3. 肝再生療法の確立，実用化にむけて

上述のごとく，海外ではさまざまな幹細胞および幹細胞を含む細胞，前駆細胞を用いた肝硬変に対する再生療法開発の臨床試験が計画，実施されているが，我が国において，安全で有効な肝再生療法を確立し，実用化するためには，関連する規制，法規に従い臨床試験を行う必要がある。国民皆保険の我が国において標準治療として認められるには，治療法の保険適応が必要である。また，細胞あるいは細胞製剤等について薬事承認が必要な医薬品・医療機器を扱う再生療法開発においては，治験による開発が必要となる。再生療法に用いられる可能性のある幹細胞，前駆細胞は，培養しない自己由来の細胞，培養した細胞，同種細胞，さらに人工的に製造される iPS 細胞とさまざまであり，実用化にむけた臨床試験では，早期から規制当局と相談を行い，評価項目，方法を確認し，臨床研究，臨床試験を実施していくことが極めて重要である。前術の如く，肝硬変の病態は，肝機能の低下のみならず，生活の質を低下させるさまざまな合併症を生じる慢性疾患の終末状態である。肝再生療法の有効性の評価には，短い期間では，肝予備能低下，門脈圧亢進症，炎症の程度が臨床試験での評価項目と考えられるが，長期間における生存率の改善に，生活の質を低下させる合併症の改善効果も重要な指標と考えられ，実用化後においての評価の視点となる。

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REGULAR ARTICLE

Improvement of liver fibrosis by infusion of cultured cells derived from human bone marrow

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Abstract We develop "autologous bone marrow cell infusion (ABM*i*) therapy" for the treatment of human decompensated liver cirrhosis and confirm the efficacy and safety of this treatment in multicenter clinical studies. With the goal of further expanding the applications of ABM*i*, we first cultured human bone marrow cells and then determined whether a cell fraction found to be effective in improving liver fibrosis can be amplified. Cells harvested after two passages (P2 cells) consistently contained approximately 94 % mesenchymal stem cells (MSCs); conversely, the cells harvested after only medium change (P0 cells) contained many macrophages. MSCs (2.8×10^8) in P2 cells were harvested from 3.8×10^8 bone marrow-derived mononuclear cells after 22 days. DNA-chip analysis also showed during the culturing step that bone marrow-derived cells decreased with macrophage phenotype. The infused 5×10^5 P2 cells significantly improved liver fibrosis in the nonobese diabetic/severe combined immunodeficient (NOD-SCID) mouse carbon tetrachloride (CCl_4) liver cirrhosis model and induced the expression of matrix metalloproteinase (MMP)-9 and suppressed expressions of alpha smooth muscle actin (αSMA), tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ) in the liver. Cultured human bone marrow-derived cells (P2 cells) significantly inhibited liver fibrosis. The increase of MMP-9 and suppressed activation of hepatic stellate cells (HSCs) through the regulation of humoral factors (TNFα and TGFβ) contribute to the improvement of liver fibrosis by MSCs comprising about 94 % of P2 cells. MSCs in cultured human bone marrow-derived mononuclear cells (BM-MNCs) proliferate sufficiently in cell therapy, so we believe our cultured bone marrow-derived cell therapy can lead to expanded clinical applications and enable outpatient therapy.

Keywords Autologous bone marrow cell infusion · Nonobese diabetic/severe combined immunodeficient mouse · Carbon tetrachloride · Mesenchymal stem cell · Matrix metalloproteinase

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Abbreviations

αSMA	Alpha smooth muscle actin
ABMi	Autologous bone marrow cell infusion
BM-MNC	Bone marrow-derived mononuclear cell
CCl_4	Carbon tetrachloride
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HSC	Hepatic stellate cell
IHC	Immunohistochemistry
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NOD-SCID	Nonobese diabetic/severe combined immunodeficient
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription polymerase chain reaction
TNFα	Tumor necrosis factor alpha
TGFβ	Transforming growth factor beta

Introduction

In the past, fluorescence in situ hybridization has been used to confirm the presence of the Y chromosome in liver and gastrointestinal tissues in autopsies of patients (XX) with blood disorders who had undergone bone marrow grafts (XY) and these findings suggested the presence of pluripotent stem cells in bone marrow cells (Alison et al. 2000; Theise et al. 2000). In addition, adherent cells ($CD90^+$, $CD44^+$ $CD14^-$, $CD34^-$ and $CD45^-$) have been isolated from human bone marrow aspirate, which suggests that these pluripotent cells are MSCs (Pittenger et al. 1999).

In our laboratory, we began basic research in mice concerning autologous bone marrow cell infusion (ABM*i*) therapy for liver cirrhosis by focusing on the presence in bone marrow of these pluripotent cells that engraft in the liver. We established a "murine green fluorescent protein (GFP)/CCl_4 model" to evaluate bone marrow cell differentiation and proliferation in liver cirrhosis (Terai et al. 2003). We found that liver fibrosis was improved in the same liver cirrhosis model because the infused bone marrow cells produce MMP-9 and other substances (Sakaida et al. 2004). Using the results of this basic research in mice as a foundation, in 2003, we began a clinical study entitled "ABM*i* therapy for decompensated liver cirrhosis" and we were the first to report on the efficacy and safety of this therapy (Terai et al. 2006). In joint research with this laboratory, researchers at Yonsei University in Korea have recently found that the therapeutic effect of ABM*i* lasts for at least 1 year in patients with hepatitis B-induced liver cirrhosis (Kim et al. 2010). In addition, in joint research with Yamagata University, we found that improved liver function in patients with alcoholic liver cirrhosis continues for 6 months after ABM*i* therapy (Saito et al. 2011). Cell therapy using bone marrow cells is a promising therapy for liver cirrhosis (Terai et al. 2012; Takami et al. 2012).

However, current ABM*i* therapy requires the collection of bone marrow aspirate under general anesthesia, so there are strict usage criteria regarding the general health condition of patients. Therefore, with the goal of expanding the therapeutic applications of ABM*i*, in our laboratory, we cultured human bone marrow cells and evaluated whether the cell fraction that has an improving effect on liver fibrosis can be amplified. We found that, from the standpoint of growth capability and pluripotency, the second passage (P2) cells comprise a fraction that is clearly more important for liver cirrhosis therapy. In addition, we set out to analyze the mechanism of liver fibrosis improvement using mouse livers in which an improvement in fibrosis brought about by cell infusion had already been confirmed. Here, we report our findings on the effect on liver fibrosis of cultured human bone marrow-derived cells.

Materials and methods

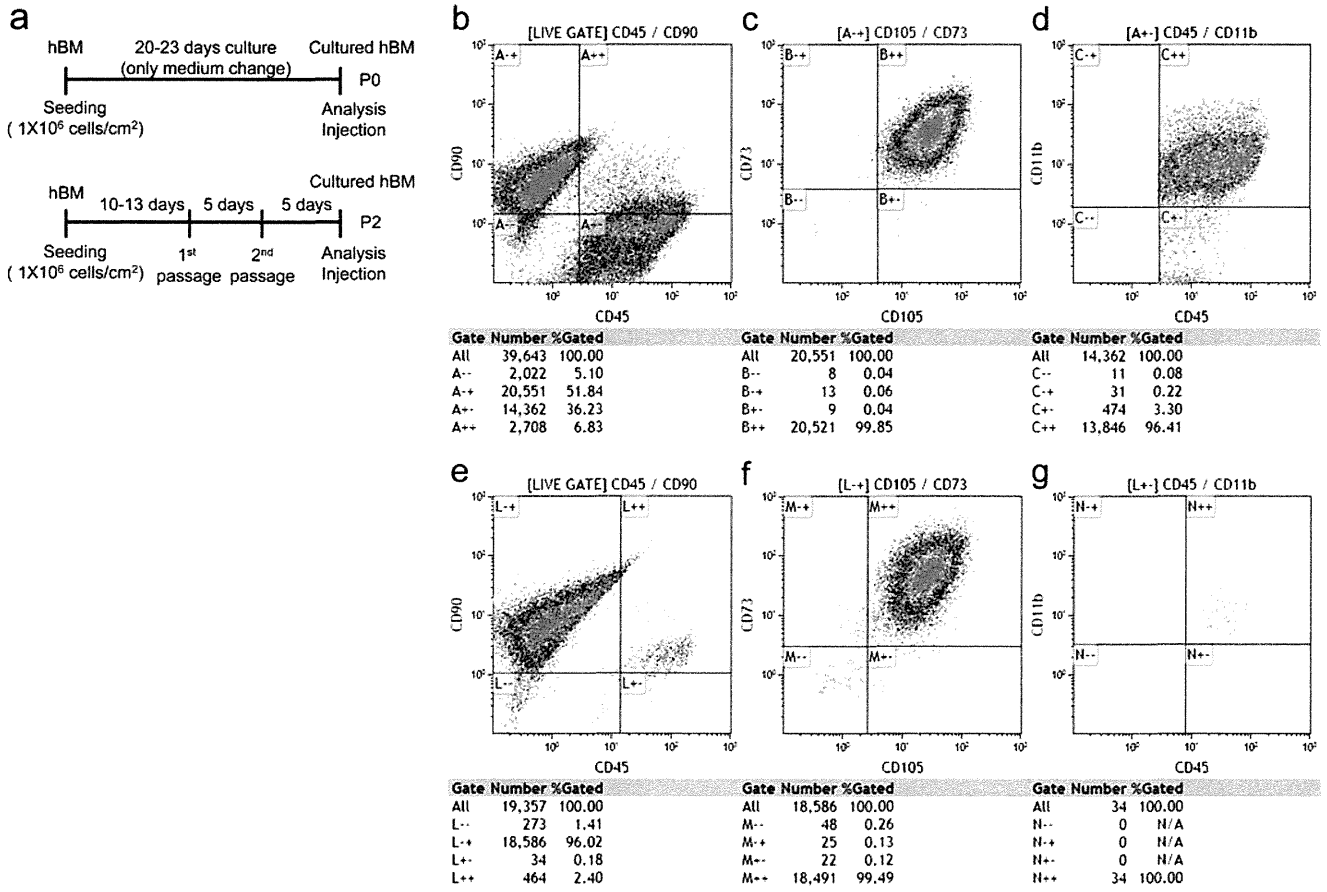
Preparation of culture of human BM-MNCs and human MSCs

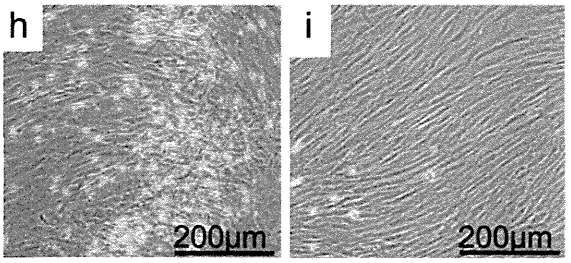
Human BM-MNCs (Code:2M-125A, male, HIV/HBV/HCV-negative) were purchased from Lonza (Basel, Switzerland). Human BM-MNCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (GIBCO) and penicillin/streptomycin (GIBCO) on non-coated dishes (Becton and Dickinson, NJ, USA). Human BM-MNCs were plated at a density of 1×10^6 cells/cm^2 and incubated at 37 °C with 5 % CO_2. After 20–23 days culture, proliferated cells grown under only medium change every 3 days (P0 cells) or through two successive passages on days 10–13 and 5 additional days before confluence (P2 cells) were harvested (Fig. 1a). The cells were detached with trypsin-EDTA (0.05 % trypsin, 0.53 mM EDTA-4Na) (GIBCO) for 5 min at 37 °C. Human MSCs were cultured in the same manner and 2–3 successive passages were performed. Before infusion, these cells were trypsinized and washed twice with phosphate-buffered saline (PBS) (GIBCO).

Characterization of cultured human BM-MNCs

The cell marker expressions of P0 and P2 cells were analyzed by flow cytometry (FACS Calibur; Becton and Dickinson). Cells were stained using the following preconjugated antibodies: CD45, CD90, CD105 (eBioscience, CA, USA), CD73 and

Fig. 1 The majority of cultured human bone marrow-derived mononuclear cells (BM-MNCs) (P2 cells) were phenotypically mesenchymal stem cells (MSCs). **a** Human BM-MNCs (hBM) were plated at a density of 1×10^6 cells/cm^2 on non-coated dishes in 10 % FBS-DMEM and incubated. After 20–23 days of culture, proliferated cells with only medium change every 3 days (P0 cells) or two successive passages on days 10–13 and 5 additional days before confluence (P2 cells) were harvested. **b–d** Cellular characteristics of P0 cells. Typical data from analysis by flow cytometry are shown. P0 cells were approximately fractionated into two subgroups: MSCs and macrophages. CD45 (+) cells (hematopoietic cells) accounted for 43.1 % of P0 cells and macrophages (CD45 and CD11b positive and CD90 (−) cells) accounted for 34.9 %; conversely, 51.8 % were MSCs (CD45 (−) and CD90, CD105 and CD73 positive). **e–g** Cellular characteristics of P2 cells. In contrast to P0 cells, the majority of cultured human BM-MNCs after two passages (P2) were MSCs (95.5 %), 2.6 % were CD45 (+) cells and 0.2 % were macrophages. **h** On the photomicrograph (×100), P0 cells were contaminated with many round hematopoietic cells. Conversely, **i** on the photomicrograph (×100), P2 cells were homogenous fibroblastic-shaped cells. **j** The mean MSC percentage of P2 cells from four healthy men was 94.1±2.6 % and individual differences were small. **k** After 13 days of plating of human BM-MNCs at a density of 1×10^6 cells/cm^2 (3.8×10^6 cells/3.8 cm^2) and incubation in 10 % FBS-DMEM, adhesive cells had proliferated to 0.7± 0.3×10^5 cells/3.8 cm^2 ($n=5$) and under two successive passages (P2) grew to $28.3\pm15.9\times10^5$ cells/3.8 cm^2 ($n=5$)





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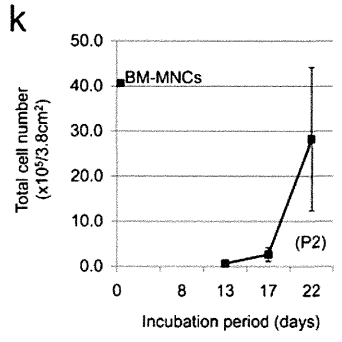
	BM-MNCs	day13 (pre passage)	day17 (1st passage)	day22 (P2) (2nd passage)
Alived cell number (x10⁵/3.8cm²/well) (n=5)	38.0	0.66±0.31	2.7±1.5	28.3±15.9



	P0	P2
MSC(%) (CD90+/CD105+/CD73+/CD45-) (n=4)	66.2±11.7	94.1±2.6

CD11b (Beckman Coulter, CA, USA). Through fluorescence-activated cell sorting analysis, the phenotypical MSC ratio (%) was examined. P2 cells were stained with additional antibodies to confirm MSC phenotypically: CD34, CD13, CD45, CD73, CD90, HLA-DR, HLA-ABC, iso IgG (BD Pharmingen, CA, USA), CD44, CD105, CD11b (Beckman Coulter) and CD117 (Beckton and Dickinson).

Differentiation and DNA-chip analysis

P2 cells were cultured with each differentiation medium and evaluated for adipogenesis (Oil-Red O, anti-mouse FABP-4 antibody), osteogenesis (Alizarin Red) and chondrogenesis (anti-human aggrecan antibody) with a human MSC functional identification kit (Invitrogen, NY, USA). We compared DNA expression between P0 and P2 cells using the DNA-chip system (Agilent Technology, CA, USA). We next also analyzed the expression pattern using the IPA software system (Ingenuity Systems, CA, USA).

Experimental protocol for the NOD-SCID mouse CCl_4 liver cirrhosis model

All animals received humane care and the experiments were approved by the Animal Experiment Committee of Yamaguchi University School of Medicine according to the National Institutes of Health criteria. NOD.CB17-Prkdc^{scid}/J female mice 5 weeks of age purchased from Charles River Laboratories (MA, USA) were properly anesthetized during the experiments.

NOD.CB17-Prkdc^{scid}/J female mice 6 weeks of age were treated with CCl_4 (Wako, Tokyo, Japan) dissolved in corn oil (Wako) (1:3) twice a week for 6 weeks, only once with 0.5 mL/kg body (0.25 μg/g) CCl_4, from the second time with 1.0 mL/kg body weight (0.5 μg/g), and for the last 4 weeks with 1.5 mL/kg body weight (0.75 μg/g). These were used as the control group of the NOD-SCID mouse CCl_4 liver cirrhosis model. Treatment with 1.5 mL/kg body weight CCl_4 was continued for a further 4 weeks. Then, 5.0×10^5 P2 cells for the P2-administration group ($n=18$) and 5.0×10^5 P0 cells for the P0-administration group ($n=7$) were infused through the tail vein, while in the control group ($n=13$), only PBS was injected.

Quantitative analysis of liver fibrosis

After 4 weeks of administration of P2 or P0 cells, the livers of the NOD-SCID mouse CCl_4 liver cirrhosis model were fixed in 4 % formaldehyde and 3-μm paraffin sections were used for analysis. The liver fibrosis area was quantified with Sirius red staining and assessed by application software (BIOREVO microscope BZ-9000, BZ-II; KEYENCE, Osaka, Japan) at a magnification of ×100. The mean value of 10 randomly selected areas per sample was used as the expressed percent area of fibrosis.

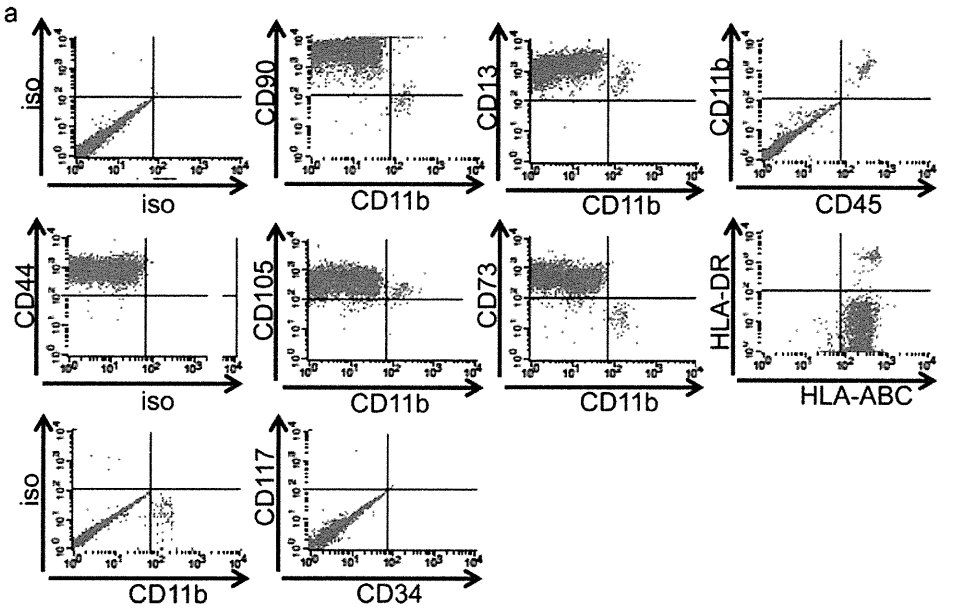
Fig. 2 P2 cells were phenotypically and functionally mesenchymal stem cells (MSCs). **a** The phenotypical character of P2 cells was consistent with that of MSCs. Hematopoietic stem cell marker (CD34)-positive cells in P2 cells were only 0.06 %. **b**–**e** P2 cells (×200) (**b**) differentiated into adipocytes (×200) (**c**), osteocytes(×200) (**d**) and chondrocytes (× 40) (**e**). The control group cells (**b**) were cultured in only 10 % FBS-DMEM. **f** Category of up-regulated genes in P2 cells than those in P0 cells. **g** Category of down-regulated genes in P0 cells than those in P2 cells

Immunohistochemistry of MMP-9 and αSMA

Three-micrometer paraffin sections of livers 2 or 4 weeks after administration of P2 cells were used for immunostaining. MMP-9 and αSMA detection required antigen retrieval with Vector Antigen Unmasking Solution (Vector Laboratories, CA, USA) and the bound antibodies were detected using the avidin-biotin complex method staining kit (Vector Laboratories). Primary antibodies were used at the following dilutions: 1:100 for MMP-9 (R and D Systems, MN, USA) and 1:300 for αSMA (Abcam, Cambridge, UK). Biotinylated antibody was used as the secondary antibody. The number of MMP-9(+) cells was counted at a magnification of ×200 and the mean value of six randomly selected areas per sample was assessed. The αSMA(+) area (%) was quantified at a magnification of ×100 and the mean value of ten randomly selected areas per sample was assessed. The same application software described above was used.

Quantification of messenger RNA levels by real-time reverse-transcription polymerase chain reaction (PCR)

Total RNA was extracted from the liver of mice 4 weeks after P2 cell infusion using RNA extraction solution (ISOGEN; Nippon Gene, Tokyo, Japan) and complementary DNA was generated from 500 ng of RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, IN, USA). Primers for the messenger RNA (mRNA) expression of MMP-9, αSMA, TNFα and TGFβ were evaluated using real-time PCR. Real-time PCR was performed with SYBR Green Master Mix (Roche Diagnostic, Basel, Switzerland). The primers used for MMP-9 were 5'-TCT CTA CGG CCG GCT TTG CT-3' (forward) and 5'-GGC AAG TCT TCA GAG TAG TT-3' (reverse), those for αSMA were 5'-ACT CTC TTC CAG CCA TCT TTC A-3' (forward) and 5'-ATA GGT GGT TTC GTG GAT GC-3' (reverse), those for TNFα were 5'-CAG GTT CTG TCC CTT TCA CTC ACT-3' (forward) and 5'-GTT CAG TAG ACA GAA GAG CGT GGT-3' (reverse), those for TGFβ1 were 5'-TGG AGC AAC ATG TGG AAC TC-3' (forward) and 5'-CAG CAG CCG GTT ACC AAG-3' (reverse) and those for β-actin were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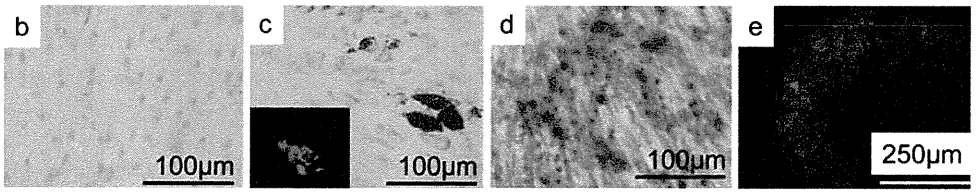
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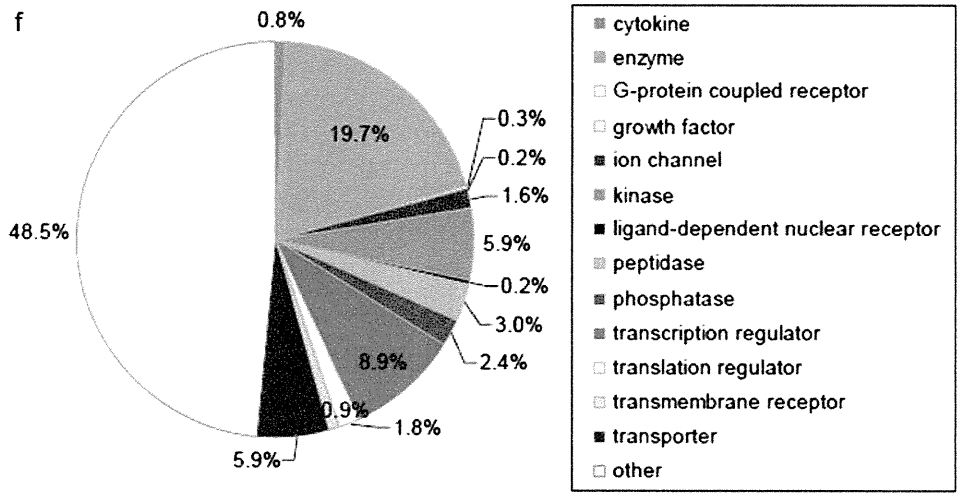
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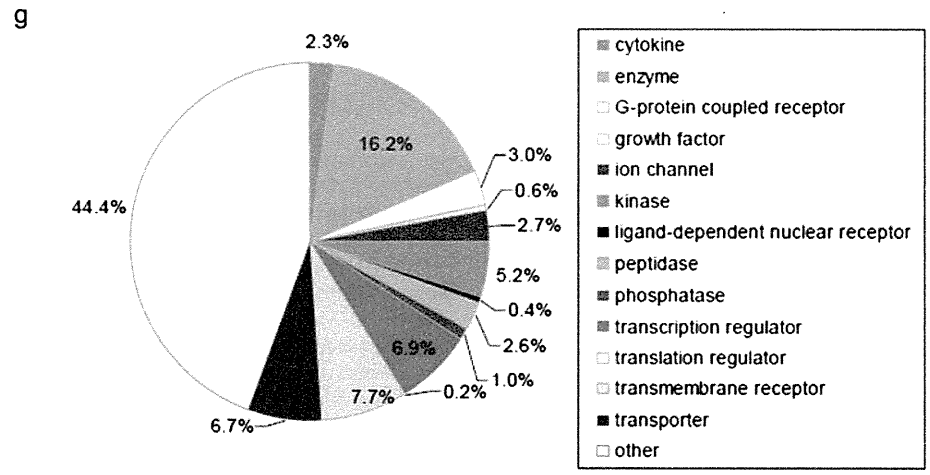
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5'-TGA CAG GAT GCA GAA GGA GA-3' (forward) and 5'-GCT GGA AGG TGG ACA GTG AG-3' (reverse).

Statistics

Data are presented as mean ± standard error of the mean. The two-tailed Student's *t* test was used to analyze parametric data.

Results

The majority of P2 cells were phenotypically and functionally MSCs

Cultured human BM-MNCs without passage (P0 cells) were approximately fractionated into two subgroups: MSCs and macrophages. CD45 (+) cells (hematopoietic cells) accounted for 43.1 % of P0 cells and macrophages [CD45 and CD11b positive and CD90 (−) cells] accounted for 34.9 %; conversely, 51.8 % were MSCs [CD45 (−) and CD90, CD105 and CD73 positive] (Fig. 1b–d). In contrast to P0 cells, the majority of cultured human BM-MNCs under two passages (P2 cells) were MSCs (95.5 %), 2.6 % were CD45 (+) cells and only 0.2 % were macrophages (Fig. 1e–g). On the photomicrograph (×100), P0 cells were contaminated with many round hematopoietic cells; conversely, P2 cells were homogenous fibroblastic-shaped cells (Fig. 1h–i). The mean MSC percentage of P2 cells from four healthy men were 94.1±2.6 % and individual differences were small (Fig. 1j). After 13 days of plating of human BM-MNCs at a density of 1×10^6 cells/cm^2 (3.8×10^6 cells/3.8 cm^2) and incubation in 10 % FBS-DMEM, adhesive cells grew to $0.7\pm0.3\times10^5$ cells/3.8 cm^2 ($n=5$) and, under two successive passages, P2 cells grew to $28.3\pm15.9\times10^5$ cells/3.8 cm^2 ($n=5$) (Fig. 1k). The phenotypical character of P2 cells was consistent with that of MSCs. Hematopoietic stem cell marker (CD34)-positive cells in P2 cells accounted for only 0.06 % (Fig. 2a). P2 cells differentiated into adipocytes, osteocytes and chondrocytes (Fig. 2b–e).

Comparison of DNA expressions between P2 cells and P0 cells

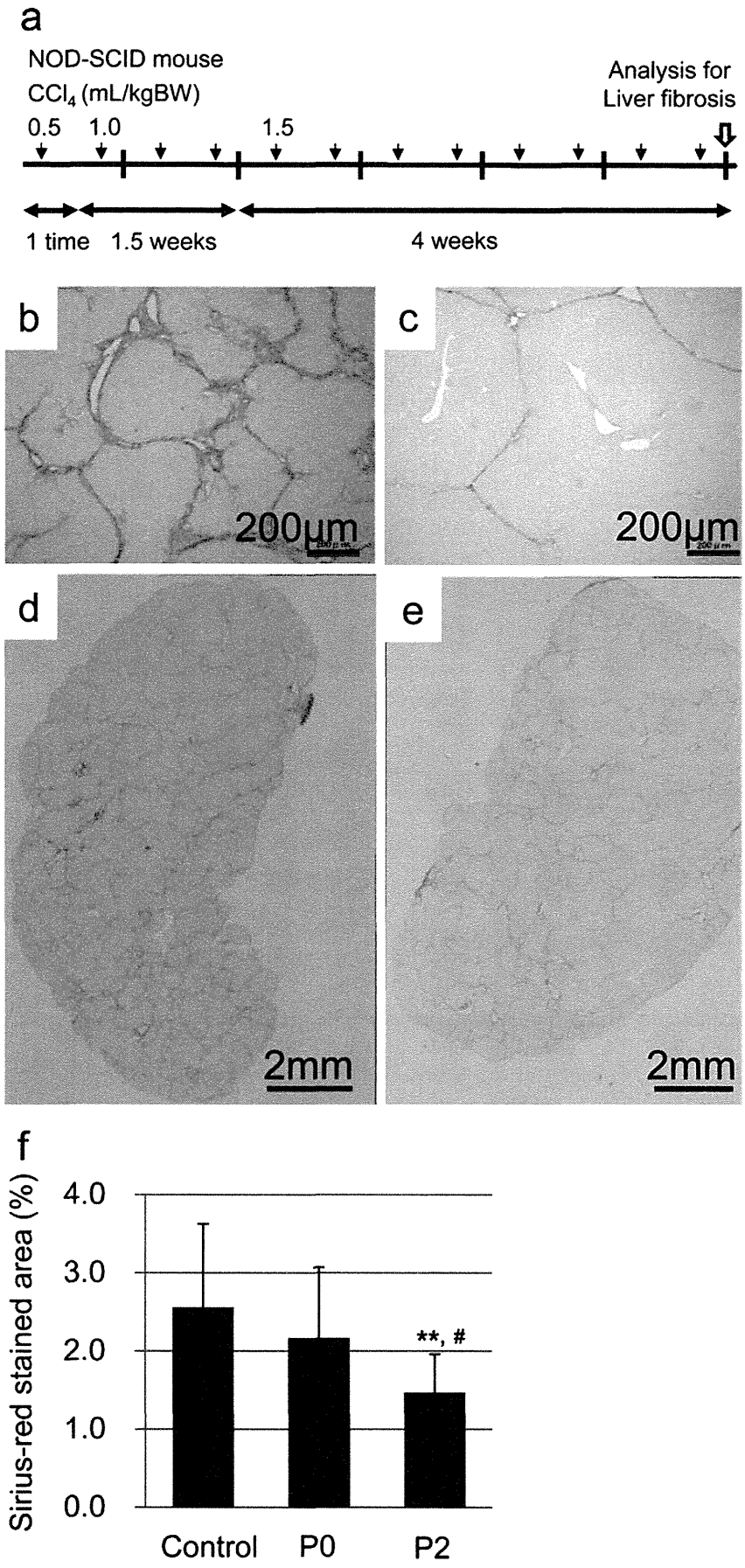
We performed DNA-chip analysis using an IPA system. The majority gene that is upregulated is 1,569 probe. In P2 cells, genes related with cell cycle, G2/M DNA damage checkpoint regulated genes were up-regulated. On the other hand, genes related with function of blood cells, proliferation of blood cells and leukocyte migration were down-regulated, indicating that the macrophage fraction was decreased (Fig. 2f, g). Moreover, in P2 cells, we also confirmed that many clusters of differentiation (CD) markers related to monocytes and lymphocytes were down-regulated, consistent with the decreased macrophage phenotype in P2 cells (Table 1).

Table 1 Down-regulated CD markers in P2 cells versus P0 cells

Symbol	Ratio (log_2)	*P* value	Entrez gene ID	Type of cells
CD2	−2.937	1.63E-02	914	Subcortical/cortical/ medullary thymocyte
CD9	−1.308	4.31E-02	928	Pre-B cell
CD14	−3.379	9.57E-03	929	Promonocyte
CD19	−2.839	3.72E-02	930	Pro-B cell, pre-pre-B cell, pre-B cell, early B cell
CD33	−4.022	1.04E-03	945	CFU-GEMM myeloid stem cell, BFU-E, CFU-M, CFU-G, CFU-Eo, promonocyte, myelocyte
CD36	−3.025	1.65E-02	948	CFU-E
CD37	−3.454	2.02E-02	951	Early B cell
CD52	−4.706	3.00E-03	1,043	Early B cell, cortical/ medullary thymocyte
CD53	−3.348	1.30E-02	963	Monocyte, B cell
CD68	−2.351	1.06E-02	968	Monocyte, DC, granulocyte, B subset
CD74	−3.092	2.21E-03	972	Pre-B cell, early B cell
CD82	−0.743	2.35E-02	3,732	Many hematopoietic cells except RBC
CD83	−1.762	3.24E-02	9,308	MatDC, langerhans cell
CD84	−3.063	3.07E-02	8,832	B cell, monocyte, macrophage, platelet
CD163	−4.059	1.21E-03	9,332	Monocyte, macrophage
CD209	−3.706	6.98E-03	30,835	Monocyte, macrophage
CD302	−1.753	2.05E-02	9,936	DC
CD163L1	−3.496	3.04E-02	283,316	Unknown
CD300A	−3.220	2.20E-02	11,314	Unknown
CD300C	−2.264	3.00E-03	10,871	Unknown

Many CD markers related to monocytes and lymphocytes were down-regulated

Fig. 3 Advanced liver fibrosis was induced in NOD.CB17-Prkdcs^{scid}/J ▶ mice by chronic administration of CCl_4 (NOD-SCID mouse CCl_4 liver cirrhosis model). Liver fibrosis in this model was improved by infusion of P2 cells. **a** NOD.CB17-Prkdcs^{scid}/J female mice 6 weeks of age were treated with CCl_4 dissolved in corn oil (1:3) twice a week for 6 weeks, only once with 0.5 mL/kg body weight CCl_4, from the second time with 1.0 mL/kg body weight and for the last 4 weeks with 1.5 mL/kg body weight. In the NOD-SCID mouse CCl_4 liver cirrhosis model, P2 or P0 cells were infused. Treatment with 1.5 mL/kg body weight CCl_4 was continued further for 4 weeks. **b**–**c** Photomicrograph showing Sirius red staining for hepatic collagens after 4 weeks 5×10^5 P2 cells infusion into the liver cirrhosis model mouse (×100) (**c**) and control PBS-infusion (×100) (**b**). **d**–**e** Photomicrograph of the right lobe of the liver (control group **d** and P2 cells infused group **e**) (original magnification ×100). **f** P2 cells (5×10^5) infused in the liver cirrhosis model mouse resulted in a significant reduction in fibrosis measured by Sirius red quantification after 4 weeks of infusion. The infusion of P2 cells improved liver fibrosis in this liver fibrosis model [$P=0.009$, $^{**}P<0.01$, 1.5±0.5 % ($n=11$) vs. control 2.6±1.1 % ($n=7$); $P=0.048$, $^{\#}P<0.05$ vs. P0-administration group 2.2±0.9 % ($n=7$)]


a
NOD-SCID mouse
CCl4 (mL/kgBW)
0.5 1.0 1.5
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Liver fibrosis
1 time 1.5 weeks 4 weeks
b
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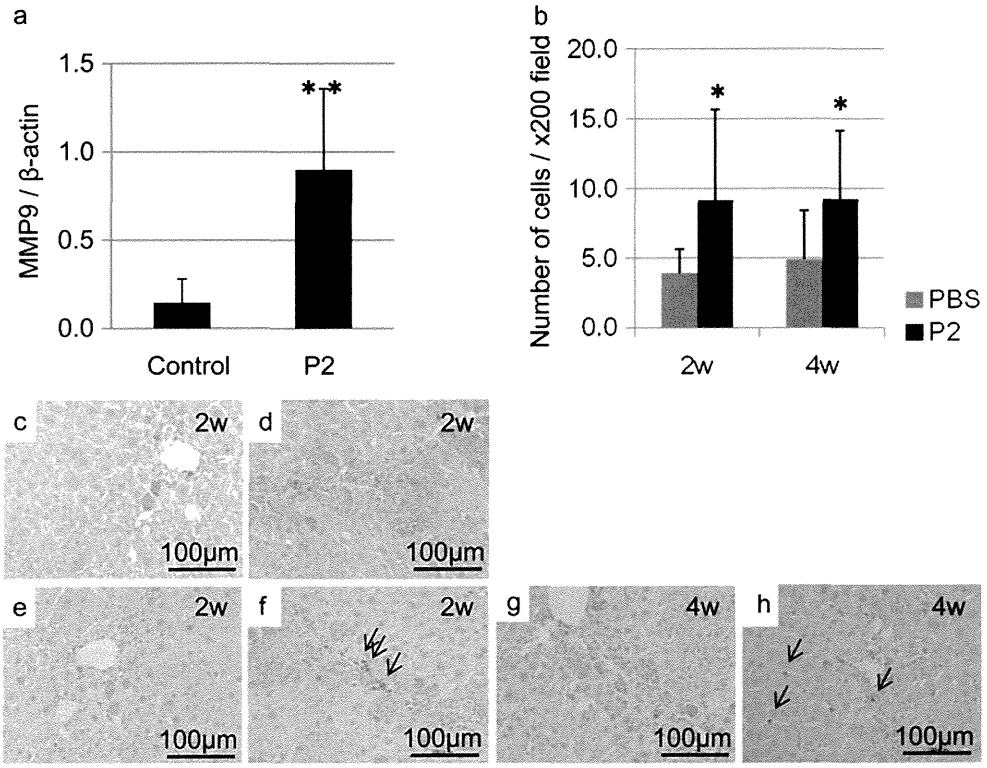

Fig. 4 Matrix metalloproteinase (MMP)-9 expression in P2 cells infused cirrhosis liver was up-regulated. **a** mRNA expression of MMP-9 in the liver after 4 weeks of P2 cells infusion was significantly up-regulated [$P=0.003$, $^{**}P<0.01$, MMP-9/β-actin 0.9±0.5 ($n=6$) vs. 0.2±0.1 control ($n=6$)]. **b** P2 cells also significantly up-regulated MMP-9 protein expression in the liver after 2 and 4 weeks of P2 cells-infusion [$P=0.031$, $^{*}P<0.05$ and $P=0.047$, $^{*}P<0.05$, MMP-9(+) cell number 9.2±6.5 ($n=10$) vs. 3.9±1.7 control ($n=9$), 9.2±4.9 ($n=12$) vs. 4.9±3.5 control ($n=8$)]. **c–f** Photomicrograph of MMP-9-positive cells in the liver after 2 weeks of P2 cells infusion (**f**) (original magnification ×400). *Arrow* indicated. IgG control of PBS group (**c**) and of P2 group (**d**) and MMP-9 expression of PBS control group (**e**). **g–h** Photomicrograph of MMP-9-positive cells in the liver 4 weeks after P2 cells infusion (**h**) (original magnification ×400). *Arrow* indicated. MMP-9 expression of PBS control (**g**)

Cultured human bone marrow-derived cell (P2 cells) infusion improves liver fibrosis

P2 cells (5×10^5) infused into the liver cirrhosis model mice resulted in a significant reduction in fibrosis measured by Sirius red quantification after 4 weeks of the infusion (Fig. 3a). The infusion of P2 cells improved liver fibrosis in this liver fibrosis model [$P=0.009$, $^{**}P<0.01$, 1.5±0.5 % ($n=11$) vs. control 2.6±1.1 % ($n=7$); $P=0.048$, $^{\#}P<0.05$ vs. P0-administration group 2.2±0.9 % ($n=7$)] (Fig. 3b–f).

Up-regulation of MMP-9 expression in P2 cells-infused cirrhosis liver

Messenger RNA expression of MMP-9, which degrades the extracellular matrix, in the liver after 4 weeks of infusion of P2 cells was significantly up-regulated [$P=0.003$, $^{**}P<0.01$, MMP-9/β-actin 0.9±0.5 ($n=6$) vs. 0.2±0.1 control ($n=6$)] (Fig. 4a). P2 cells also significantly up-regulated MMP-9 protein expression in the liver after 2 and 4 weeks of P2 cell infusion [$P=0.031$, $^{*}P<0.05$ and $P=0.047$, $^{*}P<0.05$, MMP-9(+) cell number 9.2±6.5 ($n=10$) vs. 3.9±1.7 control ($n=9$), 9.2±4.9 ($n=12$) vs. 4.9±3.5 control ($n=8$), ×200] (Fig. 4b–h).

Suppressed activation of hepatic stellate cells (HSCs) in P2 cells-infused cirrhosis liver

Messenger RNA expression of αSMA, a marker of activated HSCs, in the liver after 4 weeks of infusion of P2 cells was significantly reduced [$P=0.045$, $^{*}P<0.05$, αSMA/β-actin 0.6;0.2 ($n=4$) vs. 0.9;0.2 control ($n=6$)] (Fig. 5a). The amount of αSMA staining [αSMA(+) area(%)] in the P2 cells infusion group decreased [$P=0.048$, $^{*}P<0.05$, 1.0±0.5 % ($n=8$) vs. 2.0±1.1 % control ($n=6$), ×100] (Fig. 5b–d).

Reduction of TNFα and TGFβ in P2 cells recipients

Messenger RNA expression of TNFα, an inflammatory cytokine, in the liver after 4 weeks of P2 cells infusion was significantly reduced [$P=0.019$, $^{*}P<0.05$, TNFα/β-actin 0.2±0.1 ($n=8$) vs. 2.3±2.3 control ($n=9$)] (Fig. 6a). Messenger RNA expression of TGFβ, which activates HSCs, in the liver after 4 weeks of P2 cells infusion was significantly

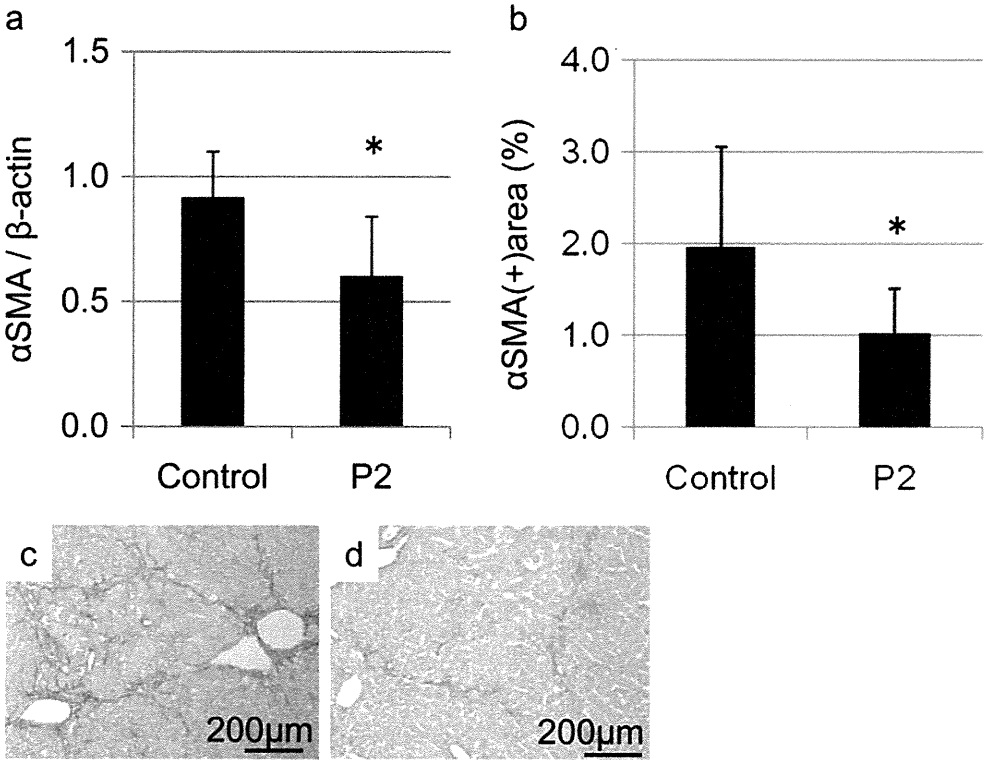

Fig. 5 P2 cells delivery causes a reduction of alpha smooth muscle actin (αSMA)-positive hepatic stellate cells (HSCs). **a** mRNA expression of αSMA in the liver after 4 weeks of P2 cells infusion was significantly reduced [P=0.045, *P<0.05, αSMA/β-actin 0.6±0.2 (n=4) vs. 0.9±0.2 control (n=6)]. **b** The amount of αSMA staining [αSMA(+) area(%)] in the P2 cells-infused group decreased [P=0.048, *P<0.05, 1.0±0.5 % (n=8) vs. 2.0±1.1 % control (n=6)]. **c**–**d** Photomicrographs demonstrate the reduction in αSMA(+) HSCs after 4 weeks of P2 cells delivery (**d**) and PBS control (**c**) (original magnification ×100)

reduced [P=0.049, *P<0.05, TGFβ/β-actin 0.9±0.6 (n=13) vs. 1.5±1.0 control (n=12)] (Fig. 6b).

Discussion

We reported in 2004 that we had administered whole bone marrow cells from a GFP transgenic mouse to model mice with cirrhosis induced by repeated administration of CCl_4 and that an improvement in liver fibrosis, accompanied by improvements in liver function and survival, was obtained with donor-derived bone marrow cells that adhered to the fibrotic regions of the cirrhotic livers and produced fibrolytic enzymes including MMP-9 (Sakaida et al. 2004). With regard to clinical research, we were the first in the world to begin ABM*i* therapy, in 2003 and we demonstrated that it improved liver function in patients with cirrhosis without serious adverse events. At that time, we also found an increase in the number of proliferating

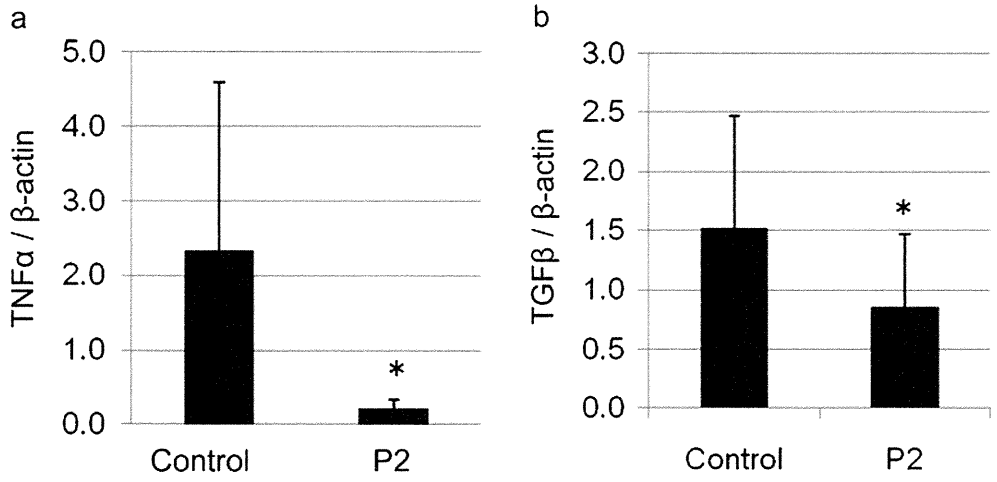

Fig. 6 P2 cells delivery causes a reduction of tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ). **a** mRNA expression of TNFα, an inflammatory cytokine, in the liver after 4 weeks of P2 cells infusion was significantly reduced [P=0.019, *P<0.05, TNFα/β-actin 0.2±0.1 (n=8) vs. 2.3±2.3 control (n=9)]. **b** mRNA expression of TGFβ in the liver, which activates HSCs, after 4 weeks of P2 cells infusion was significantly reduced [P=0.049, *P<0.05, TGFβ/β-actin 0.9±0.6 (n=13) vs. 1.5±1.0 control (n=12)]

cell nuclear antigen-positive cells after infusion of the bone marrow (Terai et al. 2006). Furthermore, based on joint research with our laboratory, Kim et al. of Yonsei University reported that the efficacy of ABM*i* therapy continued for at least 1 year and that, in liver biopsies taken over time, activation of the hepatic progenitor cell fraction was confirmed (Kim et al. 2010). Additionally, based on joint research with our laboratory, Saito et al. of Yamagata University reported that, in patients with alcohol-induced liver cirrhosis, ABM*i* was effective in improving liver function and their findings indicated that bone marrow may activate this process (Saito et al. 2011). As noted above, ABM*i* therapy has been shown to improve the pathological condition of cirrhosis in human clinical research but the cell fraction necessary for this therapeutic effect is still unknown. Meanwhile, ABM*i* therapy has been limited in its application because it requires the collection of 400 mL of bone marrow aspirate under general anesthesia. In reports from other laboratories on cultured bone marrow cells, bone marrow-derived macrophages have been shown to improve liver fibrosis in studies using murine bone marrow cells (Thomas et al. 2011). The proportion of macrophages in murine bone marrow is inherently large and is also large in cultured bone marrow cells. It is possible that the liver fibrosis-improving effect of cultured murine bone marrow cells comes mainly from macrophages. We also performed analyses using a mice model and found that bone marrow cells easily differentiated macrophages and the infusion of the macrophage fraction improved liver fibrosis (Iwamoto et al. 2013; Phinney et al. 1999). Basically, in the mice model, macrophages are easily cultured (Phinney et al. 1999). Huang et al. showed that MSC is also effective in improving liver fibrosis (Huang et al. 2013). So, we believe that both macrophages and MSC might be important for improving liver fibrosis. These are mice data, so we analyzed and cultured human bone marrow cells and then determined whether a cell fraction with this fibrosis-improving effect could be amplified to set up a clinical study.

In anticipation of clinical use in humans, we cultured human BM-MNCs in a medium that contained only 10 % FBS without the addition of growth factors. We carried out subculturing twice and, after approximately 3 weeks, we obtained a sufficient number of cells to expect an effect on liver fibrosis. We collected populations of P2 cells with almost no individual differences, in which the cell fractions were stable and contained roughly 94 % MSCs, a few percent hematopoietic cells consisting mainly in macrophages and less than 0.1 % hematopoietic stem cells (Fig. 1b–i). We found that, in cultured human bone marrow cells, the main component of the P2 cells were MSCs and that there was a clear difference in the proportion of MSCs between P2 and P0. DNA-chip analysis also showed that bone marrow-derived cells also decreased the macrophage fraction. Therefore, the characteristics of culturable bone marrow-derived cells may differ between mice and humans.

Next, P2 cells were infused via the caudal vein and they significantly improved liver fibrosis in an immunodeficient liver cirrhosis mouse model (NOD-SCID mouse CCl_4 liver cirrhosis model) that we developed for this study. Furthermore, we were also able to confirm that cultured human MSCs brought about a significant improvement in liver fibrosis using the same mouse model. Therefore, this study demonstrates that the liver fibrosis-improving effect in the cultured human bone marrow-derived cells originates in MSCs.

In human clinical research, Mohamadnejad and Kharaziha have shown that MSC from bone marrow can improve the pathological condition of liver cirrhosis by infusion both intravenously and via the portal vein (Mohamadnejad et al. 2007; Kharaziha et al. 2009). In addition, Pai et al. have demonstrated that liver function is improved when CD34-positive cells induced from bone marrow cells by granulocyte colony-stimulating factor are grown in vitro and then administered via the hepatic vein (Pai et al. 2008). In contrast, our research has demonstrated that a sufficient number of homogenous cells with almost no individual differences can be recovered after two passages of human BM-MNCs to which only serum was added without the involvement of growth factors, as well as that cultured cell infusion therapy, that can be expected to improve liver fibrosis, is possible through intravenous infusion. In addition, we developed an animal model that enables the evaluation of human cell function, proved the liver fibrosis-improving effect of MSCs in cultured human bone marrow-derived cells (P2) and demonstrated cultured cell collection conditions that are simple and provide a stable effect. In the future, we plan to assess cultured bone marrow-derived cell fractions from patients with liver cirrhosis and evaluate the effect on improving liver fibrosis in this animal model to enable the collection and infusion of even better cells and to determine the prognosis of therapeutic efficacy in patients.

In this study, we have shown that the mechanism of the improvement in liver fibrosis brought about by cultured human bone marrow-derived cells occurs via the enhanced expression of MMP-9, which is important for fibrolysis and a decrease in fiber production brought about by a decrease in HSC activity. Moreover, we have shown that this mechanism acts by controlling the production of cytokines such as TGFβ and TNFα. As shown in Fig. 6a, b, TNFα and TGFβ expressions were decreased. MSC infusion might induce the decrease of these cytokines and improve liver fibrosis. These results might be similar with our previous analysis and show rapid cytokine change after autologous bone marrow cell infusion (Mizunaga et al. 2012). We also report that TNFα signal is important to regulate the improvement of liver fibrosis after bone marrow cell infusion (Hisanaga et al. 2011). These cytokine changes after cultured human bone marrow cell infusion might be important to improve liver fibrosis.

In our ABM*i* therapy, we collected 400 mL of autologous bone marrow aspirate (BM-MNC fraction average, 7.8×10^9

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.

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Original Article

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

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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]

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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 ($P < 0.001$)

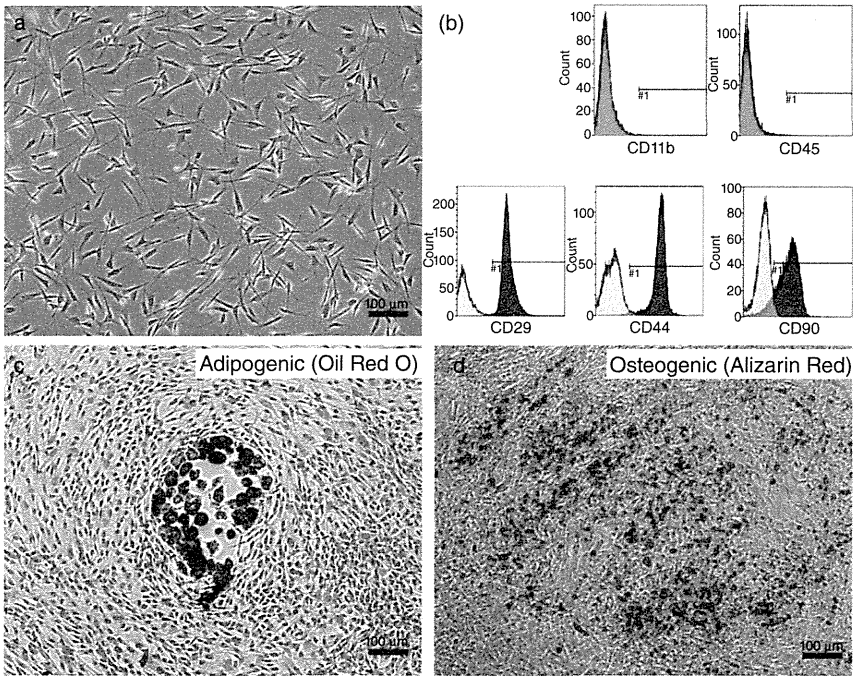

Figure 1 Canine mesenchymal stem cells (cMSC) characterization. (a) cMSC adhered to plastic and showed fibroblast-like morphology (original magnification ×10). (b) cMSC used for infusion showed no CD45 or CD11b expression and were positive for CD90, CD29 and CD44. cMSC showed (c) adipogenic and (d) osteogenic 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

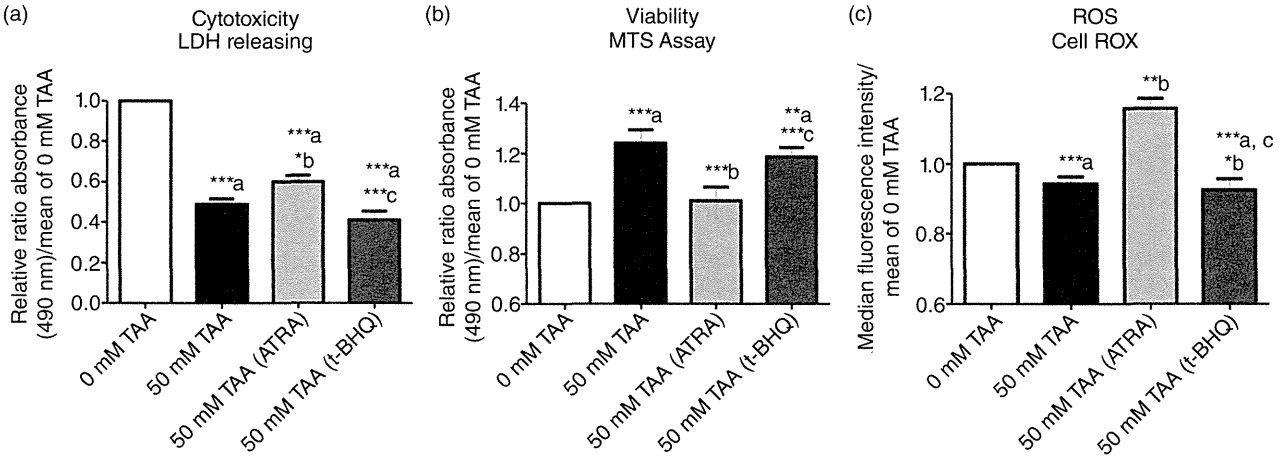

Figure 2 Canine mesenchymal stem cells (cMSC) treated with thioacetamide (TAA). cMSC resisted TAA-induced oxidative stress as verified by (a) lower lactate dehydrogenase (LDH) release into the culture medium (Cytotoxicity), (b) increased viability (MTS assay) and (c) lower cellular ROS levels (Cell Rox 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).

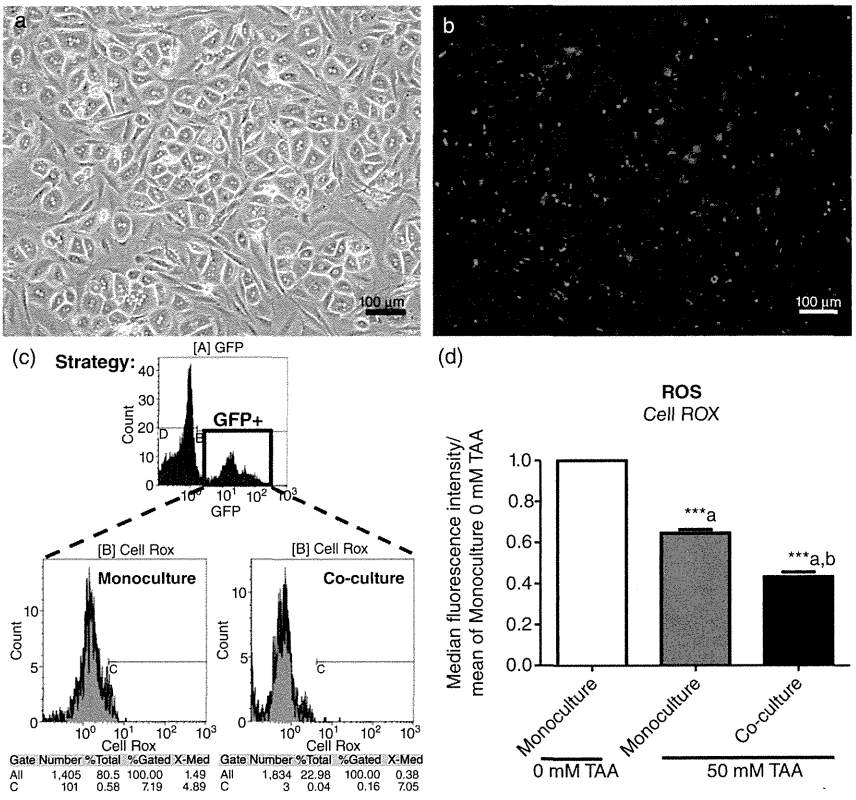

Figure 3 Co-culture with canine mesenchymal stem cells (cMSC) protected hepatocytes from oxidative stress. Murine green fluorescent protein (GFP) positive hepatocytes (green) were cultured alone or in co-culture with CM-DiI-labeled cMSC (red). (a) Bright field and (b) fluorescence microscopy of the co-culture 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.

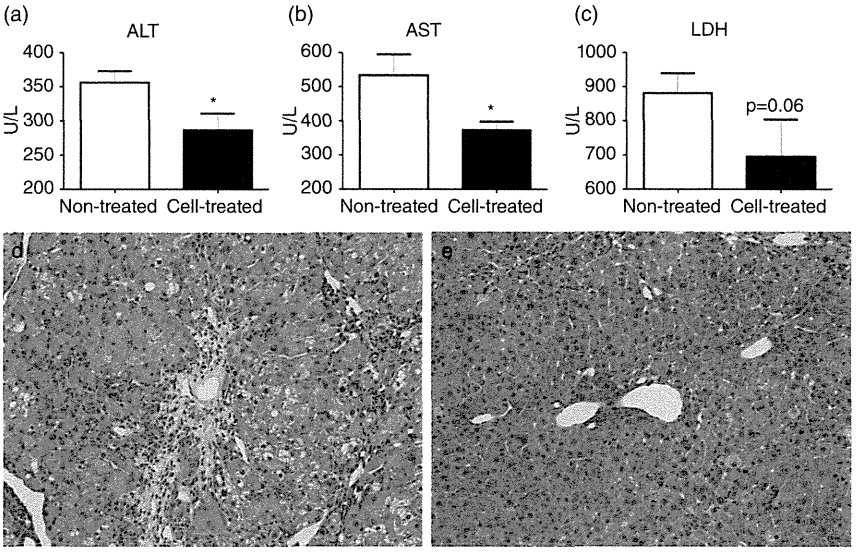

Figure 4 Canine mesenchymal stem cells (cMSC) transplantation protects against thioacetamide (TAA)-induced liver injury. The injury markers (a) alanine aminotransferase (ALT), (b) aspartate aminotransferase (AST) and (c) lower lactate dehydrogenase (LDH) were measured to evaluate the extent of liver 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, $26.9 \pm 19.2\%$ vs $61.4 \pm 13.0\%$ 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