative stress resolution.[34] Importantly, NF-E2-related factor 2 (Nrf2) has emerged as a crucial transcription factor that is capable of inducing a large array of enzymes involved in oxidative stress resolution.[35,36] Maintenance of the cellular redox balance by Nrf2 has multiple activation pathways and has been shown to be essential in combating many inflammatory diseases.[37–43] Some molecules such as all-trans retinoic acid (ATRA) and *tert*-butylhydroquinone (t-BHQ) have shown the ability to significantly reduce (ATRA) or induce (t-BHQ) Nrf2 functions, which modify the expression of antioxidant response element (ARE)-driven genes.[44,45]

Thioacetamide (TAA) is one the most popular chemical toxins used worldwide to generate experimental liver injury.[46,47] Its toxicity results from its biotransformation by a mixed-function oxidase system (e.g. cytochrome P450 enzymes and FAD monooxygenases), which leads to the formation of reactive metabolites including ROS.[48–53] ROS production resulting from TAA administration is related to the consequences of oxidative damage including lipid peroxidation.[54,55]

Given the above concerns and the recent evidence for the effectiveness of cell-based therapy in liver diseases involving oxidative stress, we hypothesized that MSC could ameliorate the deleterious effects of TAA-induced oxidative stress injury in liver. In this study, we tested the ability of canine MSC (cMSC) to overcome TAA-induced oxidative stress *in vitro* and verified whether these cells could protect against oxidative stress damage in isolated hepatocytes. In addition, we evaluated whether cMSC could reduce the effects of TAA-induced chronic injury *in vivo*. An important note is that few studies have used cells derived from medium-sized animals. Results from such studies will be important for supporting new clinical trials.

METHODS

Animals and ethics

ALL ANIMALS USED in this study were maintained and used in accordance with the Animal Care Guidelines of Yamaguchi University. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were purchased from Kyudo (Saga, Japan).

Cells and culture conditions

Canine bone marrow-derived MSC (Cyagen, Sunnyvale, CA, USA) were seeded onto 10-cm dishes (Iwaki, Tokyo, Japan) and cultured in OriCell Mesenchymal Stem Cell Growth Medium (Cyagen) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA), penicillin (100 U/mL; Life Technologies) and streptomycin (100 μg/mL; Life Technologies) in a 5% CO_2 incubator at 37°C. After seeding, non-adherent cells were removed when the medium was replaced. The culture medium was changed every 2 days. Cells from the fourth to sixth passages were used in this study.

Cellular characterization

Adherent cells were dissociated with 0.05% trypsin-ethylenediaminetetraacetic acid (Life Technologies) and resuspended in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% FBS. Then, they were washed once with phosphate-buffered saline (PBS; Life Technologies) and incubated in PBS containing 2% canine serum (AbD Serotec, Oxford, UK) for 20 min on ice. After incubation, cells were incubated for 20 min on ice with monoclonal antibodies against

CD11b (AbD Serotec), CD14-PE-Cy7 (BD Biosciences, San Jose, CA, USA), CD29-PE (Abcam, Cambridge, UK), CD34-PE (Abcam), CD44-PE-Cy7 (Biolegend, San Diego, CA, USA), CD45-e-fluor (ebioscience, San Diego, CA, USA), CD90-APC (ebioscience) or CD133-PercP-eFluor 710 (ebioscience). Secondary detection of the CD11b antibody was performed using goat polyclonal secondary antibody to mouse IgG–H&L (DyLight 488; Abcam). Isotype-identical antibodies were used as controls. Flow cytometry analyses were performed utilizing Gallios equipment (Beckman Coulter, Danvers, MA, USA). Propidium iodide (PI; Sigma-Aldrich, St Louis, MO, USA) was used to exclude dead cells from analyses. Assessment of each sample was performed at least in triplicate. Data were analyzed using Kaluza software (Beckman Coulter). To confirm the differentiation potential, the cells were grown in osteogenic and adipogenic canine differentiation media (Cell Applications, San Diego, CA, USA) in accordance with the manufacturer's instructions. After 2 weeks, lipid droplets were observed following oil red O staining (Sigma-Aldrich), and deposition of bone mineral was observed following alizarin red staining (AppliChem, Darmstadt, Germany).

CM-DiI labeling

Cultured cMSC were CM-DiI stained following the manufacturer's instructions. In brief, cMSC were suspended in 2 μM CM-DiI (Molecular Probes, Eugene, OR, USA) and incubated for 5 min at 37°C followed by an additional incubation at 4°C for 15 min. Then, labeled cells were washed three times and resuspended in PBS. Cell labeling was confirmed with fluorescence microscopic observation.

Monoculture system

To evaluate the ability of cMSC to challenge TAA-induced oxidative stress, cMSC were seeded onto 96- or six-well plates (Corning, NY, USA) at a density of 1.0×10^5 cells/cm^2 in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 5% CO_2 incubator at 37°C. Non-adherent cells were removed by washing with PBS. The medium was replaced with non-supplemented DMEM with or without 50 mM TAA with additional overnight incubation.

Cells in 96-well plates were washed three times, and the viability was measured indirectly using CellTiter 96 AQueous One Solution (Promega, Madison, WI, USA). Cytotoxicity was measured with lactate dehydrogenase (LDH) quantification using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. Values were normalized using media exposed to the same culture conditions without cells. Finally, to quantify cellular ROS, cells in six-well plates were stained with the CellRox Reagent (Molecular Probes) following the manufacturer's instructions. Stained live cells were quantified using Gallios equipment. Additionally, to reduce or increase the antioxidant response by Nrf2, cMSC were pretreated for 1 h with non-supplemented DMEM containing 10 μM ATRA (Sigma-Aldrich) or 10 μM t-BHQ (Sigma-Aldrich), respectively. ATRA is an inhibitor, and t-BHQ is an inducer of ARE-driven gene induction that is mediated by Nrf2. After incubation, the same volume of non-supplemented DMEM was added (final concentration, 50 mM TAA). After overnight incubation, we performed a viability test, LDH quantification and ROS measurement as described above.

Co-culture system

For co-culture, primary green fluorescent protein (GFP) positive murine hepatocytes were isolated from C57BL/6 Tg14 (act-EGFP) OsbY01 mice as previously described[56] (with modifications). In brief, livers were perfused via the portal vein with pre-warmed liver perfusion medium (Life Technologies) at a flow rate of 6 mL/min for 5 min with additional perfusion with 0.05% type IV collagenase (Sigma-Aldrich) containing 20 U/mL deoxyribonuclease I (Sigma-Aldrich) at 37°C. Whole livers were carefully harvested, the gall bladder was removed and further mechanical digestion was performed in a glass dish. The released cells were filtered through 100-μm nylon mesh (BD Falcon, San Jose, CA, USA) and washed twice with centrifugation at 50 *g* at 4°C for 1 min. Finally, the pellet was resuspended in PBS and diluted in stock isotonic Percoll solution (GE Lifesciences, Uppsala, Sweden) with further centrifugation at 60 *g* for 10 min. The pellet was washed twice with PBS, and the viability and cell number were determined with a Trypan blue exclusion test (Molecular Probes). Then, approximately $3–5 \times 10^5$ isolated hepatocytes were seeded onto collagen I-coated six-well plates (Life Technologies) using rodent hepatocyte plating medium (Zenbio, Durham, NC, USA) in a 5% CO_2 incubator at 37°C. After 4 h, the same number of CM-DiI-labeled cMSC was seeded in experimental plates (monoculture was used as control). After overnight incubation, non-adherent cells were removed by washing with PBS, and the medium was replaced with rodent hepatocyte maintenance medium (Zenbio) with or without 50 mM TAA.

After overnight incubation, hepatocytes in monoculture or direct co-culture were analyzed. For ROS quantification, six-well plates were stained with the CellRox Deep Red Reagent for 30 min. After staining, cells were harvested, centrifuged for 1 min at 50 *g*, and resuspended in PBS containing 1% FBS and 2 μg/mL PI for flow cytometry analysis. The cellular ROS levels of PI negative live GFP positive cells were analyzed. Figure 3(a–c) illustrates our experimental strategy.

Experimental model of liver injury

For liver injury induction, 6-week-old female NOD/SCID mice ($n = 16$) were given TAA (Sigma-Aldrich) injection (250 mg/kg i.p.) twice a week for 13 weeks. Beginning on the 10th week, 2.0×10^6 cMSC diluted in 200 μL PBS (cell-treated group; $n = 8$) or the same volume of PBS only (non-treated group; $n = 8$) were slowly injected weekly via the tail vein using a metal hub needle (31/2″/2) and a 250-μL syringe (Hamilton, Reno, NV, USA). Three days after the last cMSC/PBS injections, mice were killed.

Biochemical analyses

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and LDH were measured in duplicate using an automated analyzer for clinical chemistry (SPOTCHEM EZ SP-4430; Arkray, Kyoto, Japan).

Histological staining

Paraffin-embedded liver samples were sectioned (5 μm) and stained with hematoxylin–eosin and Sirius red according to standard protocols.

Fibrosis level

Histomorphometry was performed using an imaging system coupled to a fluorescence microscope (Biorevo BZ9000; Keyence, Osaka, Japan). The fibrosis index was estimated by the percent of the area that was stained with Sirius red of the total area of the histological fields, which were examined with a BZ Analyzer II (Keyence).

Hyaluronic acid (HA), procollagen N-terminal peptide (PIIIP) and AST/ALT ratio are valuable markers for fibrosis measurement.[57] Here, serum HA and PIIIP levels were measured by using an enzyme-linked immunosorbent assay (ELISA; Mybiosource.com, San Diego, CA, USA) in accordance with the manufacturer's instructions.

Antioxidant effects of cMSC

Total antioxidant activity was measured in serum samples using an antioxidant assay kit (Cayman Chemicals, Ann Arbor, MI, USA), which is based on the ability of all antioxidant components in the sample to inhibit the oxidation of ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS+ by metmyoglobin. The amount of ABTS+ produced was monitored by reading the absorbance at 750 nm using Infinite M200 (Tecan, Männedorf, Switzerland). The capacity of antioxidants in the sample to prevent ABTS oxidation was compared with that of TROLOX, a water-soluble tocopherol analog. The result is presented as the percent of the maximum antioxidant activity.

Lipid peroxidation was assessed in liver tissues using a malondialdehyde (MDA) assay kit (Abcam), which is based on colorimetric quantification of MDA, a natural product of lipid peroxidation in the samples.

Statistical analysis

Data were analyzed using Student's paired *t*-test or one-way ANOVA as appropriate. Values of $P < 0.05$ were considered statistically significant. Data are presented as the mean ± standard deviation.

RESULTS

Cellular characterization

THE CELLS USED in this study adhered to plastic and showed homogeneous distribution with a fibroblastoid shape (Fig. 1a). Flow cytometry analyses showed that cultured cMSC were positive for CD29, CD44 and CD90. On the other hand, these cells were negative for the pan-leukocyte marker CD45 and the monocyte/macrophage marker CD11b (Fig. 1b). These cells also had the potential to differentiate into adipogenic and osteogenic lineages (Fig. 1c,d), indicating a typical MSC phenotype.[58]

cMSC have a high capacity to overcome TAA-induced oxidative stress *in vitro*

Surprisingly, when cultured in medium containing TAA, cMSC exhibited an elevated ability to resist this condition by showing lower levels of LDH release (cytotoxicity) into the culture medium ($P < 0.001$), consistent with better viability (MTS assay, $P < 0.001$) and, also, they showed a lower cellular ROS level ($P < 0.001$). Additionally, because the Nrf2 pathway is considered to be an important factor in oxidative stress protection and resolution,[44] we used ATRA to abrogate and t-BHQ to induce the antioxidant effects mediated by Nrf2. In the presence of ATRA, pre-treated cMSC showed higher LDH release ($P < 0.05$), lower cell viability ($P < 0.001$)

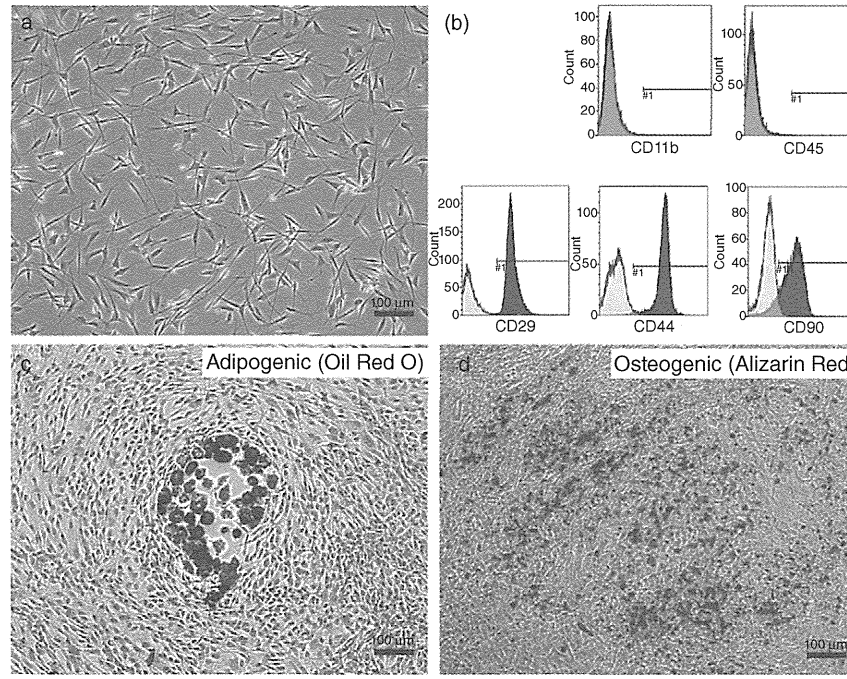

Figure 1 Canine mesenchymal stem cells (cMSC) characterization. (a) cMSC adhered to plastic and showed fibroblast-like morphology (original magnification ×10). (b) cMSC used for infusion showed no CD45 or CD11b expression and were positive for CD90, CD29 and CD44. cMSC showed (c) adipogenic and (d) osteogenic differentiation potential. Bar indicates 100 µm. ▒, isotype control; ■, CD antibody.

and higher ROS levels ($P < 0.01$) compared to cMSC cultured in medium containing TAA only. On the other hand, t-BHQ reversed these ATRA effects (Fig. 2). Also, to verify whether ROS itself was responsible for viability changes, we added hydrogen peroxide (H_2O_2; Wako Pure Chemical Industries, Osaka, Japan) or *N*-acetyl-L-cystein (NAC; Sigma-Aldrich) to the cultures to increase (H_2O_2) or decrease (NAC) the ROS levels. Even though

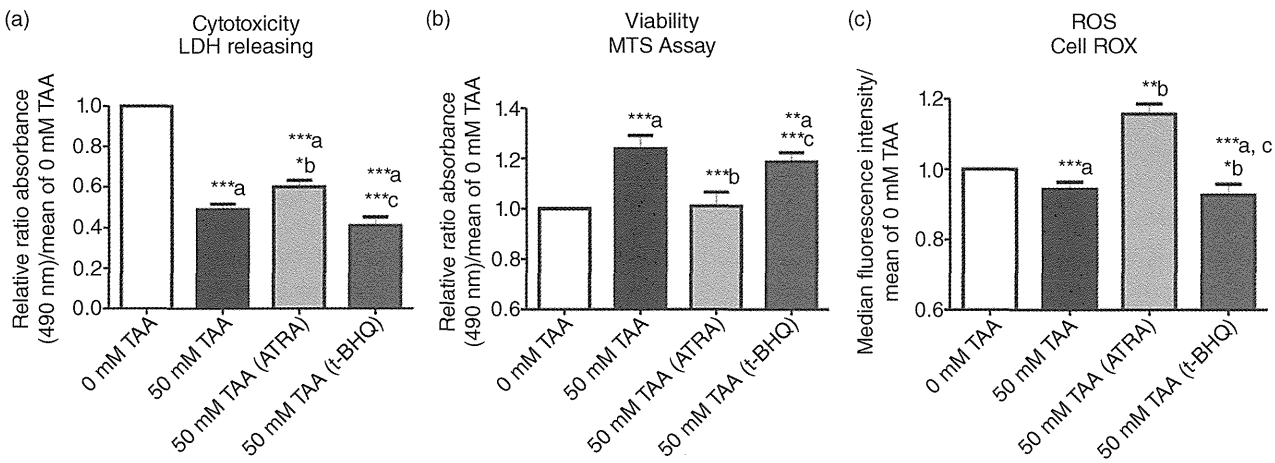

Figure 2 Canine mesenchymal stem cells (cMSC) treated with thioacetamide (TAA). cMSC resisted TAA-induced oxidative stress as verified by (a) lower lactate dehydrogenase (LDH) release into the culture medium (Cytotoxicity), (b) increased viability (MTS assay) and (c) lower cellular ROS levels (Cell Rox assay). An NF-E2-related factor 2 (Nrf2) inhibitor (all-trans retinoic acid [ATRA]) abrogated the values achieved by cMSC exposed to TAA. On the other hand, a Nrf2 inducer (*tert*-butylhydroquinone [t-BHQ]) showed the opposite results compared to ATRA. (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$; [a] vs 0 mM TAA, [b] vs 50 mM TAA and [c] vs 50 mM TAA [ATRA]).

ROS levels changed as expected, no direct association was observed with viability at the time points tested (data not shown).

cMSC protect hepatocytes against oxidative stress *in vitro*

To evaluate whether cMSC could exert antioxidant effects on hepatocytes, we co-cultured both types of cells (CM-DiI-labeled cMSC and GFP positive murine hepatocytes; Fig. 3a,b). Cellular ROS in GFP positive hepatocytes was evaluated with flow cytometric analysis (Fig. 3c). Interestingly, hepatocyte monoculture in TAA showed lower levels of cellular ROS. The co-culture system induced further reduction in the cellular ROS level in hepatocytes treated with 50 mM TAA (Fig. 3d).

Cell-therapy ameliorates TAA-induced liver injury

Biochemical analyses were performed to verify the extent of liver injury in TAA-treated NOD/SCID mice by measuring serum ALT, AST and LDH levels. The cell-treated group showed reduced levels of serum ALT (non-treated group vs cell-treated group, 356.1 ± 48.1 vs 286.4 ± 69.3 U/L; $P < 0.05$) and AST (non-treated group vs cell-treated group, 553.0 ± 174.9 vs 372.8 ± 71.1 U/L; $P < 0.05$). LDH also tended to be lower in the cell-treated group, but the difference was not statistically significant (non-treated group vs cell-treated group, 880.0 ± 164.9 vs 695.6 ± 305.8 U/L; $P = 0.06$) (Fig. 4).

In accordance, tissues harvested from non-treated mice showed more necrotic areas and increased inflammatory infiltration compared to the cell-treated group (Fig. 4d,e).

Fibrosis quantification

Morphometric analysis showed a decrease in the liver collagen content in the cell-treated group (5.1 ± 2.9%) compared to the non-treated group (8.3 ± 1.7%) (Fig. 5a,b). Moreover, the cell-treated group exhibited better indirect fibrosis parameters such as HA (non-treated vs cell-treated group, 40.0 ± 7.8 vs 29.7 ± 11.6 ng/mL, $P < 0.05$; Fig. 5c), PIIIP (non-treated vs cell-treated group, 71.7 ± 3.6 vs 56.9 ± 14.0 pg/mL, $P = 0.06$; Fig. 5d) and AST/ALT ratio (non-treated vs cell-treated group, 1.5 ± 0.3 vs 1.3 ± 0.03, $P < 0.05$; Fig. 5e).

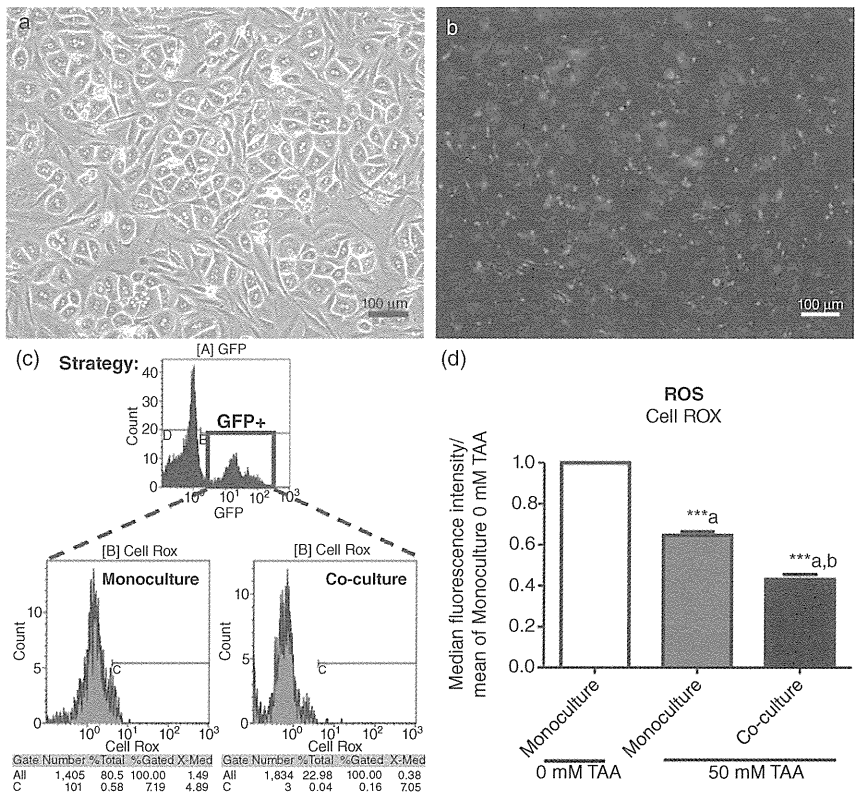

Figure 3 Co-culture with canine mesenchymal stem cells (cMSC) protected hepatocytes from oxidative stress. Murine green fluorescent protein (GFP) positive hepatocytes (green) were cultured alone or in co-culture with CM-DiI-labeled cMSC (red). (a) Bright field and (b) fluorescence microscopy of the co-culture system are shown. (c) Strategy to analyze GFP positive cells. (d) Cell ROX analysis of GFP positive cells revealed that hepatocytes have reduced reactive oxygen species (ROS) levels when cultured in the presence of thioacetamide (TAA). When co-cultured with cMSC, they showed a further decrease in intracellular ROS (***$P < 0.001$; [a] vs monoculture without TAA and [b] vs monoculture with TAA). Bar indicates 100 µm.

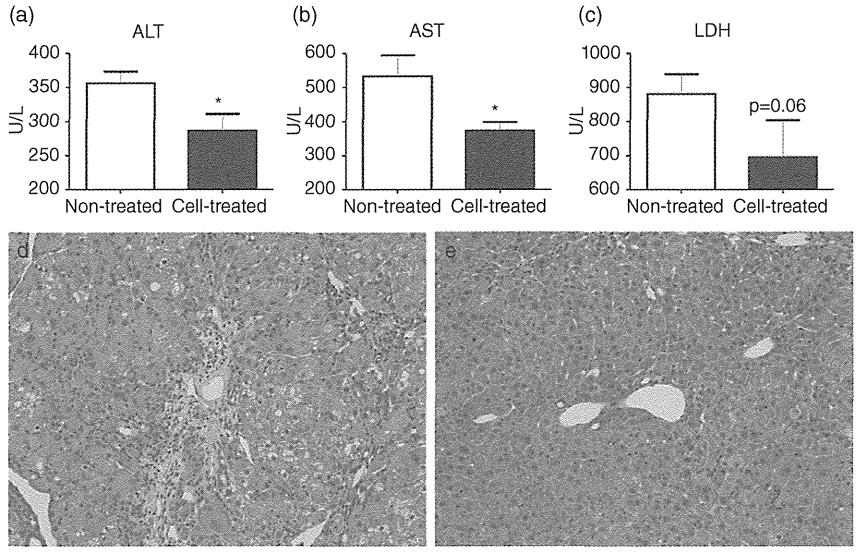

Figure 4 Canine mesenchymal stem cells (cMSC) transplantation protects against thioacetamide (TAA)-induced liver injury. The injury markers (a) alanine aminotransferase (ALT), (b) aspartate aminotransferase (AST) and (c) lower lactate dehydrogenase (LDH) were measured to evaluate the extent of liver injury. The cell-treated group showed lower levels in all measurements. (d–e) Representative figures showing higher levels of parenchymal disarrangement, diffuse cellular fat accumulation and necrosis in non-treated samples (d) compared with samples from the cell-treated group (e) (original magnification ×20; $^*P < 0.05$).

Cell therapy improves redox homeostasis

Based on our *in vitro* results, we investigated whether cMSC transplantation could ameliorate oxidative stress in animals with chronic TAA infusions. High total antioxidant activity was sustained in sera collected from the cell-treated group, whereas the non-treated group showed a clear decrease in this ability (non-treated group vs cell-treated group, 26.9 ± 19.2% vs 61.4 ± 13.0% of maximum values; $P < 0.001$, Fig. 6a). In addition, lipid peroxidation was lower in liver tissues harvested from cell-treated mice, indicating lower susceptibility to oxidation in tissue (non-treated group vs cell-treated group, 23.8 ± 4.2 vs 19.6 ± 3.0 nmol/mg; $P < 0.05$, Fig. 6b).

DISCUSSION

MURINE EXPERIMENTAL MODELS are commonly used to test new therapies for hepatic diseases,[59] including cell-based therapy using bone marrow-derived cells, which have shown promising results.[8–15] Among the different cell populations found in bone marrow, MSC have shown beneficial effects against liver disease.[8,12,14,25,60] Furthermore, MSC have advantages such as multiple tissue sources, fast proliferation, possible use in autologous transplantation and *in vitro* manipulation. Also, MSC were recently shown to promote an antioxidant response in injured liver.[34]

Despite good results in basic studies and clinical trials,[61–63] the mechanism of action of these cells is still being discussed. Recently, many studies have linked oxidative stress and development of liver diseases such as viral hepatitis, cirrhosis, hepatocellular carcinoma and others.[26–29] Here, we examined whether the antioxidant potential demonstrated by MSC has effects in reducing TAA-induced liver injury.

Thioacetamide is a drug that is widely used in animal models.[59] Because biotransformation of TAA produces oxidative damage associated with liver injury and this drug is usually used for systemic infusion, we examined if MSC, which are also usually injected systemically, could provide resistance to the toxic effects produced by TAA. Surprisingly, rather than resistance alone, cMSC showed a high level of tolerance to TAA (Fig. 2). Additionally, when cMSC were pretreated with ATRA or t-BHQ, they showed opposite responses regarding cytotoxicity, viability and ROS accumulation (Fig. 2). Considering that ATRA inhibits and t-BHQ induces Nrf2 effects both *in vitro* and *in vivo*,[44,45] these results indicate that cMSC have high antioxidant activity *in vitro* and suggest that the Nrf2 pathway may be involved in this process. Consistent with this hypothesis, Mohammadzadeh *et al.* recently showed that induced overexpression of Nrf2 by MSC was able to promote reduction of cell death in hypoxia, serum deprivation and oxidative stress conditions. In this study, MSC with transient overexpression of Nrf2 presented better cell viability and reduced apoptosis levels.[64] Moreover, Gorbunov *et al.* showed that MSC treated with lipopolysaccharide, which induces inflammatory responses including release of ROS, induce a number of adaptive responses including induction and nuclear

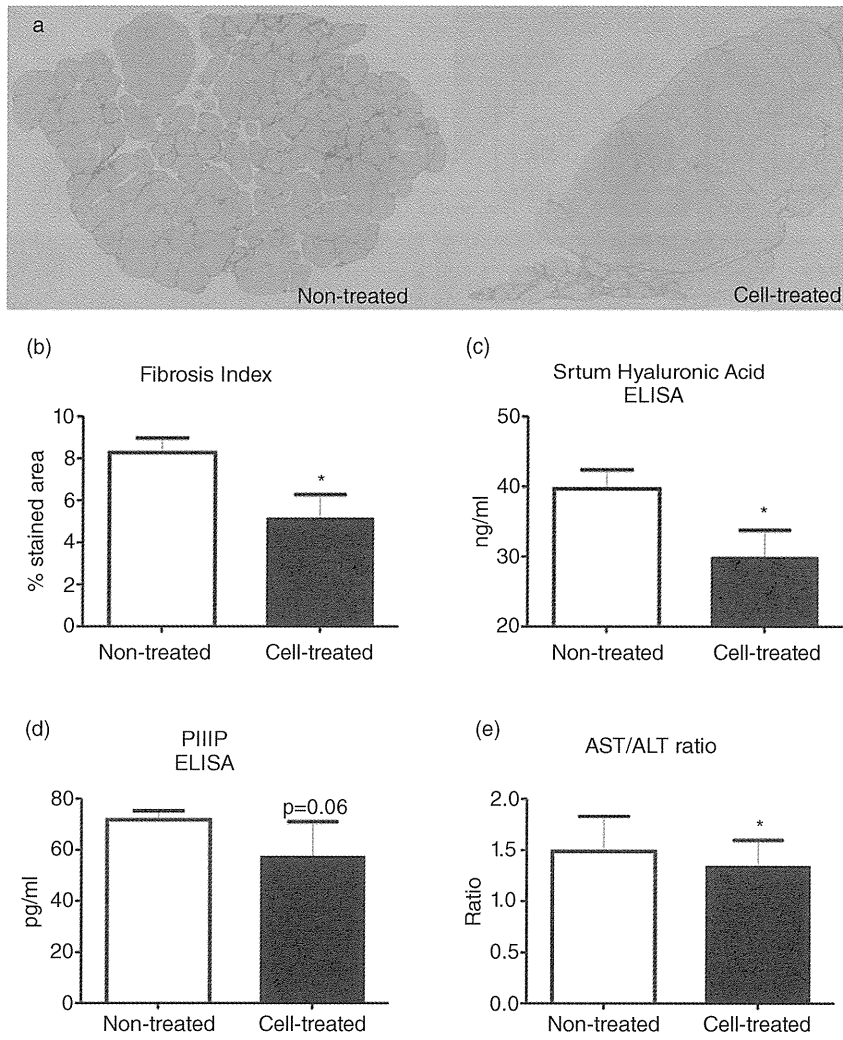

Figure 5 Fibrosis quantification. (a–b) Morphometry analysis of Sirius red-stained liver samples showed that the cell-treated group had lower levels of fibrosis (original magnification ×4). Complementarily, serum collected from cell-treated mice presented lower mean values for (c) hyaluronic acid (HA),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

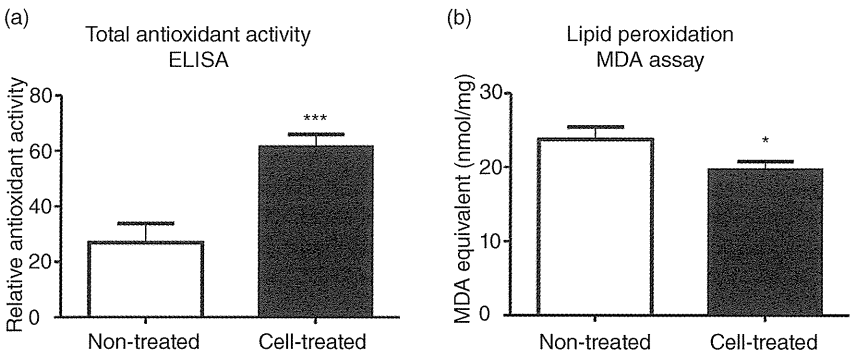

Figure 6 Antioxidant activity in cell-treated mice. (a) Total antioxidant activity was higher in the cell-treated group compared to the non-treated group ($^{***}P < 0.001$). Additionally, (b) lipid peroxidation in liver tissue was lower in the cell-treated group compared to the non-treated group ($^{*}P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

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.

ACKNOWLEDGMENTS

WE THANK MS Mariko Yamada, Ms Ihoko Fujimoto, Ms Yoko Fukusumi, Ms Hiromi Kurose and Ms Kumie Ohta for technical experimental support and Mr Bruno Diaz Paredes for graphic works. This study was supported by Grants-in-Aid for scientific research from the Japan Society for the Promotion of Science (JSPS) (nos. 22390150 to S. T. and 24790703 to T. T.); Ministry of Health, Labor and Welfare, health and labor sciences research grants; and the Japan Science and

Technology Agency (JST), the project of realization of regenerative medicine (Highway program).

REFERENCES

1 Nussler A, Konig S, Ott M *et al.* Present status and perspectives of cell-based therapies for liver diseases. *J Hepatol* 2006; **45**: 144–59.
2 Stravitz RT. Critical management decisions in patients with acute liver failure. *Chest* 2008; **134**: 1092–102.
3 Lee SW, Wang X, Chowdhury NR, Roy-Chowdhury J. Hepatocyte transplantation: state of the art and strategies for overcoming existing hurdles. *Ann Hepatol* 2004; **3**: 48–53.
4 Chung H, Kim KH, Kim JG, Lee SY, Yoon YH. Retinal complications in patients with solid organ or bone marrow transplantation. *Transplantation* 2007; **83**: 694–9.
5 Francoz C, Belghiti J, Durand J. Indications of liver transplantation in patients with complications of cirrhosis. *Best Pract Res Clin Gastroenterol* 2007; **21**: 175–90.
6 Patel H, Vogl DT, Aqui N *et al.* Posttransplant lymphoproliferative disorder in adult liver transplant recipients: a report of seventeen cases. *Leuk Lymphoma* 2007; **48**: 885–91.
7 Tamsel S, Demirpolat G, Killi R *et al.* Vascular complications after liver transplantation: evaluation with Doppler US. *Abdom Imaging* 2007; **32**: 339–47.
8 Cho KA, Ju SY, Cho SJ *et al.* Mesenchymal stem cells showed the highest potential for the regeneration of injured liver tissue compared with other subpopulations of the bone marrow. *Cell Biol Int* 2009; **33**: 772–7.
9 Ishikawa T, Terai S, Urata Y *et al.* Administration of fibroblast growth factor 2 in combination with bone marrow transplantation synergistically improves carbon-tetrachloride-induced liver fibrosis in mice. *Cell Tissue Res* 2007; **327**: 463–70.
10 Higashiyama R, Inagaki Y, Hong YY *et al.* Bone marrow–derived cells express matrix metallopeptidases and contribute to regression of liver fibrosis in mice. *Hepatology* 2007; **45**: 213–22.
11 Sakaida I, Terai S, Yamamoto N *et al.* Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. *Hepatology* 2004; **40**: 1304–11.
12 Yan Y, Xu W, Qian H *et al.* Mesenchymal stem cells from human umbilical cords ameliorate mouse hepatic injury *in vivo*. *Liver Int* 2009; **29**: 356–65.
13 Ali G, Sadia M, Mohsin K *et al.* Nitric oxide augments mesenchymal stem cell ability to repair liver fibrosis. *J Transl Med* 2012; **10**: 75.
14 Hardjo M, Miyazaki M, Sakaguchi M *et al.* Suppression of carbon tetrachloride-induced liver fibrosis by transplantation of a clonal mesenchymal stem cell line derived from rat bone marrow. *Cell Transplant* 2009; **18**: 89–99.
15 Maeda M, Takami T, Terai S, Sakaida I. Autologous bone marrow cell infusion suppress tumor initiation in hepatocarcinogenic mice with liver cirrhosis. *J Gastroenterol Hepatol* 2012; **27**: 104–11.
16 Chernykh ER, Starostina NM, Paltsev AI *et al.* Autologous bone marrow cells in treatment of cirrhosis of the liver. *Bull Exp Biol Med* 2007; **144**: 640–5.
17 Couto BG, Goldenberg RC, da Fonseca LM *et al.* Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study. *Liver Int* 2011; **31**: 391–400.
18 Lyra AC, Soares MB, da Silva LF *et al.* Infusion of autologous bone marrow mononuclear cells through hepatic artery results in a short-term improvement of liver function in patients with chronic liver disease: a pilot randomized controlled study. *Eur J Gastroenterol Hepatol* 2010; **22**: 33–42.
19 Saito T, Okumoto K, Haga H *et al.* Stem cells and development potential therapeutic application of intravenous autologous bone marrow infusion in patients with alcoholic liver cirrhosis. *Stem Cells Dev* 2011; **20**: 1503–10.
20 Terai S, Ishikawa T, Omori K *et al.* Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. *Stem Cells* 2006; **24**: 2292–8.
21 Theise ND, Nimmakayalu M, Gardner R *et al.* Liver from bone marrow in humans. *Hepatology* 2000; **32**: 11–6.
22 Alison MR, Poulsom R, Jeffery R *et al.* Hepatocytes from non-hepatic adult stem cells. *Nature* 2000; **406** (6793): 257.
23 Petersen BE, Bowen WC, Patrene KD *et al.* Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284** (5417): 1168–70.
24 Theise ND, Badve S, Saxena R *et al.* Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology* 2000; **31**: 235–40.
25 Salem HK, Thiemermann C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 2010; **28**: 585–96.
26 Clément S, Pascarella S, Negro F. Hepatitis C virus infection: molecular pathways to steatosis, insulin resistance and oxidative stress. *Viruses* 2009; **1**: 126–43.
27 Tanikawa K, Torimura T. Studies on oxidative stress in liver diseases: important future trends in liver research. *Med Mol Morphol* 2006; **39**: 22–7.
28 Ivanov AV, Smirnova OA, Ivanova ON, Masalova OV, Kochetkov SN, Isaguliants MG. Hepatitis C virus proteins activate NRF2/ARE pathway by distinct ROS-dependent and independent mechanisms in HUH7 cells. *PLoS ONE* 2011; **6**: e24957.
29 Zhu R, Wang Y, Zhang L, Guo Q. Oxidative stress and liver disease. *Hepatol Res* 2012; **42**: 741–9.
30 Cash WJ, McCance DR, Young IS *et al.* Primary biliary cirrhosis is associated with oxidative stress and endothelial dysfunction but not increased cardiovascular risk. *Hepatol Res* 2010; **40**: 1098–106.
31 Parola M, Robino G. Oxidative stress-related molecules and liver fibrosis. *J Hepatol* 2001; **35**: 297–306.

32 De Minicis S, Brenner DA. NOX in liver fibrosis. *Arch Biochem Biophys* 2007; **462:** 266–72.
33 Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; **33:** 105–36.
34 Cho KA, Woo SY, Seoh JY, Han HS, Ryu KH. Mesenchymal stem cells restore CCl_4-induced liver injury by an antioxidative process. *Cell Biol Int* 2012; **36:** 1267–74.
35 Jaiswal AK. Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radic Biol Med* 2004; **36:** 1199–207.
36 Klaassen CD, Reisman SA. Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. *Toxicol Appl Pharmacol* 2010; **244:** 57–65.
37 Hochmuth CE, Biteau B, Bohmann D, Jasper H. Redox regulation by Keap1 and Nrf2 controls intestinal stem cell proliferation in Drosophila. *Cell Stem Cell* 2011; **8:** 188–99.
38 Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med* 2008; **74:** 1526–39.
39 Brigelius-Flohe R, Flohe L. Basic principles and emerging concepts in the redox control of transcription factors. *Antioxid Redox Signal* 2011; **15:** 2335–81.
40 Hayes JD, McMahon M. NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends Biochem Sci* 2009; **34:** 176–88.
41 Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 2007; **47:** 89–116.
42 Lee JM, Johnson JA. An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J Biochem Mol Biol* 2004; **37:** 139–43.
43 Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes Cells* 2011; **16:** 123–40.
44 Magesh S, Chen Y, Hu L. Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents. *Med Res Rev* 2012; **32:** 687–726.
45 Wang XJ, Hayes JD, Henderson CJ, Wolf CR. Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha. *Proc Natl Acad Sci U S A* 2007; **104:** 19589–94.
46 Li X, Benjamin IS, Alexander B. Reproducible production of thioacetamide-induced macronodular cirrhosis in the rat with no mortality. *J Hepatol* 2002; **36:** 488–93.
47 Ledda-Collumbano GM, Coni P, Curto M *et al.* Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after single dose of thioacetamide. *Am J Pathol* 1991; **139:** 1099–109.
48 Zaragoza A, Andres D, Sarrion D, Cascales M. Potentiation of thioacetamide hepatotoxicity by phenobarbital pretreatment in rats. Inducibility of FAD monooxygenase system and age effect. *Chem Biol Interact* 2000; **124:** 87–101.
49 Sanz N, Diez-Fernandez C, Andres D, Cascales M. Hepatotoxicity and aging: endogenous antioxidant systems in hepatocytes from 2-, 6-, 12-, 18- and 30-month-old rats following a necrogenic dose of thioacetamide. *Biochim Biophys Acta* 2002; **1587:** 12–20.
50 Chilakapati J, Korrapati MC, Hill RA, Warbritton A, Latendresse JR, Mehendale HM. Toxicokinetics and toxicity of thioacetamide sulfoxide: a metabolite of thioacetamide. *Toxicology* 2007; **230:** 105–16.
51 Chilakapati J, Shankar K, Korrapati MC, Hill RA, Mehendale HM. Saturation toxicokinetics of thioacetamide: role in initiation of liver injury. *Drug Metab Dispos* 2005; **33:** 1877–85.
52 Andres D, Sanchez-Reus I, Bautista M, Cascales M. Depletion of Kupffer cell function by gadolinium chloride attenuates thioacetamide-induced hepatotoxicity. Expression of metallothionein and HSP70. *Biochem Pharmacol* 2003; **66:** 917–26.
53 Hajovsky H, Hu G, Koen Y *et al.* Metabolism and toxicity of thioacetamide and thioacetamide S-oxide in rat hepatocytes. *Chem Res Toxicol* 2012; **25:** 1955–63.
54 Lotkova H, Cervinkova Z, Kucera O, Rousar T, Krivakova P. S-Adenosylmethionine exerts a protective effect against thioacetamide-induced injury in primary cultures of rat hepatocytes. *Altern Lab Anim* 2007; **35:** 363–71.
55 Staňková P, Kučera O, Lotková H, Roušar T, Endlicher R, Cervinková Z. The toxic effect of thioacetamide on rat liver *in vitro*. *Toxicol In Vitro* 2010; **24:** 2097–103.
56 Seglen PO. Preparation of isolated rat liver cells. *Methods Cell Biol* 1976; **13:** 29–83.
57 Kawada N. Evolution of hepatic fibrosis research. *Hepatol Res* 2011; **41:** 199–208.
58 Dominici M, Le Blanc K, Mueller I *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8:** 315–7.
59 Tuñón MJ, Alvarez M, Culebras JM, González-Gallego J. An overview of animal models for investigating the pathogenesis and therapeutic strategies in acute hepatic failure. *World J Gastroenterol* 2009; **15:** 3086–98.
60 Iwamoto T, Terai S, Hisanaga T *et al.* Bone-marrow-derived cells cultured in serum-free medium reduce liver fibrosis and improve liver function in carbon-tetrachloride-treated cirrhotic mice. *Cell Tissue Res* 2013; **351:** 487–95.
61 Takami T, Terai S, Sakaida I. Novel findings for the development of drug therapy for various liver diseases: current state and future prospects for our liver regeneration therapy using autologous bone marrow cells for decompensated liver cirrhosis patients. *J Pharmacol Sci* 2011; **115:** 274–8.
62 Takami T, Terai S, Sakaida I. Stem cell therapy in chronic liver disease. *Curr Opin Gastroenterol* 2012; **28:** 203–8.
63 Terai S, Sakaida I. Autologous bone marrow cell infusion therapy for liver cirrhosis patients. *J Hepatobiliary Pancreat Sci* 2011; **18:** 23–5.

64 Mohammadzadeh M, Halabian R, Gharehbaghian A *et al.* Nrf-2 overexpression in mesenchymal stem cells reduces oxidative stress-induced apoptosis and cytotoxicity. *Cell Stress Chaperones* 2012; **17**: 553–65.
65 Gorbunov NV, Garrison BR, McDaniel DP *et al.* Adaptive redox response of mesenchymal stromal cells to stimulation with lipopolysaccharide inflammagen: mechanisms of remodeling of tissue barriers in sepsis. *Oxid Med Cell Longev* 2013; **2013**: 186795.
66 Xu W, Hellerbrand C, Köhler UA *et al.* The Nrf2 transcription factor protects from toxin-induced liver injury and fibrosis. *Lab Invest* 2008; **88**: 1068–78.
67 Okuyama H, Nakamura H, Shimahara Y *et al.* Overexpression of thioredoxin prevents acute hepatitis caused by thioacetamide or lipopolysaccharide in mice. *Hepatology* 2003; **37**: 1015–25.
68 Okuyama H, Nakamura H, Shimahara Y *et al.* Overexpression of thioredoxin prevents thioacetamide-induced hepatic fibrosis in mice. *J Hepatol* 2005; **42**: 117–23.

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 ($^*P < 0.05$).

TISSUE ENGINEERING: Part B
Volume 20, Number 3, 2014

DOI: 10.1089/ten.teb.2013.0527

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

Shuji Terai, MD, PhD,[1] Taro Takami, MD, PhD,[1] Naoki Yamamoto, MD, PhD,[2] Koichi Fujisawa, MD, PhD,[3] Tsuyoshi Ishikawa, MD, PhD,[1] Yohei Urata, MD, PhD,[1] Haruko Tanimoto, MD, PhD,[1] Takuya Iwamoto, MD, PhD,[1] Yuko Mizunaga, MD, PhD,[1] Takashi Matsuda, MD,[1] Takashi Oono, MD,[1] Miho Marumoto, MD, PhD,[1] Guzel Burganova, MD,[1,4,5] Luiz Fernando Quintanilha, PhD,[1,6] Isao Hidaka, MD, PhD,[1] Yoshio Marumoto, MD, PhD,[1] Issei Saeki, MD, PhD,[1] Koichi Uchida, MD, PhD,[1] Takahiro Yamasaki, MD, PhD,[1] Kenji Tani, MVD, PhD,[7] Yasuho Taura, MVD, PhD,[7] Yasuhiko Fujii, MD, PhD,[8] Hiroshi Nishina, PhD,[9] Kiwamu Okita, MD, PhD,[1] and Isao Sakaida, MD, PhD[1,3]

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

[1]Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan.
[2]Yamaguchi University Health Administration Center, Yamaguchi University, Yamaguchi, Japan.
[3]Center for Reparative Medicine, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan.
[4]Human Anatomy Department, Kazan State Medical University, Kazan, Russia.
[5]Institute of Fundamental Medicine and Biology, Kazan (Volga region) Federal University, Kazan, Russian Federation.
[6]Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
[7]Department of Veterinary Surgery, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan.
[8]Center of Regenerative Medicine and Cell Therapy, Yamaguchi University Hospital, Yamaguchi, Japan.
[9]Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan.

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

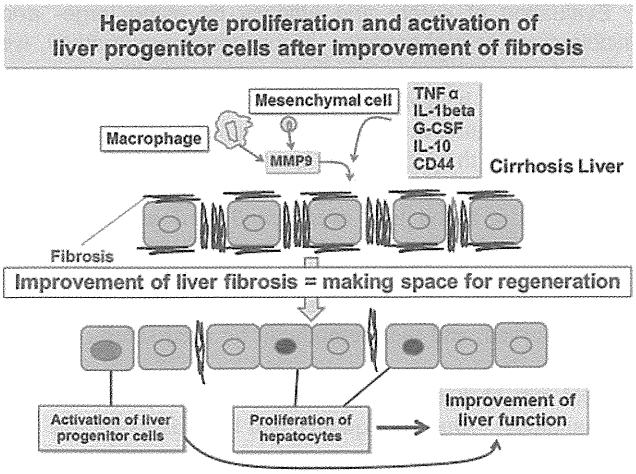

FIG. 1. Mechanism of sequential activation of liver progenitor cells and hepatocytes and improvement of liver fibrosis by BMCs (macrophages and mesenchymal cells).

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

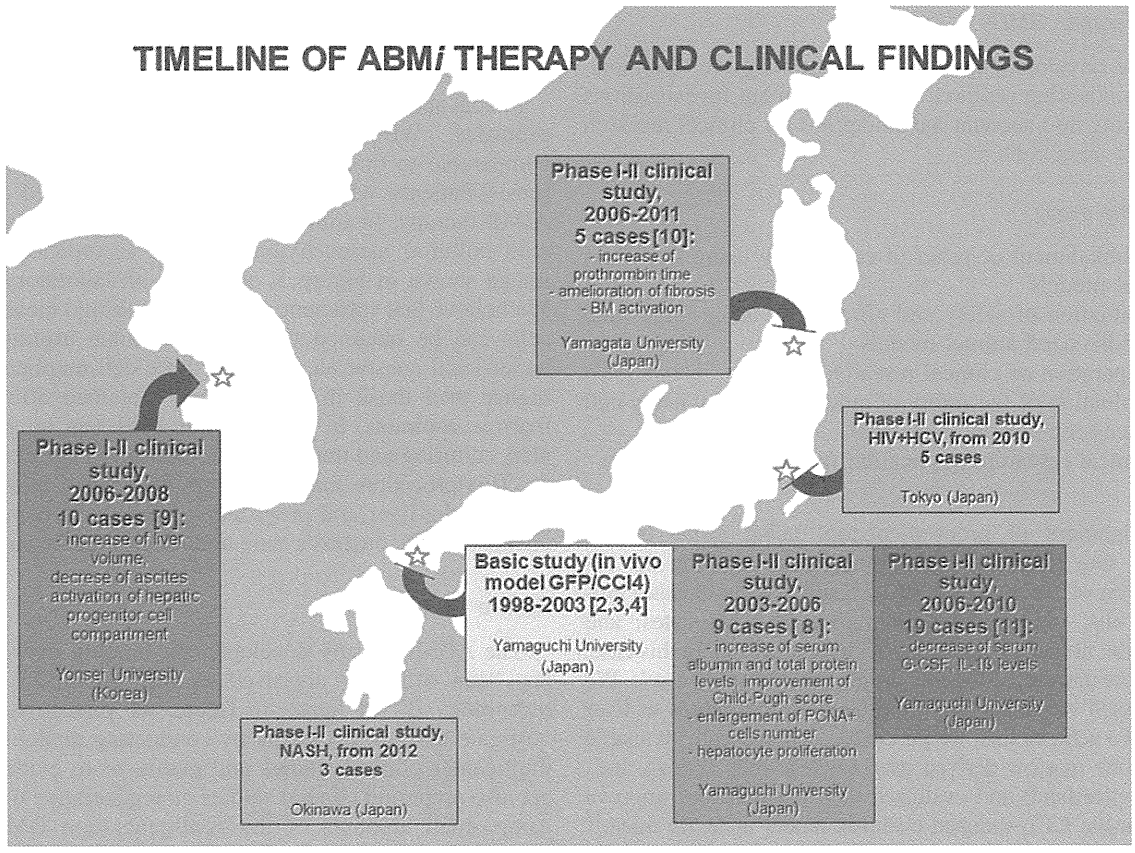

FIG. 2. Timeline of the autologous bone marrow cell infusion (ABM*i*) therapy and clinical findings.

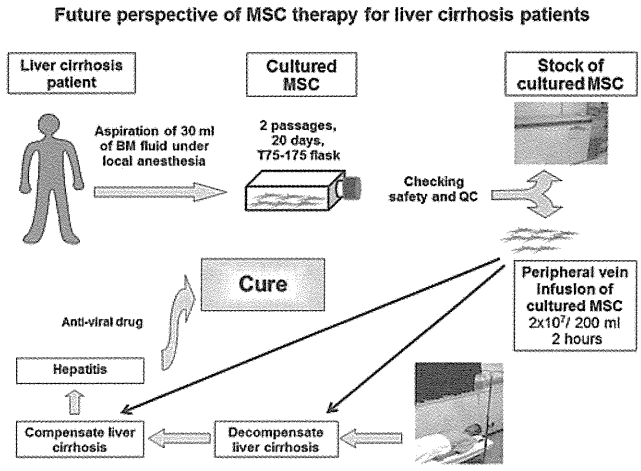

FIG. 3. Schematic representation of the therapeutic method of cultured MSC for liver cirrhosis patients.

with decompensated liver cirrhosis could be cultured and advanced into the mesenchymal stem cell stage, and then be administered through the peripheral vein. The results showed no adverse effects, and at 24 weeks, the model for end-stage liver disease score and liver volume showed improvements in two and three patients, respectively. An improvement in health-related Quality of Life parameters measured by the short form 36 [Medical Outcomes Study 36-Item Short-Form Health Survey (SF-36)] was also confirmed in this study.[17]

Status of the development of a liver-repair-and-regeneration therapy by using the next-generation BMCs

For the development of a minimally invasive treatment, we are conducting research and development investigations while taking into account the guidelines for clinical research on human stem cells.

Flow of the study

1. Establishment of proof-of-concept by using small animal models
2. Evaluation of safety and efficacy by using large- and middle-sized animal models
3. Preparation of clinical research
4. Evaluation of the safety of cultured cells by using cell processing centers (isolators)
5. Clinical research (application for human stem cells).

Establishment of proof-of-concept using small animal models. Our previous treatment regimen based on the administration of autologous BMCs should activate hepatic progenitor cells, induce the proliferation of hepatocytes, and improve the fibrosis associated with liver cirrhosis. In this case, BMCs were introduced through the peripheral blood vessels. We also assessed whether BMCs improved the symptoms of liver fibrosis. To achieve this, we purchased commercially available human bone marrow-derived mononuclear cells (Lonza, Inc., Basel, Switzerland) and evaluated their capability to improve fibrosis in the CCl_4-induced cirrhosis model in SCID mice.[18] Stable cultures of mesenchymal cells were indeed obtained, and our assessments validated that they could improve fibrosis.

Evaluation of safety and efficacy by using large- and middle-sized animal models. In our treatment strategy, we cultured mesenchymal cells from autologous bone marrow aspirated from beagle dogs. These cells were then administered to the same dogs through peripheral blood vessels in approximately three times the quantities delivered by other methods, and at 10 times the concentration used in humans. Subsequently, blood tests were conducted and the development of pulmonary embolism was monitored by computed tomography (CT) tests. No incidents of pulmonary embolism due to the administration of mesenchymal cells were observed, and the method can be used safely.

Cold run at a cell-processing center for preparation of clinical study. Once the quality had been assessed, the cell culture was established in a cell-processing center (CPC) and monitored in compliance with the standard operating procedures. In addition, if the cell culture involved fetal bovine serum (FBS), tests for the detection of residual FBS in the cell preparations were conducted before the evaluations. This stage of the study is focused on assessing whether the cell culture method established outside the CPC can be adopted and used by the CPC. Currently, we are conducting simulations of the practicality of this method for application in humans.

Quality assessment and standard criteria. The quality of the cultured mesenchymal stem cells was examined using flow cytometry with cell-specific surface markers. Cultures were tested for sterility, mycoplasma contamination, and presence of endotoxins and viruses. In addition, if the cell culture involved FBS, the cell preparations were analyzed for residual FBS before further evaluations.

Preparation and realization of a clinical research proposal. We plan to make our clinical research proposal comparable to those already implemented for treating cirrhosis patients who have already been treated with the ABM*i* therapy. In addition, we plan to conduct experiments with cultured mesenchymal cells in accordance with the image shown in Figure 3, starting with laboratory studies. We believe that sufficient amounts of cultured mesenchymal cells can be obtained from small volume aspirates taken under local anesthesia, and that those cell numbers will be higher than those in the currently obtained 30-mL bone marrow aspirates. In addition, the cells can be preserved after culturing and thus can be administered frequently (Fig. 3). This procedure may induce gradual repair and regeneration of the liver and progression from the decompensated cirrhosis to the cirrhosis state and later to the hepatitis state.

Future Prospects

The ABM*i* therapy developed for treating liver cirrhosis has been officially approved as an "Advanced medical technology B" in Japan. In future, we plan to further investigate the ABM*i* therapy by conducting randomized and multicenter clinical studies and gather more evidence. We are also preparing clinical studies on regenerative therapy by using small amounts of the next-generation BMCs (harvested through minimally invasive bone marrow aspiration) based on our previous laboratory and clinical research. A

therapeutic approach based on using somatic stem cells such as cultured BMCs for the treatment of liver cirrhosis is urgently needed. The development of such a strategy will firmly establish an effective regenerative therapy for the treatment of liver cirrhosis, potentially saving the lives of many patients.

Acknowledgments

This study was supported by Grants-in-Aid for scientific research from the Japan Society for the Promotion of Science (JSPS); the Ministry of Health, Labour and Welfare, Health and Labour sciences research grants; and the Japan Science and Technology Agency (JST), the project of realization of regenerative medicine and highway.

Disclosure Statement

No competing financial interests exist.

References

1. Theise, N.D., Badve, S., Saxena, R., Henegariu, O., Sell, S., Crawford, J.M., and Krause, D.S. Derivation of hepatocytes from BMCs in mice after radiation-induced myeloablation. Hepatology **31,** 235, 2000.
2. Terai, S., Sakaida, I., Yamamoto, N., Omori, K., Watanabe, T., Ohata, S., Katada, T., *et al.* An *in vivo* model for monitoring trans-differentiation of BMCs into functional hepatocytes. J Biochem (Tokyo) **13,** 551, 2003.
3. Terai, S., Sakaida, I., Nishina, H., and Okita, K. Lesson from the GFP/CCl4 model—translational research project: the development of cell therapy using autologous BMCs in patients with liver cirrhosis. J Hepatobiliary Pancreat Surg **12,** 203, 2005.
4. Sakaida, I., Terai, S., Yamamoto, N., Aoyama, K., Ishikawa, T., Nishina, H., and Okita, K. Transplantation of BMCs reduces CCl4-induced liver fibrosis in mice. Hepatology **40,** 1304, 2004.
5. Iimuro, Y., Nishio, T., Morimoto, T., Nitta, T., Stefanovic, B., Choi, S.K., Brenner, D.A., *et al.* Delivery of matrix metalloproteinase-1 attenuates established liver fibrosis in the rat. Gastroenterology **124,** 445, 2003.
6. Higashiyama, R., Inagaki, Y., Hong, Y.Y., Kushida, M., Nakao, S., Niioka, M., Watanabe, T., *et al.* Bone marrow-derived cells express matrix metalloproteinases and contribute to regression of liver fibrosis in mice. Hepatology **45,** 213, 2007.
7. Siller-Lopez, F., Sandoval. A., Salgado, S., Salazar, A., Bueno, M., Garcia, J., Vera, J., *et al.* Treatment with human metalloproteinase-8 gene delivery ameliorates experimental rat liver cirrhosis. Gastroenterology **126,** 1122, 2004;
8. Terai, S., Ishikawa, T., Omori, K., Aoyama, K., Marumoto, Y., Urata, Y., Yokoyama, Y., *et al.* Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells **24,** 2292, 2006.
9. Kim, J.K., Park, Y.N., Kim, J.S., Park, M.S., Paik, Y.H., Seok, J.Y., Chung, Y.E., *et al.* Autologous bone marrow infusion activates the progenitor cell compartment in patients with advanced liver cirrhosis. Cell Transplant **19,** 1237, 2010.
10. Saito, T., Okumoto, K., Haga, H., Nishise, Y., Ishii, R., Sato, C., Watanabe, H., *et al.* Potential therapeutic application of intravenous autologous bone marrow infusion in patients with alcoholic liver cirrhosis. Stem Cells Dev **20,** 1503, 2011.
11. Mizunaga, Y., Terai, S., Yamamoto, N., Uchida, K., Yamasaki, T., Nishina, H., Fujita, Y., Shinoda, K., Hamamoto, Y., and Sakaida, I. Granulocyte colony-stimulating factor and interleukin-1β are important cytokine in repair of the cirrhotic liver after bone marrow cell infusion—comparison of humans and model mice. Cell Transplant **21,** 2363, 2012.
12. Iwamoto, T., Tera, S., Mizunaga, Y., Yamamoto, N., Omori, K., Uchida, K., Yamasaki, T., *et al.* Splenectomy enhances the anti-fibrotic effect of bone marrow cell infusion and improves liver function in cirrhotic mice and patients. J Gastroenterol **47,** 300, 2012.
13. Gholamrezanezhad, A., Mirpour, S., Bagheri, M., Mohamadnejad, M., Alimoghaddam, K., Abdolahzadeh, L., Saghari, M., *et al. In vivo* tracking of 111In-oxine labeled mesenchymal stem cells following infusion in patients with advanced cirrhosis. Nucl Med Biol **38,** 961, 2011.
14. Peng, L., Xie, D.Y., Lin, B.L., Liu, J., Zhu, H.P., Xie, C., Zheng, Y.B., *et al.* Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. Hepatology **54,** 820, 2011.
15. Maeda, M., Takami, T., Terai, S., and Sakaida, I. Autologous bone marrow cell infusions suppress tumor initiation in hepatocarcinogenic mice with liver cirrhosis. J Gastroenterol Hepatol **27 Suppl 2,** 104, 2012.
16. Terai, S., Tanimoto, H., Maed, M., Zaitsu, J., Hisanaga, T., Iwamoto, T., Fujisawa, K., *et al.* Timeline for development of autologous bone marrow infusion (ABMi) therapy and perspective for future stem cell therapy. J Gastroenterol **47,** 491, 2012.
17. Mohamadnejad, M., Namiri, M., Bagheri, M., Hashemi, S.M., Ghanaati, H., Zare Mehrjardi, N., Kazemi Ashtiani, S., *et al.* Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis. World J Gastroenterol **13,** 3359, 2007.
18. Tanimoto, H., Terai, S., Takami, T., Murata, Y., Fujisawa, K., Yamamoto, N., and Sakaida, I. Improvement of liver fibrosis by infusion of cultured cells derived from human bone marrow. Cell Tissue Res **354,** 717, 2013.

Address correspondence to:
Shuji Terai, MD, PhD
Department of Gastroenterology and Hepatology
Yamaguchi University Graduate School of Medicine
Minami-kogushi 1-1-1, Ube
Yamaguchi 755-8505
Japan

E-mail: terais@yamaguchi-u.ac.jp

Received: August 28, 2013
Accepted: January 8, 2014
Online Publication Date: March 5, 2014

14. 肝臓の再生療法

山口大学大学院医学系研究科消化器病態内科学講師　高見太郎
同　准教授　寺井崇二
同　教授　坂井田功

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