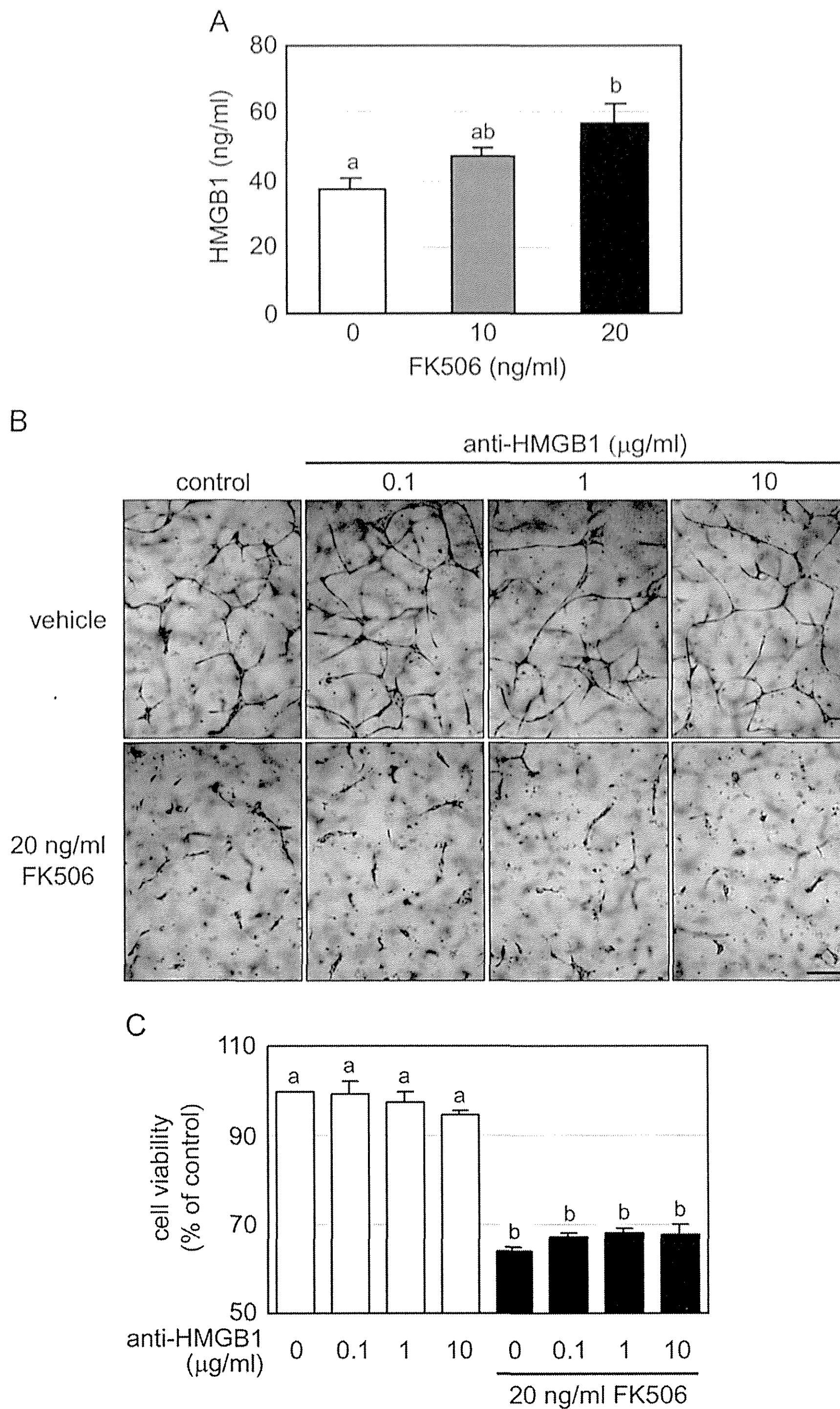

Fig. 2 – HMGB1 is not directly involved in FK506-induced endothelial cell death. (A) Protein levels of HMGB1 in 3D culture supernatants are increased by FK506 in a concentration-dependent manner. Tube-forming HUVEC were treated with FK506 at the indicated concentrations for 48 h. Supernatants in 3D cultures were harvested at 48 h after FK506 treatment, and protein levels of HMGB1 were assessed using ELISA as described in Materials and methods. Values represent means±SEs of three independent experiments. $^{a,b}P<0.05$, values with different letters are significantly different (Bonferroni test). (B) FK506-induced tube breakdown is not suppressed by an anti-HMGB1 neutralizing antibody. Tube-forming HUVEC were incubated with the indicated treatments for 48 h. The bar indicates 100 μm. Representative data of three independent experiments with similar results are shown. (C) The anti-HMGB1 neutralizing antibody fails to suppress FK506-induced cell death. Tube-forming HUVEC were incubated with the indicated treatments for 48 h, and cell viability was assessed using WST-8. Values represent means±SEs of three independent experiments. $^{a,b}P<0.05$, values with different letters are significantly different (Bonferroni test).

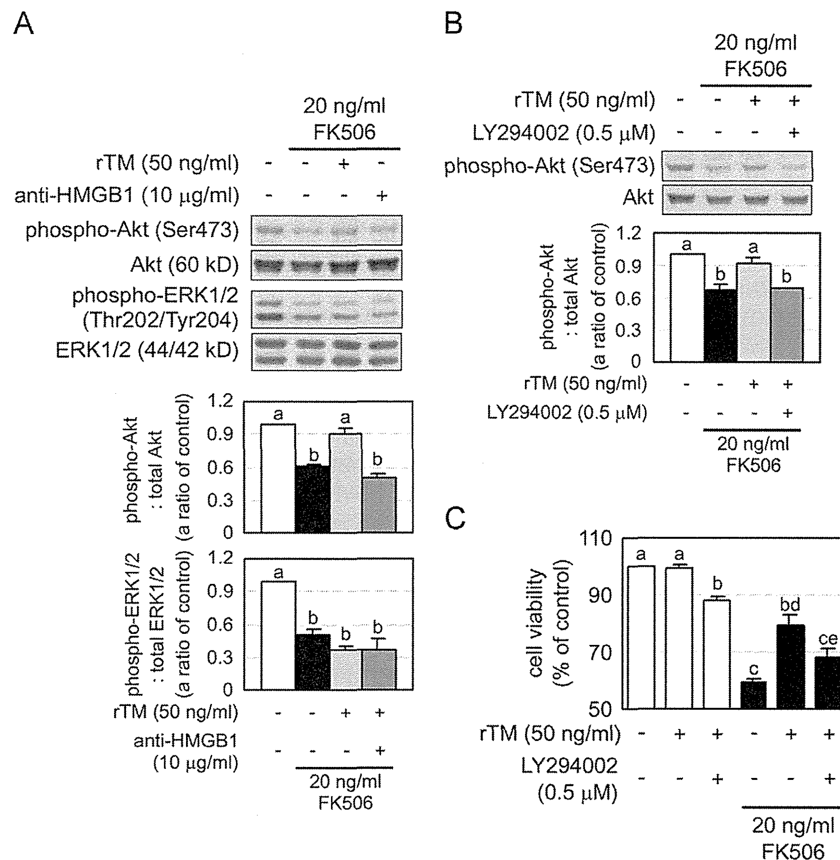

Fig. 3 – rTM prevents FK506-induced Akt inactivation, leading to suppression of FK506-induced endothelial cell death in 3D cultures. (A) rTM suppresses FK506-induced inactivation of Akt, but not of ERK1/2. Cell extracts were prepared from tube-forming HUVEC treated with 20 ng/ml FK506 alone, or together with either 50 ng/ml rTM or 10 μg/ml anti-HMGB1 neutralizing antibody for 48 h. Western blotting was performed three times and the representative figures are shown. Values represent means ± SEs of three independent experiments. $^{a,b}P < 0.05$, values with different letters are significantly different (Bonferroni test). (B) LY294002 abrogates the preventive effect of rTM on FK506-induced Akt inactivation. Cell extracts were prepared from tube-forming HUVEC incubated with the indicated treatments for 48 h. Western blotting was performed three times and the representative figures are shown. Values represent means ± SEs of three independent experiments. $^{a,b}P < 0.05$, values with different letters are significantly different (Bonferroni test). (C) LY294002 also abrogates the suppressive effect of rTM on FK506-induced cell death. Tube-forming HUVEC were incubated with the indicated treatments for 48 h, and cell viability was assessed using WST-8. Values represent means ± SEs of three independent experiments. $^{a,b,c,d,e}P < 0.05$, values with different letters are significantly different (Bonferroni test).

Tube formation and maintenance in 3D cultures

We defined *tube formation* as the first 48 h of incubation, and *tube maintenance* as the following 48 h as described previously [4]. The 96-well culture plates for morphological observation and cell viability analysis, and the 48-well culture plates for Western blotting and Enzyme-linked immunosorbent assay (ELISA) were used. Tube-induction medium was replaced every 24 h, and HUVEC were then incubated for up to 48 h. After tube formation, the fresh tube-induction medium supplemented with 20 ng/ml FK506 or vehicle (dimethylsulfoxide, DMSO) was replaced every 24 h, and incubation continued for an additional 48 h. rTM (Asahi Kasei Pharma, Tokyo, Japan), a polyclonal anti-human HMGB1 antibody (Shino-test, Kanagawa, Japan) or LY294002 (Enzo Life Sciences, Farmingdale, NY), was added to the fresh tube-induction medium supplemented with FK506 or DMSO.

Morphological observation

Morphological observation was performed as described previously [4]. Briefly, HUVEC in 3D cultures were fixed with 1% glutaraldehyde overnight at 4 °C after the treatments described above. HUVEC were stained with 0.1% toluidine blue in 30% methanol, destained, and observed under a light microscope (Nicon, Tokyo, Japan) for bright field images.

Cell viability analysis

Cell viability was determined using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) as described previously [4]. Briefly, Water tetrazolium salt 8 (WST-8), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, was added in the 3D cultures. The compound was

reduced by cellular dehydrogenases to form a water-soluble orange-colored formazan dye. The intensity of color developed was quantified using a micro plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, CA). Experiments were repeated in triplicate.

ELISA for HMGB1

Supernatants of the tube-induction medium were harvested at 48 h after FK506 treatment. A microtiter plate was precoated with a polyclonal anti-human HMGB1 antibody (Shino-test). The supernatant samples and pig HMGB1 (Shino-test) were added to the plate for 24 h at 37 °C. After five washes with 0.05% Tween 20 in phosphate-buffered saline, 100 μl peroxydase-linked anti-HMGB1 monoclonal antibody was added to each well for 2 h at 25 °C. Detection was performed with tetramethyl-benzidine, dihydrochloride, dihydrate, and hydrogen peroxide. Measurements were repeated in triplicate, and sensitivity was 1 ng/ml. The intensity of color developed was quantified using a micro plate reader (SPECTRAmax PLUS384).

Western blotting and antibodies

After the treatments described above, Western blotting was performed as described previously [4]. All antibodies were purchased from Cell Signaling Technology (Beverly, MA). Signal intensities were quantified using the Scion Image 4.0.3 program. The relative changes in the signal intensities of Akt and ERKs were determined by calculating the ratios of phospho-Akt (Ser473): total Akt, and phospho-ERK1/2 (Thr202/Tyr204): total ERK1/2, respectively.

Statistical analyses

All data are presented as the mean ± standard error (SE) of three independent experiments. Differences in mean values among groups were subjected to one-way Factorial analysis of variance (ANOVA) with Bonferroni test for multiple comparisons, and were considered significantly different at $P<0.05$.

Results and discussion

We examined the effects of rTM on FK506-induced endothelial dysfunction using a 3D culture blood vessel model. rTM showed little effect on tube maintenance of the tube structures induced by bFGF and VEGF (Fig. 1A, upper panels). FK506 caused breakdown of tube structures as we reported previously [4], while rTM at 0.05–5 μg/ml, which is within the range of therapeutic windows as an anticoagulatnt [12], showed no effect on FK506-induced tube breakdown (Fig. 1A, lower panels). rTM had no effect on cell viability (Fig. 1B), and FK506 caused cell death as reported [4]. Intriguingly, FK506-induced cell death was significantly suppressed by rTM at 0.05–5 μg/ml (Fig. 1B). These results suggest that rTM suppressed FK506-induced endothelial cell death but not tube breakdown, and it could be postulated that FK506-induced tube breakdown and cell death are regulated through different pathways.

rTM shows anti-inflammatory effects including degradation of HMGB1, a mediator of inflammation, and antibodies that neutralize HMGB1 confer protection against HMGB1-mediated tissue injury [7,8]. To clarify the role of HMGB1 in the FK506-induced cell death, we measured protein levels of HMGB1 in the 3D culture supernatants using ELISA. HMGB1 levels were increased by FK506 in a concentration-dependent manner (Fig. 2A). However, the anti-HMGB1 neutralizing antibody did not suppress the FK506-induced tube breakdown (Fig. 2B) and cell death (Fig. 2C). These results suggest that HMGB1 is released from FK506-treated HUVEC, but HMGB1 is not directly involved in FK506-induced endothelial dysfunction.

To investigate how rTM suppresses FK506-induced endothelial cell death, we analyzed signal transduction in tube-forming HUVEC. It has been reported that a portion of the extracellular domain of TM, which lacks an N-terminal lectin-like domain, facilitates angiogenesis through activation of Akt and ERK1/2 [13,14]. However, we observed that rTM had no direct effect on tube formation induced by bFGF and VEGF (data not shown), which is consistent with the finding that rTM has little effect on migration of HUVEC [15]. As previously reported [4], we confirmed that FK506 induced inactivation of Akt and ERK1/2 (Fig. 3A). rTM, but not the anti-HMGB1 neutralizing antibody, significantly prevented FK506-induced Akt inactivation (Fig. 3A), while rTM alone had no effect on Akt activation induced by bFGF and VEGF (data not shown). However, neither rTM nor anti-HMGB1 neutralizing antibody prevented FK506-induced ERK1/2 inactivation (Fig. 3A). Furthermore, LY294002, an inhibitor of the phosphatidylinositol 3-kinase/Akt pathway, significantly abrogated the preventive effect of rTM on FK506-induced Akt inactivation (Fig. 3B), and the suppressive effect of rTM on FK506-induced endothelial cell death (Fig. 3C). These results suggest that rTM prevents FK506-induced Akt inactivation, leading to suppression of FK506-induced endothelial cell death. Our present study also suggests that Akt regulates endothelial cell death, and a possibility that ERK1/2 may regulate tube breakdown (Fig. 4).

Conclusions

We clarified for the first time using a 3D culture blood vessel model that rTM attenuates FK506-induced endothelial dysfunction through

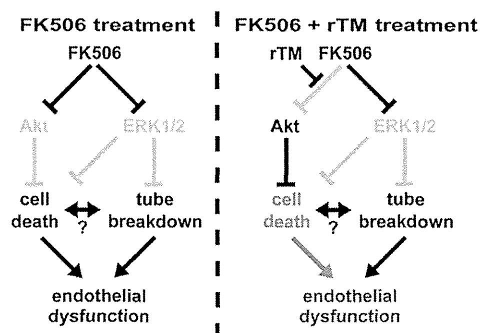

Fig. 4 – Scheme of the effect of attenuation by rTM on FK506-induced endothelial dysfunction. FK506 induces inactivation of Akt and ERK1/2, leading to endothelial dysfunction. FK506-induced endothelial cell death is caused by Akt inactivation. Concurrently, FK506-induced tube breakdown is not mediated by Akt inactivation. rTM suppresses FK506-induced Akt inactivation followed by the prevention of FK506-induced endothelial cell death, leading to attenuation of endothelial dysfunction.

prevention of Akt inactivation (Fig. 4). This novel finding may provide a therapeutic rationale of rTM for FK506-induced endothelial dysfunction.

Conflict of interest

The authors declare no conflict of interest.

Funding and Acknowledgments

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Comparison of Angiogenic, Cytoprotective, and Immunosuppressive Properties of Human Amnion- and Chorion-Derived Mesenchymal Stem Cells

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Abstract

Although mesenchymal stem cells (MSCs) can be obtained from the fetal membrane (FM), little information is available regarding biological differences in MSCs derived from different layers of the FM or their therapeutic potential. Isolated MSCs from both amnion and chorion layers of FM showed similar morphological appearance, multipotency, and cell-surface antigen expression. Conditioned media obtained from amnion- and chorion-derived MSCs inhibited cell death caused by serum starvation or hypoxia in endothelial cells and cardiomyocytes. Amnion and chorion MSCs secreted significant amounts of angiogenic factors including HGF, IGF-1, VEGF, and bFGF, although differences in the cellular expression profile of these soluble factors were observed. Transplantation of human amnion or chorion MSCs significantly increased blood flow and capillary density in a murine hindlimb ischemia model. In addition, compared to human chorion MSCs, human amnion MSCs markedly reduced T-lymphocyte proliferation with the enhanced secretion of PGE2, and improved the pathological situation of a mouse model of acute graft-versus-host disease. Our results highlight that human amnion- and chorion-derived MSCs, which showed differences in their soluble factor secretion and angiogenic/immuno-suppressive function, could be ideal cell sources for regenerative medicine.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Mesenchymal stem cells (MSCs) residing within various tissues, including bone marrow [1] and adipose tissue [2], are reported to differentiate into various types of cells including osteoblasts, chondrocytes, and adipocytes. This multipotency renders MSCs an attractive therapeutic source for regenerative medicine. However, because an invasive procedure is required to obtain autologous bone marrow or adipose tissue-derived MSCs, an alternative source of MSCs that can be obtained non-invasively is desirable.

Appendages of the fetus, which consist of the placenta, umbilical cord, and fetal membrane (FM), are normally discarded after delivery as medical waste. A large quantity of MSCs could be obtained without harm from the human FM because of its size (> 40×40 cm), which represents an advantageous characteristic as a source of cell therapy. We have previously reported the therapeutic potential of rat FM-derived MSCs using various rat models including hindlimb ischemia, autoimmune myocarditis, glomerulonephritis, renal ischemia-reperfusion injury, and myocardial infarction [3–8]. Although the FM is composed of the amnion and chorion, and both layers contain MSCs [9], it is technically difficult to separate these membranes as well as their MSCs in rat.

Thus, the purposes of this study were: 1) to isolate and characterize MSCs from human amnion and chorion; 2) to examine their differences in the expression profile of growth factors and cytokines; and 3) to investigate the therapeutic potential and difference of these MSCs using murine hindlimb ischemia and acute graft-versus-host disease (GVHD) models.

Materials and Methods

Ethics Statement

The study protocol and informed consent procedure were approved by the ethics committee of the National Cerebral and

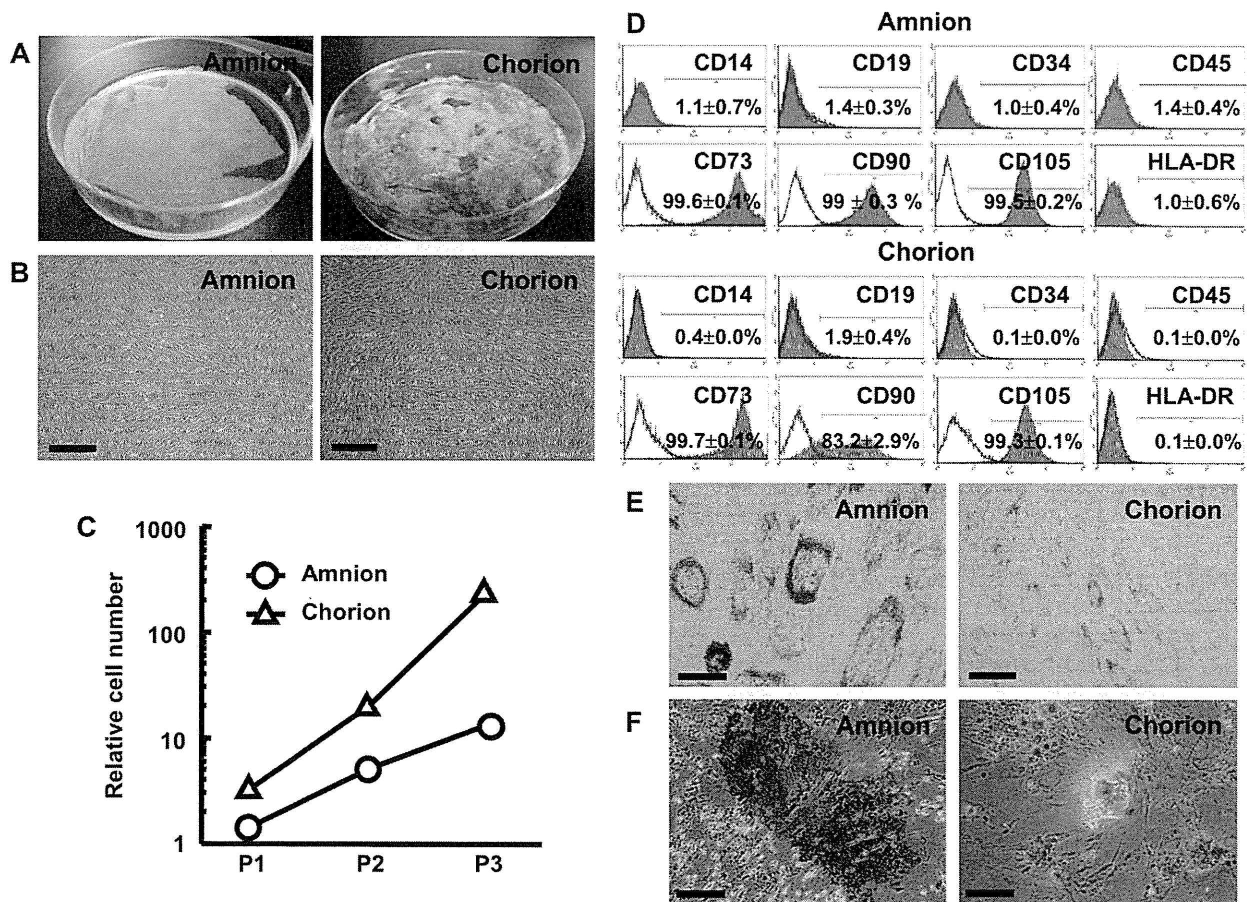

Figure 1. Characterization of human amnion- and chorion-derived MSCs. (A) Representative photographs of human amnion and chorion. (B) Photographs of cultured MSCs obtained from human amnion and chorion at passage 3. Scale bars = 500 μm. (C) Relative cell number of amnion- and chorion-derived MSCs at each passage. (D) FACS analysis of amnion and chorion MSCs. (E, F) Differentiation of amnion and chorion MSCs into adipocytes (E) and osteocytes (F). Scale bars = 100 (E) and 50 (F) μm.
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Cardiovascular Center (Permit Number: M18-042-4). Animal protocols were approved by the Animal Care Committee of the National Cerebral and Cardiovascular Center Research Institute (Permit Number: 13052). Animal studies were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

Isolation and Expansion of Amnion- and Chorion-derived MSCs from Human FMs

After obtaining written informed consent, FMs were obtained following cesarean section of healthy donor mothers. Amnion and chorion were separated by mechanical peeling of the FM, and digested with type-II collagenase solution (5 ml/g tissue and 300 U collagenase/mL, Worthington Biochemicals, Lakewood, NJ) for 1 h at 37°C in a waterbath shaker. After filtration with a mesh filter, cells were suspended in α-minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen), and incubated at 37°C with 5% CO_2 after plating on a dish. The adherent, spindle-shaped MSCs developed visible symmetric colonies by days 1 to 2.

Characterization of Human Amnion and Chorion MSCs

For defining FM-MSCs, we referred to the criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [10].

Cultured MSCs were analyzed by FACSCalibur (BD Biosciences). Cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal against human CD14 (clone M5E2), CD19 (clone HIB19), CD34 (clone 581), CD45 (clone HI30), CD73 (clone AD2), CD90 (clone 5E10), CD105 (clone 266), or HLA-DR (clone G46-6 (L243)), all purchased from BD Biosciences. Isotype identical antibodies served as controls.

To induce differentiation into osteocytes, MSCs were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan) according to the manufacturer's instructions. After 14–17 days of differentiation, cells were fixed and stained with Alizarin Red S (Sigma-Aldrich, St. Louis, MO).

To induce adipocyte differentiation, MSCs were cultured with adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methyl-

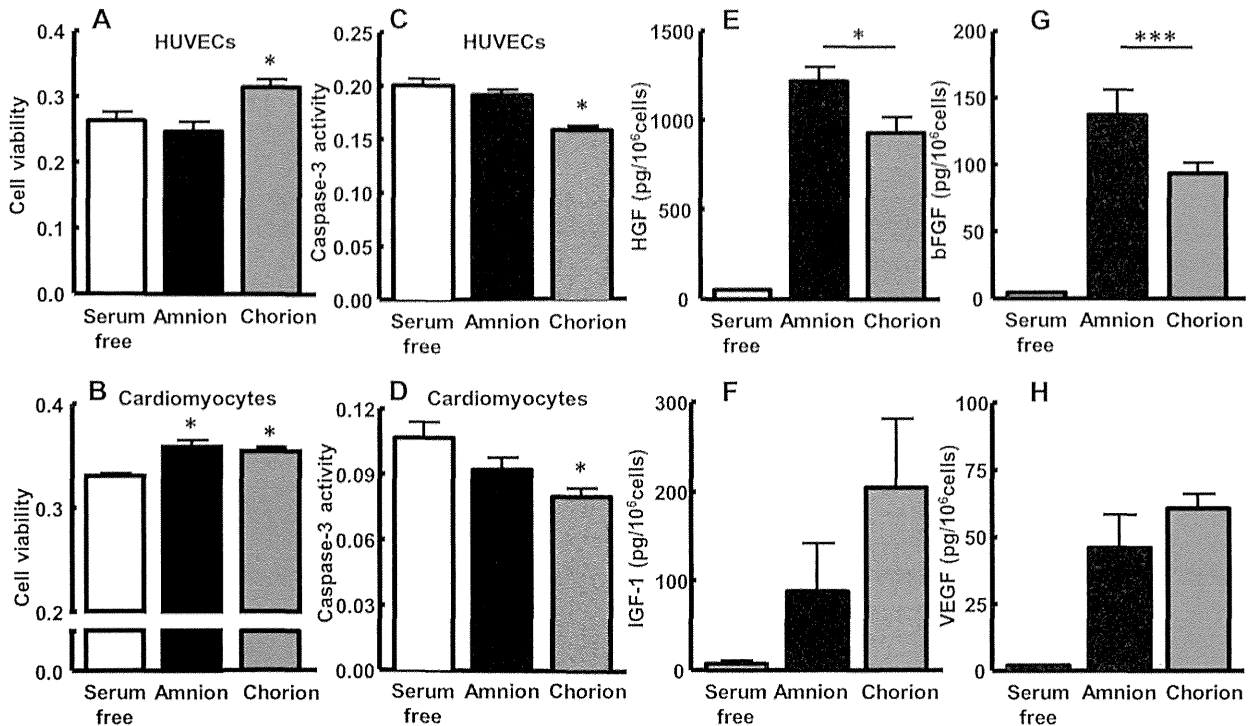

Figure 2. Growth factor secretion and the cytoprotective effect of amnion and chorion MSCs. (A–D) Cytoprotective effect of FM MSC-derived conditioned medium was analyzed by the MTS assay (A, B) and caspase-3 activity (C, D) in HUVECs (A, C) and cardiomyocytes (B, D). Values are mean ± SEM. *$p<0.05$ vs. serum-free. (E–H) Conditioned medium obtained from FM-derived MSCs was collected after incubation for 24 h. The concentration of HGF (E), IGF-1 (F), bFGF (G), and VEGF (H) in serum free conditioned medium was measured by ELISA. *$p<0.05$ and ***$p<0.001$. doi:10.1371/journal.pone.0088319.g002

xanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μM dexamethasone (Wako), 50 μM indomethacin (Wako), and 10 μg/mL insulin (Sigma-Aldrich) in α-MEM supplemented with 10% FCS. After 21 days of differentiation, adipocytes were stained with Oil Red O (Sigma-Aldrich).

Conditioned Medium Analysis of FM-MSC-associated Cytoprotective Function

Human umbilical vascular endothelial cells (HUVECs; Lonza, Basel, Switzerland) were seeded onto a collagen-coated plate and incubated in medium 199 (Invitrogen) supplemented with 20% FCS for 24 h. Neonatal rat cardiomyocytes were isolated from Lewis rats on postnatal day 1, as described previously [11], and seeded onto a laminin-coated plate followed by incubation in α-MEM supplemented with 10% FCS for 24 h. Cells were then subjected to serum deprivation with/without hypoxia (1% O_2) by culturing with serum-free medium or serum-free conditioned medium obtained from FM-MSCs cultured for 24 h. The cellular level of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium (MTS), indicative of cell viability, as well as caspase-3 activity, was measured with a CellTiter96 AQueous One Solution Kit (Promega, Madison, WI) and a CaspACE™ Assay System Kit (Promega), according to the manufacturer's instructions.

Analysis of FM-MSC Production of Growth Factors and Prostaglandin E2

Conditioned media were collected from MSCs cultured in α-MEM with/without 10% FCS for 24 h (n = 4–6). The concentrations of the following growth factors were measured using ELISA kits: hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE2), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

FM-MSC Transplantation in the Hindlimb Ischemia Model

Six-week-old male KSN nude mice were anesthetized with pentobarbital, and the right common iliac artery was resected. After surgery, amnion MSCs (1×10^6 cells/50 μL PBS), chorion MSCs (1×10^6 cells/50 μL PBS), or PBS (50 μL PBS) was injected into the ischemic muscle with a 30-gauge needle at five different sites (n = 15 in each group). A laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) was used to measure serial hindlimb blood flow for 7 days, as previously described [12].

Five and seven days after MSC transplantation, ischemic hindlimb tissues were obtained and snap-frozen. Frozen tissue sections were stained with anti-mouse CD31 antibody (BD Biosciences) to detect capillary endothelial cells. Ten fields were randomly selected to count the number of capillaries. The adjusted capillary number per muscle fiber was used to compare the differences in capillary density between the three groups.

In vitro CD4+ T cell Proliferation Assay

Peripheral blood mononuclear cells were prepared from buffy coats obtained from healthy donors by centrifugation through Ficoll-Paque (GE healthcare, Uppsala, Sweden). CD4+ T cells were isolated by magnetic bead depletion of CD8, CD14+,

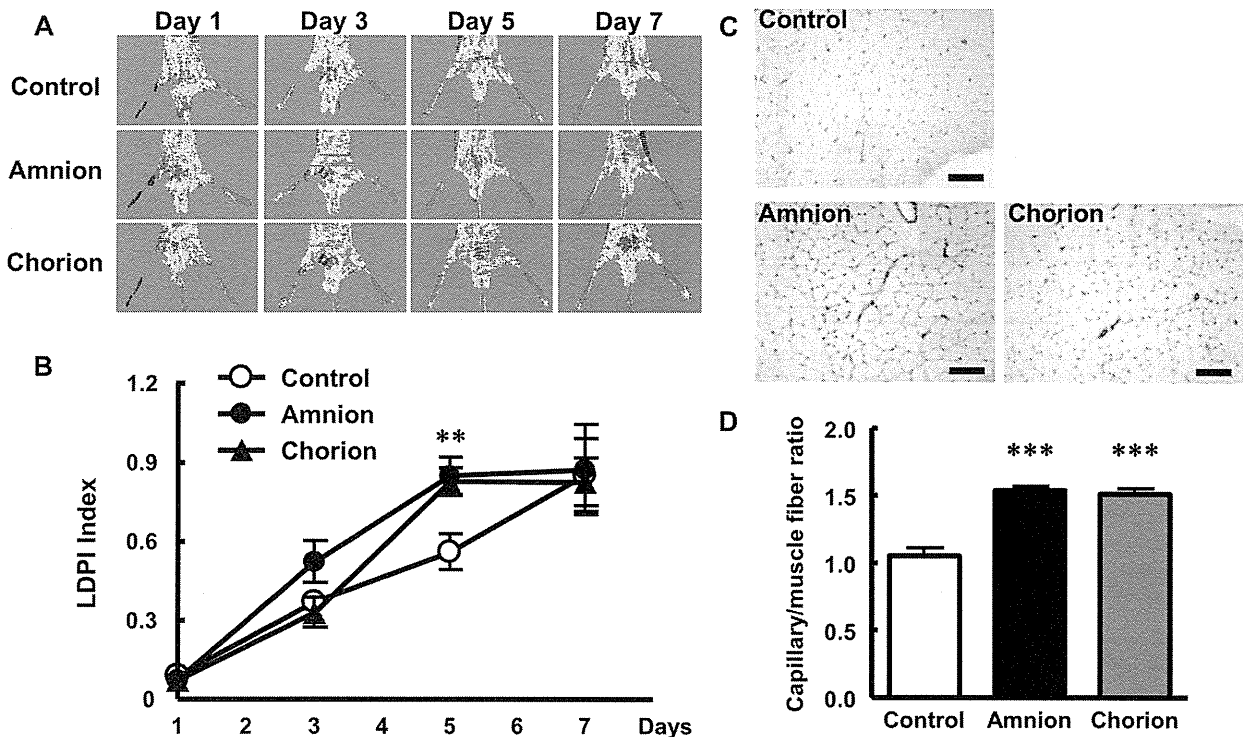

Figure 3. Angiogenic potential of amnion and chorion MSCs against hindlimb ischemia. (A) Representative images of serial hindlimb blood perfusion. Blood perfusion of ischemic hindlimb increased in the amnion and chorion MSC groups at day 5. (B) Quantitative analysis of hindlimb blood perfusion with the LDPI index, the ratio of ischemic to non-ischemic hindlimb blood perfusion. (C) Representative photographs of immunohistochemistry with anti-CD31 antibody. Scale bars = 100 μm. (D) Quantitative analysis of capillary density in ischemic hindlimb muscle at day 5 among the control, amnion, and chorion MSC groups. Capillary density is shown as the capillary-to-muscle-fiber ratio. Data are mean ± SEM. **$p<0.01$ and ***$p<0.001$ vs. control.

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CD15+, CD16+, CD19+, CD36+, CD56+, CD123+, T cell receptor-gamma/delta, and glycophorin A–positive cells (CD4+ T Cell Isolation Kit) on an AutoMACS instrument (Miltenyi Biotec). CD4+ T cells (5×10^5 cells/well) were cultured with X-VIVO medium (Lonza, Walkersville, MD) containing 2% FBS and 5 μg/ml anti-CD28 antibody (clone CD28.2, BioLegend, San Diego, CA) in anti-CD3-precoated 24-well culture plates (clone OKT3, BioLegend). During in vitro proliferation of CD4+ T cells, human amnion-, chorion-, or bone marrow-derived (Lonza) MSCs were co-cultured at 5×10^4 cells/well. After 5 days of co-culturing, T cells were separated from the monolayer MSCs and counted with an automated cell counter (Countess, Invitrogen).

FM-MSC Transplantation into the Acute GVHD Model

Seven- to eight-week-old female B6C3F1 (recipient; C57BL/6×C3H/He; H-$2^{b/k}$) and BDF1 (donor; C57BL/6×DBA/2; H-$2^{b/d}$) mice were purchased from Japan SLC (Shizuoka, Japan). Recipient mice were lethally irradiated with 15 Gy total body irradiation (TBI; X-ray) split into two doses separated by 2 h. On the following day, donor-derived cells (1×10^7 bone marrow cells and 3×10^7 spleen cells) were suspended in 0.2 mL RPMI-1640 medium (Invitrogen) and transplanted via the tail vein into the post-irradiation recipient mice. On days 14, 17, 21, and 25 after hematopoietic stem cell transplantation, 1×10^5 amnion or chorion MSCs in 0.1 mL RPMI medium were transplanted via the tail vein. In the control group, the same amount of RPMI was infused via the tail vein. The severity of GVHD was evaluated by measuring the body weight of mice.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (S.E.M). Comparisons of parameters for more than three groups were made by one-way analysis of variance (ANOVA) followed by the Newman-Keuls' test. Comparisons of the time-course of the LDPI index were made by two-way ANOVA for repeated measures, followed by Bonferroni tests. A p value <0.05 was considered statistically significant.

Results

Characterization of Amnion and Chorion MSCs

From each human FM, 23.5±3.7 g amnion and 37.6±2.5 g chorion could be separated (n = 5 and n = 3, respectively) (Figure 1A). By enzymatic digestion, over one million cells per gram of the amnion ($1.9\pm0.2\times10^6$/g, n = 5) or chorion ($1.3\pm0.3\times10^7$/g, n = 3) were obtained. At passage 3, cultured cells from both layers were fibroblast-like, spindle-shaped cells, and there was no difference in morphology according to the origin of layers (Figure 1B). Cell-doubling time of amnion MSCs (32.2±1.13 h) was equal to that of chorion MSCs (34.1±1.94 h) (Figure 1C).

Both amnion- and chorion-derived MSCs expressed CD73, CD90, and CD105, but not CD14, CD19, CD34, CD45, or HLA-

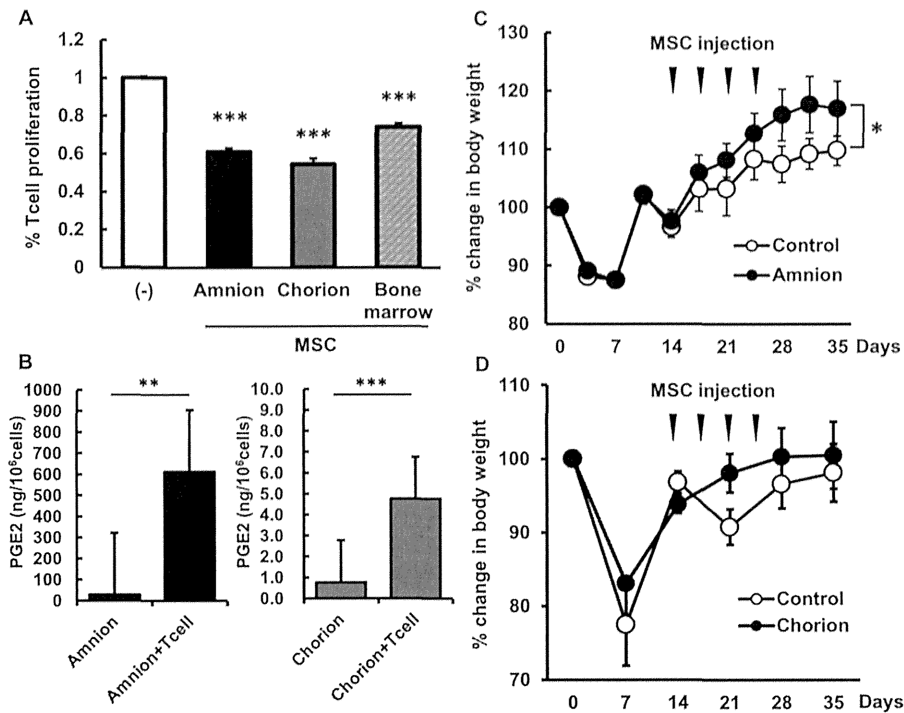

Figure 4. Immunosuppressive property of amnion and chorion MSCs. (A) Inhibition of human CD4+ T cell proliferation upon co-culture with human amnion, chorion, and bone marrow MSCs. (B) The concentration of PGE2 in FM-MSC-conditioned medium was measured by ELISA. Amnion MSCs secreted a significant amount of PGE2 compared with chorion MSCs. (C, D) Effect of human amnion (C) or chorion (D) MSC transplantation in a murine GVHD model. Treatment with amnion MSCs significantly reduced recipient weight loss in a mouse model of GVHD. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

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DR (Figure 1D), which satisfied the criteria for identifying MSCs [10]. In addition, amnion and chorion MSCs could differentiate into adipocytes and osteocytes, as demonstrated by positive Oil Red O and Alizarin Red S staining, respectively (Figure 1E and 1F).

Cytoprotective Effects of Amnion and Chorion MSCs on Endothelial Cells and Cardiomyocytes

To evaluate the cytoprotective effect of amnion and chorion MSCs, we examined cell viability and apoptosis of HUVECs and neonatal rat cardiomyocytes cultured under serum deprivation. In the MTS assay, cell viability of cardiomyocytes was significantly increased when cultured with conditioned medium obtained from amnion and chorion MSCs (absorbance value: serum-free control 0.331±0.002, amnion MSCs 0.359±0.006; $p<0.001$, and chorion MSCs 0.355±0.004; $p<0.01$ vs. control) (Figure 2B). Cell viability of HUVECs also increased when cultured with chorion MSC-derived conditioned medium (serum-free control 0.263±0.013, amnion MSCs 0.247±0.014, and chorion MSCs 0.313±0.012; $p<0.05$ vs. control) (Figure 2A). Similarly, conditioned medium obtained from chorion MSCs significantly decreased the caspase-3 activity of HUVECs (absorbance value: serum-free control 0.201±0.006 vs. chorion MSCs 0.159±0.004; $p<0.001$) and cardiomyocytes (control 0.106±0.007 vs. chorion MSCs 0.079±0.004; $p<0.05$) (Figure 2C, D). Amnion MSC-derived conditioned medium also showed a tendency to decrease the caspase-3 activity of these cells, but without statistical significance.

Secretion of Growth Factors from Cultured Amnion- and Chorion-derived MSCs

To investigate the secretion of major growth factors from MSCs, we performed ELISA of HGF, IGF-1, bFGF, and VEGF. The differences in the cellular expression profile of the growth factors were observed in these FM-derived MSCs (Figure 2E–H). Among these growth factors, amnion MSCs secreted significant amounts of HGF (1217.2±80.2 pg/10^6 cells; $p<0.001$ vs. chorion-MSC) and bFGF (137.2±18.5 pg/10^6 cells; $p<0.05$ vs. chorion-MSC) compared with chorion MSCs (HGF: 932.5±85.3 pg/10^6 cells, bFGF: 93.6±8.1 pg/10^6 cells) (Figure 2E, G). There was no significant difference between amnion and chorion MSCs in the level of secreted IGF-1 (88.8±53.4 pg/10^6 cells and 205±77.0 pg/10^6 cells, respectively) and VEGF (46.1±12.3 pg/10^6 cells and 60.7±5.3 pg/10^6 cells, respectively) (Figure 2F, H).

Augmentation of Angiogenesis in the Ischemic Hindlimb after Human FM-MSC Transplantation

Analysis of LDPI revealed that accelerated limb perfusion was observed in the amnion and chorion MSC-transplanted groups (Figure 3A). The LDPI index was significantly higher in the amnion and chorion MSC groups (amnion MSCs: 0.85±0.07; $p<0.01$, chorion MSCs: 0.83±0.05; $p<0.01$) than in the control group (0.56±0.07) 5 days after transplantation (Figure 3B). At 7 days after transplantation, there was no difference between the treated and control groups.

Immunostaining with the endothelial marker CD31 showed significant augmentation of capillaries in the amnion and chorion

MSC-treated groups compared with the control group (Figure 3E). The capillaries-to-muscle-fiber ratio of ischemic muscle at day 5 after transplantation was significantly increased in the amnion and chorion MSC groups (amnion MSCs: 1.53±0.03/muscle fiber; $p<0.001$, chorion MSCs: 1.51±0.04/muscle fiber; $p<0.001$) compared with the control group (1.05±0.06/muscle fiber; Figure 3F). At day7, the capillaries-to-muscle-fiber ratio of ischemic muscle was also increased in the amnion or chorion MSC-transplanted mice (amnion MSCs: 1.67±0.17/muscle fiber, chorion MSCs: 1.43±0.09/muscle fiber) compared to the control mice (1.36±0.11/muscle fiber).

Immunosuppressive Property of Human FM-MSCs

Although the number of T cells was markedly increased under proliferating conditions of human CD4+ T cells stimulated with anti-CD3 and -CD28 antibodies, the increase was significantly suppressed when co-cultured with amnion-, chorion-, or bone marrow-derived MSCs (61.1±1.8%, 54.6±3.0%, 74.0±2.1%, respectively. $p<0.001$ vs. control) (Figure 4A).

PGE2 is a well-known immune modulator in bone marrow MSCs [13] and we confirmed that amnion MSCs in culture secreted a significant amount of PGE2 (29.7±7.8 ng/10^6 cells), particularly when co-cultured with human CD4+ T cells (613.1±139.9 ng/10^6 cells; $p<0.01$ vs. amnion MSCs) (Figure 4B). In chorion MSCs, however, the concentration of PGE2 was relatively low (0.77±0.13 ng/10^6 cells) but significantly increased in co-culture with CD4+ T cells (4.76±0.47 ng/10^6 cells; $p<0.001$ vs. chorion MSCs). The experiments were repeated with two or three independent MSC/CD4+ T cell donor pairs and the data are presented as the measured mean levels.

In addition, to evaluate the potential of FM-MSCs to suppress acute GVHD, mice underwent allogeneic hematopoietic stem cell transplantation and treatment with human FM-MSCs. As shown in Figure 4C, the loss in body weight of recipient mice after allogeneic hematopoietic stem cell transplantation was significantly reduced with concomitant transplantation of human amnion-derived MSCs. In human chorion MSC-transplanted group, however, no significant changes in body weight was observed during the observation period (Figure 4D).

Discussion

Human MSCs derived from bone marrow or adipose tissue exert a regenerative effect in animal models and human patients [14]. In addition, several reports have described the therapeutic potential of transplanted cells derived from the appendages of the fetus, including amniotic epithelium cells [15], and amniotic fluid- [16], amnion-, and chorion-derived MSCs [17,18]. We have previously demonstrated the therapeutic potential of rat FM-MSCs using various rat models including hindlimb ischemia, autoimmune myocarditis, glomerulonephritis, renal ischemia-reperfusion injury, and myocardial infarction [3–8]. Recent studies including ours also revealed the angiogenic and immunosuppressive property of human fetal appendage-derived MSCs [14,18–20], but comparative studies of the therapeutic effects among these MSCs are lacking. Therefore, in this study, we examined the differences in the cellular function and therapeutic properties between human FM-derived amnion and chorion MSCs.

It is known that MSCs exert their regenerative effects through differentiation into specific cell types, but recent studies suggest that their ability to stimulate regenerative effects is mainly induced via paracrine effects [3,4,8,21]. This theory is substantiated by several reports that MSCs secrete various growth factors and cytokines including VEGF, IGF-1, HGF, adrenomedullin (AM), and PGE2 [3–5,8,21,22]. In this study, we first confirmed that chorion MSCs as well as amnion MSCs secreted significant amount of these soluble factors, which would contribute to accelerating regenerative effects. Compared with chorion MSCs, amnion MSCs secreted significantly larger amounts of HGF and bFGF. However, amnion MSCs secreted less IGF-1 compared to chorion MSCs. We assume that these differences in the cytokine expression profile might reflect the angiogenic and cytoprotective properties of amnion and chorion MSCs, as we observed difference in the effect on endothelial cells and cardiomyocytes in our conditioned-medium analysis. However, the actual function of amnion or chorion MSC-derived cytokines should be further investigated *in vivo* because both human amnion and chorion MSC transplantation similarly induced angiogenesis in the hindlimb ischemia model.

Previous reports have shown that PGE2 is a major modulator of the MSC-induced anti-inflammatory response [13]. In this study, a noteworthy finding was a distinctly high concentration of PGE2 in amnion MSCs in comparison with chorion MSCs, particularly when co-cultured with CD4+ T cells. Because of their high PGE2 production, human amnion MSCs might be a better cell source from an immunosuppressive point of view. In fact, we proved for the first time that human amnion MSCs, but not chorion MSCs, improved the pathological situation of an acute GVHD model. Because our previous study demonstrated that human amnion MSCs markedly inhibited differentiation as well as proliferation of Th1/Th17 cells [6], human amnion MSCs could effectively suppress Th1/Th17 immunity and improve outcome in GVHD.

The merit of using FMs lies in that they are free form ethical concern and that a large number of MSCs can be obtained considering the size of FM. As more than one or ten million MSCs per gram of the amnion or chorion could be obtained, more than 10^9 or 10^{10} MSCs could theoretically be obtained at passage 3 within one month, respectively. Now we are planning to initiate clinical studies with human amnion MSCs in acute GVHD and Crohn's disease, and we need more than 10^{10} MSCs for the treatment of one patient. We are convinced that human FM-MSCs are an attractive source for cell therapy because of their easy availability compared with other somatic, embryonic stem, and iPS cells.

In conclusion, both amnion and chorion MSCs have angiogenic, cytoprotective, and immunomodulatory effects. Because of high PGE2 production and immunosuppressive properties, human amnion MSCs have the advantage for the treatment of immune-related diseases. In addition, since a large number of MSCs could be obtained from FMs, human amnion and chorion MSCs would be a useful cell source for regenerative medicine.

Author Contributions

Conceived and designed the experiments: KY AT TS HO JY MHS KK TI. Performed the experiments: KY KH MO SI SO HT KO SK JY TI. Analyzed the data: KY KH MO TI. Contributed reagents/materials/analysis tools: KY KH MO TI. Wrote the paper: KY KH MO TI.

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ORIGINAL ARTICLE

Allogeneic transplantation for primary myelofibrosis with BM, peripheral blood or umbilical cord blood: an analysis of the JSHCT

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To determine whether a difference in donor source affects the outcome of transplantation for patients with primary myelofibrosis (PMF), a retrospective study was conducted using the national registry data on patients who received first allogeneic hematopoietic cell transplantation (HCT) with related BM ($n = 19$), related PBSCs ($n = 25$), unrelated BM ($n = 28$) or unrelated umbilical cord blood (UCB; $n = 11$). The 5-year OS rates after related BM, related PBSC and unrelated BM transplantation were 63%, 43% and 41%, respectively, and the 2-year OS rate after UCB transplantation was 36%. On multivariate analysis, the donor source was not a significant factor for predicting the OS rate. Instead, performance status (PS) $\geqslant 2$ (vs PS 0–1) predicted a lower OS ($P = 0.044$), and RBC transfusion $\geqslant 20$ times before transplantation (vs transfusion $\leqslant 9$ times) showed a trend toward a lower OS ($P = 0.053$). No advantage of nonmyeloablative preconditioning regimens in terms of decreasing nonrelapse mortality or increasing OS was found. Allogeneic HCT, and even unrelated BM and UCB transplantation, provides a curative treatment for PMF patients.

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Keywords: idiopathic myelofibrosis; hematopoietic SCT; donor source; engraftment; survival

INTRODUCTION

Primary myelofibrosis (PMF) is a clonal stem cell disorder characterized by anemia, BM fibrosis, progressive splenomegaly, constitutional symptoms and a significant risk of evolution into acute leukemia.[1,2] The median age at diagnosis is $\sim$65 years, with a median survival of $\sim$5 years after diagnosis, depending on the presence or absence of clinically defined prognostic factors, such as those defined by the International Prognostic Scoring System (IPSS), Dynamic IPSS and Dynamic IPSS plus.[3–5] No available conventional drug therapies for PMF have been shown to prolong survival. Palliative therapeutic options include agents such as hydroxyurea, prednisone, EPO, androgens, thalidomide and lenalidomide, and nonpharmacological approaches such as blood transfusion, splenic irradiation and splenectomy.[6,7] The impact of new agents, such as Janus kinase 2 (JAK2) inhibitors, pomalidomide and histone deacetylase inhibitors, on the long-term management of PMF is under investigation.[7,8] The only known curative therapy for PMF is allogeneic hematopoietic cell transplantation (HCT).[9]

The largest retrospective study of PMF patients undergoing allogeneic BM or PBSC transplantation reported OS of 30–40% at 5 years after transplantation with nonrelapse mortality (NRM) of 24–43% at 1 year after transplantation.[10] The prospective study in patients with PMF or secondary myelofibrosis to evaluate a nonmyeloablative preconditioning regimen followed by mainly PBSC transplantation achieved an OS of 51% at 5 years after transplantation with NRM of 16% at 1 year after transplantation.[11] The issues of the choice of stem cell source, the choice of conditioning regimen and the timing of transplantation are currently under debate.[6–9,12,13]

To determine whether a difference in stem cell source affects the outcome of HCT for PMF patients, a retrospective study was conducted using the national registry data on patients who received first allogeneic HCT in Japan with BM, PBSCs or umbilical cord blood (UCB).

PATIENTS AND METHODS

Patients

Clinical data for patients with PMF who received first allogeneic HCT in Japan were extracted from the Transplant Registry Unified Management Program (TRUMP) system, which is a registry of the outcomes of Japanese transplant patients.[14] Patients who had progressed to myelofibrosis from polycythemia vera, essential thrombocythemia, leukemia or other disease were excluded. This study was approved by the Data Management Committee of the Japan Society for Hematopoietic Cell Transplantation (JSHCT) and by the ethics committee of the Nagoya University School of Medicine (no. 2012-0270).

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Definitions

Hematopoietic recovery was defined as time to ANC $\geqslant 0.5 \times 10^9$/L, time to reticulocytes $\geqslant 10$‰ and time to platelets $\geqslant 50 \times 10^9$/L for 3 consecutive days. Engraftment failure was defined as no neutrophil recovery by day 60. Acute and chronic GVHD were diagnosed and graded according to established criteria.[15,16] Based on the report by the Center for International Blood and Marrow Transplant Research (CIBMTR),[17] the conditioning regimens were classified as myeloablative if TBI >8 Gy, oral BU $\geqslant 9$ mg/kg, i.v. BU $\geqslant 7.2$ mg/kg or melphalan >140 mg/m^2 was included in the conditioning regimen, whereas other conditioning regimens were classified as nonmyeloablative.

End points

The primary end point was OS. The secondary end points were engraftment, GVHD, relapse and NRM.

Statistical analysis

The probabilities of hematopoietic recovery, acute and chronic GVHD, relapse and NRM were estimated on the basis of cumulative incidence curves.[18] The probability of OS was estimated according to the Kaplan–Meier method.[19] The groups were compared using the log-rank test. Competing risk regression analysis was used to identify factors associated with NRM. The adjusted probability of OS was estimated using Cox's proportional hazards model, with consideration of other significant clinical variables in the final multivariate models.[20] All variables significant at $P<0.10$ on univariate analysis were included in multivariate stepwise analyses. All tests were two sided, and $P<0.05$ was considered significant. The data were analyzed by STATA version 12 statistical software (StataCorp, College Station, TX, USA).

RESULTS

Patient and transplantation characteristics

A total of 83 patients met the inclusion criteria. Patient and transplantation characteristics are summarized in Table 1. The median age at transplantation was 53 years, and most patients (66%) were male. Transplants were performed between 1993 and 2009, but the majority (90%) of them were performed after 2000. This population consisted of 47 BM transplants, 25 PBSC transplants and 11 UCB transplants. Of the 44 related donor transplants, 40 (91%) were performed from serological HLA-A, B and DR 6/6 matched donor; 28 unrelated BM transplants included 16 (57%) HLA-A, B and DRB1 alleles 6/6 matched donors and 11 (39%) HLA-A, B and DRB1 alleles 5/6 matched donors; all (100%) unrelated UCB transplants were performed from serological HLA-A, B and DR 5/6 or 4/6 matched donors. Most patients (76%) received a nonmyeloablative regimen. The median follow-up for living patients was 40 (range, 0.4–150) months.

Table 1. Patient and transplantation characteristics ($n=83$)

	N (%)
Age at transplant, evaluable n	83
21–39 Years	9 (11)
40–49 Years	22 (27)
50–59 Years	37 (44)
60–79 Years	15 (18)
Median age (range), years	53 (21–79)
Sex, evaluable n	83
Female	28 (34)
Male	55 (66)
Transplant year, evaluable n	83
1993–1999	8 (10)
2000–2004	22 (27)
2005–2009	53 (63)
Performance status at transplant, evaluable n	70
0–1	54 (77)
⩾2	16 (23)
Time from diagnosis to transplant, evaluable n	80
<1 Years	33 (41)
1–2 Years	16 (20)
⩾2 Years	31 (39)
Median (range), years	1.5 (0.1–21.0)
Frequency of RBC transfusion before transplant, evaluable n	51
⩽9	26 (51)
10–19	8 (16)
⩾20	17 (33)
Frequency of PLT transfusion before transplant, evaluable n	51
⩽9	38 (74)
10–19	4 (8)
⩾20	9 (18)
Use of JAK2 inhibitor before transplant, evaluable n	77
Yes	0 (0)
No	77 (100)
Splenectomy before transplant, evaluable n	78
Yes	2 (3)
No	76 (97)
DIPSS at transplant	78
Low	8 (10)
Intermediate-1	17 (22)
Intermediate-2	50 (64)
High	3 (4)
Splenomegaly at transplant	78
Yes	59 (76)
No	19 (24)
CMV serostatus, evaluable n	58
Negative	5 (9)
Positive	53 (91)
Donor source, evaluable n	83
Related BM	19 (23)
Related PBSCs	25 (30)
Unrelated BM	28 (34)
Unrelated umbilical cord blood	11 (13)
Sex matching between patient and donor, evaluable n	71
Match	35 (49)
Female patient and male donor	15 (21)
Male patient and female donor	21 (30)
ABO matching between patient and donor, evaluable n	65
Match	34 (52)
Mismatch	31 (48)
Preconditioning regimen, evaluable n	71
Myeloablative	17 (24)
Nonmyeloablative	54 (76)
Prophylaxis for GVHD, evaluable n	81
CsA based	37 (46)
Tacrolimus based	42 (52)
Others	2 (2)
Use of JAK2 inhibitor after transplant, evaluable n	78
Yes	0 (0)
No	78 (100)

Abbreviations: DIPSS = Dynamic International Prognostic Scoring System; JAK2 = Janus kinase 2.

Engraftment

Seven patients (8%) died without engraftment within 60 days after transplantation, including heart failure on day 5 after UCB transplant ($n=1$), primary disease on day 7 after related PBSC transplant ($n=1$), infection on day 11 after unrelated BM transplant ($n=1$), multiple organ failure on day 12 after unrelated BM transplant ($n=1$), heart failure on day 18 after unrelated BM transplant ($n=1$), infection on day 30 after unrelated BM transplant ($n=1$) and thrombotic microangiopathy on day 56 after UCB transplant ($n=1$). Another patient (1%) received a second transplant on day 28 because of lack of engraftment signs at that time.

Neutrophil recovery on day 60 occurred in 92% (95% confidence interval (CI), 57–99%) of related BM, 92% (71–98%) of related PBSCs, 79% (58–90%) of unrelated BM and 82% (45–95%) of unrelated UCB (Figure 1a). Unrelated BM and unrelated UCB (vs related BM) transplantations were significantly associated with a lower probability of neutrophil recovery ($P=0.015$ and $P=0.016$, respectively), whereas related PBSC transplantation was

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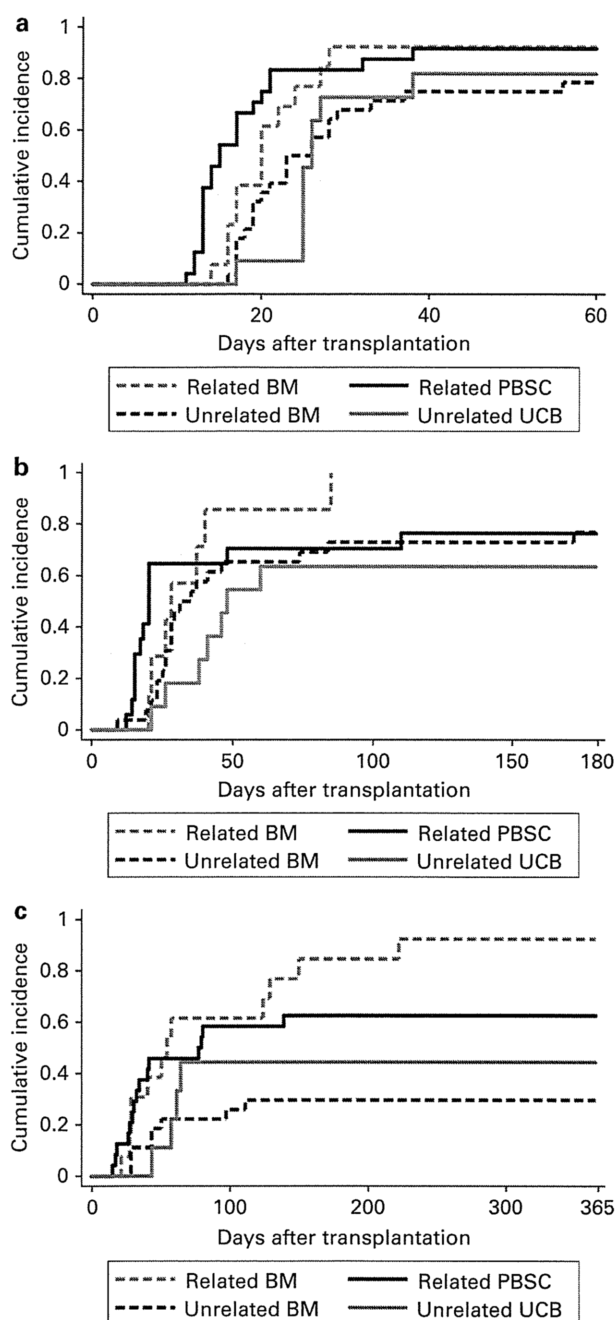

Figure 1. Hematopoietic recoveries after transplantation in PMF patients. (**a**) Cumulative incidences of neutrophil recovery after related BM (gray and dash line), related PBSC (black and solid line), unrelated BM (black and dash line) and unrelated UCB (gray and solid line) transplantations are shown. (**b**) Cumulative incidences of reticulocyte recovery after related BM (gray and dash line), related PBSC (black and solid line), unrelated BM (black and dash line) and unrelated UCB (gray and solid line) transplantations are shown. (**c**) Cumulative incidences of platelet recovery after related BM (gray and dash line), related PBSC (black and solid line), unrelated BM (black and dash line) and unrelated UCB (gray and solid line) transplantations are shown.

not significantly different from related BM transplantation ($P = 0.46$). The median days for neutrophil recovery in patients receiving related BM, related PBSCs, unrelated BM and unrelated UCB were 20, 14, 21 and 25, respectively.

Reticulocyte recovery on day 180 occurred in 100% of related BM, 75% (46–90%) of related PBSC, 77% (56–89%) of unrelated BM and 64% (30–85%) of unrelated UCB transplantations (Figure 1b). Unrelated UCB (vs related BM) transplantation was significantly associated with a lower probability of reticulocyte recovery ($P = 0.012$), whereas related PBSC and unrelated BM transplantations were not significantly different from related BM transplantation ($P = 0.57$ and $P = 0.076$, respectively). The median days for reticulocyte recovery in patients receiving related BM, related PBSCs, unrelated BM and unrelated UCB were 28, 17, 28 and 41, respectively.

Platelet recovery on day 365 occurred in 92% (57–99%) of related BM, 63% (40–78%) of related PBSC, 30% (14–47%) of unrelated BM and 44% (14–72%) of unrelated UCB transplantations (Figure 1c). Unrelated BM and unrelated UCB transplantations (vs related BM) were significantly associated with a lower probability of platelet recovery ($P < 0.001$ and $P = 0.027$, respectively), whereas related PBSC transplantation was not significantly different from related BM transplantation ($P = 0.20$). The median days for platelet engraftment in patients receiving related BM, related PBSCs, unrelated BM and unrelated UCB were 50, 32, 43 and 57, respectively.

GVHD

The incidences of grade II–IV and III–IV acute GVHD on day 100 were 17% (95% CI, 4–37%) and 6% (0–22%) in related BM, 32% (15–50%) and 16% (5–33%) in related PBSC, 29% (14–46%) and 14% (4–30%) in unrelated BM and 10% (1–36%) and 0% in unrelated UCB transplantations, respectively. There was no significant difference in the incidence of grade II–IV acute GVHD among stem cell sources, whereas the incidence of grade III–IV acute GVHD was significantly lower after unrelated UCB transplantation than after related BM transplantation ($P < 0.001$).

The incidences of chronic GVHD at 2 years after transplantation were 35% (95% CI, 14–57%) in related BM, 52% (31–69%) in related PBSC, 25% (11–42%) in unrelated BM and 18% (3–44%) in unrelated UCB transplantations. There was no significant difference in the incidence of chronic GVHD among stem cell sources.

Relapse

Relapse rates at 2 and 5 years after transplantation were 5% (95% CI, 0–21%) and 12% (2–33%) in related BM, 8% (1–22%) and 12% (3–28%) in related PBSC and 4% (0–18%) and 4% (0–18%) in unrelated BM transplantations, respectively. No patient relapsed after UCB transplantation, in which the longest follow-up was 48 months.

NRM

NRM rates at 2 and 5 years after transplantation were 33% (95% CI, 13–54%) and 33% (13–54%) in related BM, 45% (24–63%) and 50% (28–69%) in related PBSC and 61% (38–77%) and 61% (38–77%) in unrelated BM transplantations, respectively (Figure 2). NRM at 2 years after unrelated UCB transplantation was 64% (30–85%), and NRM at 5 years after UCB transplantation was not evaluable because of lack of patients alive beyond 5 years after transplantation. NRM rates after related PBSC and unrelated BM transplantation were not significantly different from that after related BM transplantation ($P = 0.28$ and $P = 0.068$, respectively), whereas unrelated UCB transplantation (vs related BM) was significantly associated with a significantly higher NRM ($P = 0.021$).

To identify predictive factors for higher NRM, multivariate analysis for all clinical features listed in Table 1 was performed, and the final multivariate model is shown in Table 2. PS $\geqslant 2$ and unrelated BM were predictive factors for higher NRM. For patients with performance status (PS) 0–1 ($n = 54$), NRM rates at 2 and 5 years after transplantation were 37% (23–50%) and 40% (26–54%),

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respectively. For patients with PS ⩾2 (n = 16), NRM at 2 years was 77% (45–92%), and NRM at 5 years was not evaluable because of lack of patients alive beyond 5 years after transplantation.

OS

OS rates at 2 and 5 years after transplantation were 63% (95% CI, 38–80%) and 63% (38–80%) in related BM, 48% (28–66%) and 43% (23–61%) in related PBSC and 41% (21–59%) and 41% (21–59%) in unrelated BM transplantations, respectively (Figure 3). The OS rate at 2 years after unrelated UCB transplantation was 36% (11–63%), and the OS rate at 5 years after UCB transplantation was not evaluable because of a lack of patients alive beyond 5 years after transplantation (longest follow-up, 48 months). There was no significant difference among stem cell donor sources (P = 0.15).

Cox's proportional hazards model was used with all clinical features listed in Table 1, and the final multivariate model is shown in Table 2. After adjustment by PS and frequency of RBC transfusion, which were significant on univariate analysis, donor source was not a significant factor for predicting OS. Instead, PS ⩾2 predicted a lower OS rate, and RBC transfusion ⩾20 times before transplantation showed a trend toward a lower OS. We confirmed that there was no significant difference in the frequencies of PS ⩾2 between patients receiving different stem cell sources (2 of 13 related BM, 6 of 24 related PBSC, 5 of 27 unrelated BM and 3 of 6 unrelated UCB transplantations; P = 0.30). Similarly, we confirmed that there was no significant difference in the frequencies of RBC transfusion ⩾20 times between patients receiving different stem cell sources (2 of 8 related BM, 5 of 18 related PBSC, 8 of 20 unrelated BM and 2 of 5 unrelated UCB transplantations; P = 0.80).

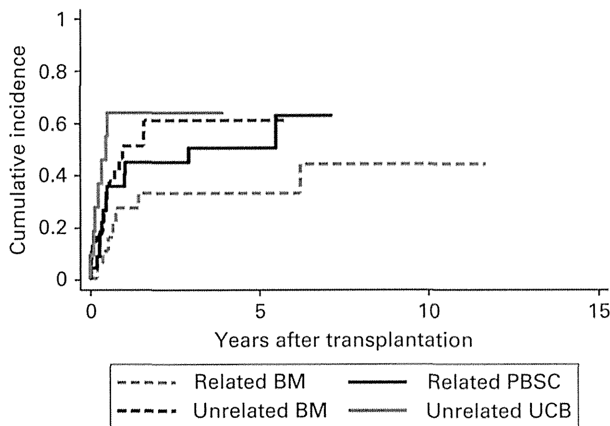

Figure 2. NRM after transplantation in PMF patients. Cumulative incidences of NRM after related BM (gray and dash line), related PBSC (black and solid line), unrelated BM (black and dash line) and unrelated UCB (gray and solid line) transplantations are shown.

Causes of death

The causes of death after transplantation are summarized in Table 3. For patients after related donor transplantation (n = 23), the most common cause of death was primary disease (n = 9, 39%), followed by infection (n = 4, 17%) and organ failure (n = 3, 13%). For patients after unrelated donor transplantation (n = 22), the most common causes of death were infection (n = 7, 32%) and organ failure (n = 7, 32%), followed by GVHD (n = 3, 14%), and only 1 patient (5%) died of primary disease.

DISCUSSION

The present study confirmed 5-year OS of 63%, 43% and 41% after related BM, related PBSC and unrelated BM transplantations, respectively. These results are comparable to previous reports in which long-term survival rates in patients with PMF or secondary myelofibrosis were 30–67% after transplantation.[10,11,21–26] This is the first report of UCB transplantation for more than 10 patients with PMF, and a 2-year OS of 36% was confirmed.

Several investigators have examined factors to predict outcomes after allogeneic HCT for PMF patients. The largest retrospective study of PMF patients from the CIBMTR demonstrated that Karnofsky score of <90% and the presence of blasts in peripheral blood, but not donor source, predicted lower disease-free survival of patients who had received BM or PBSC transplantation from related or unrelated donors.[10] Other retrospective studies including both PMF and secondary myelofibrosis demonstrated negative predictors for OS of higher patient age, nonchronic phase disease, RBC transfusion >20 times, increased comorbidity score, intermediate-2 and high scores of the Dynamic IPSS and non-HLA-matched sibling donor.[11,21,24,26,27] In the present study, multivariate analysis demonstrated that PS ⩾2 predicted a lower OS and that RBC transfusion ⩾20 times before transplantation showed a trend toward a lower OS (Table 2). Unexpectedly, the stem cell source was not a significant factor for OS. One possibility is that a significant association between stem cell source and OS was not detected because of a lack of statistical power, namely, the small

Table 2. Significant factors in multivariate analyses for nonrelapse mortality and OS after transplantation

	Nonrelapse mortality HR (95% CI)	P-*value*	*Overall survival HR (95% CI)*	P-*value*
Performance status at transplant				
0–1	1.0		1.0	
⩾2	3.36 (1.42–7.95)	0.006	2.67 (1.03–6.95)	0.044
Frequency of RBC transfusion[a]				
⩽9	NA		1.0	
10–19	NA		0.48 (0.97–2.36)	0.37
⩾20	NA		2.42 (0.99–5.93)	0.053
Donor source				
Related BM	1.0		1.0	
Related PBSCs	2.43 (0.73–8.07)	0.15	3.86 (0.81–18.44)	0.091
Unrelated BM	3.58 (1.07–12.01)	0.039	3.13 (0.66–14.79)	0.15
Unrelated umbilical cord blood	2.71 (0.49–14.86)	0.25	3.79 (0.60–23.91)	0.16

Abbreviations: CI = confidence interval; HR = hazard ratio; NA = not applicable.
[a]Frequency of RBC transfusion before transplantation.

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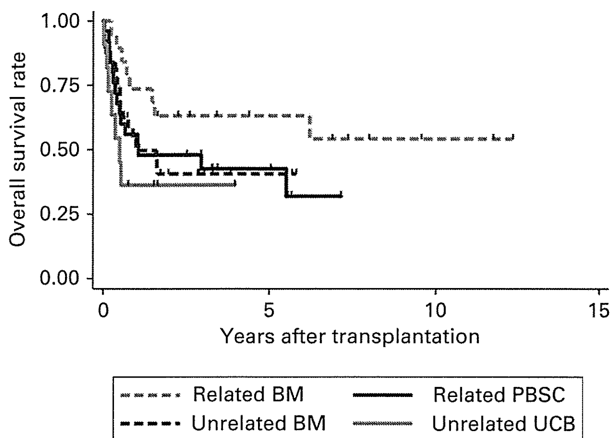

Figure 3. OS rates after transplantation in PMF patients. OS rates after related BM (gray and dash line), related PBSC (black and solid line), unrelated BM (black and dash line) and unrelated UCB (gray and solid line) transplantations are shown.

Table 3. Causes of death

	Related BM, n *(%)*	*Related PBSC*, n *(%)*	*Unrelated BM*, n *(%)*	*Unrelated UCB*, n *(%)*
Primary disease	2 (25)	7 (46)	1 (7)	0
Infection	1 (13)	3 (20)	6 (40)	1 (14)
Interstitial pneumonitis	2 (25)	0	1 (7)	0
ARDS	0	0	1 (7)	0
GVHD	1 (13)	1 (7)	2 (13)	1 (14)
Organ failure	1 (13)	2 (13)	3 (20)	4 (58)
Graft failure	1 (13)	0	0	0
Bleeding	0	1 (7)	1 (7)	0
Other	0	1 (7)	0	1 (14)
Total	8 (100)	15 (100)	15 (100)	7 (100)

Abbreviations: ARDS = acute respiratory distress syndrome; UCB = umbilical cord blood.

number of patients in each group, and the short-term follow-up. In particular, the number of patients with UCB transplantation was very small, and therefore, careful interpretation of these data is required. Further analysis with data including more patients undergoing UCB transplantation is required in order to determine the effect of UCB transplantation on outcomes of PMF patients. Another possibility is that the HCT outcome for PMF patients is more adversely affected by the deterioration in a patient's systemic condition as a consequence of multiple transfusions of blood and so on, rather than by the difference in stem cell sources.

In practice, UCB transplantation may be avoided in the treatment of PMF patients because of delayed engraftment and a higher probability of graft failure.[9] The present study demonstrated that UCB transplantation was significantly associated with a lower probability of hematopoietic recovery in comparison with related BM transplantation (Figure 1). The incidences of neutrophil recovery at 60 days and platelet recovery at 1 year were 82% and 44% for UCB transplantation, respectively. In a recent report of nonmyeloablative UCB transplantation for 14 patients with myelofibrosis, including 1 patient with PMF and 13 patients with secondary myelofibrosis, the incidences of neutrophil recovery at 60 days and platelet recovery at 100 days were 93% and 43%, respectively.[28] Thus, careful management is required for PMF patients, especially in the early period after unrelated UCB transplantation.

NRM was 30–60% (Figure 2), which is higher than in previous studies from large, well-known transplant center(s).[22–24,26,27,29–32] This may be explained by the large number of the participating centers, the heterogeneity of patients' clinical features and the fact that 18% of patients were ⩾60 years in the present study.

Nonmyeloablative preconditioning regimens have advantages of less NRM and a broader applicability in elderly patients and may, therefore, be appropriate for PMF patients. After small studies demonstrated the feasibility of allogeneic HCT with nonmyeloablative preconditioning for myelofibrosis,[33–35] Kröger *et al.*[11] prospectively treated 103 patients with PMF or post essential thrombocythemia and post polycythemia vera myelofibrosis with BU and fludarabine-based nonmyeloablative preconditioning. They reported encouraging 1-year NRM of 16% and 5-year OS of 67%. The Swedish group compared results from 10 patients undergoing nonmyeloablative transplant with 17 patients undergoing myeloablative transplant for secondary myelofibrosis. NRM was lower in the nonmyeloablative group than in the myeloablative group (10% vs 30%). With a median follow-up of 55 months, 9 (90%) of 10 patients undergoing nonmyeloablative transplant and 9 (55%) of 16 patients undergoing myeloablative transplant survived.[36] In contrast, the present study could not find any advantage of nonmyeloablative preconditioning in terms of decreasing NRM or increasing OS (Table 2). Other retrospective studies, including a large study ($n = 289$), also did not find any favorable affect with nonmyeloablative preconditioning.[10,22,24] In retrospective studies, drugs and doses of preconditioning regimens were heterogeneous, which could partly explain the failure to detect an advantage of nonmyeloablative preconditioning. There has been no randomized study to compare the efficacy of nonmyeloablative and myeloablative preconditioning for patients with PMF. The advantage of nonmyeloablative preconditioning for patients with PMF remains in question.

The molecular assessment of the *JAK2* mutation was performed in a very limited number of patients (six cases for pretransplant mutation and four cases for post transplant mutation). Therefore, we were unable to analyze association between the presence of pretransplant *JAK2* mutation and transplant outcomes or between the minimum residual disease and relapse after transplant. However, the present study clearly demonstrated that allogeneic BM and PBSC transplantations provide long-term survival for PMF patients and suggested the feasibility of UCB transplantation for PMF patients. Given the constant improvement in supportive care for transplant patients and the beginning of the use of molecular targeted therapy for myelofibrosis, the NRM and relapse rates may be further decreased. Allogeneic HCT should be considered in the treatment plan for PMF patients. The indications for allogeneic HCT in PMF patients have to be defined in a future study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX

Institutes participating in this study: Japanese Red Cross Asahikawa Hospital; Hokkaido University Hospital; Sapporo Medical University Hospital; Sapporo Hokuyu Hospital; Akita University Hospital; Iwate Medical University; Tohoku University Hospital; Fukushima Medical University Hospital; Nagaoka Red Cross Hospital; Gunmaken Saiseikai Maebashi Hospital; Tsukuba Memorial Hospital; Chiba University Hospital; Kameda Medical Center; National Defense Medical College Hospital; Saitama Medical Center, Jichi Medical University; Keio University Hospital; Tokyo Metropolitan Cancer and Infectious diseases Center, Komagome Hospital; Toranomon Hospital; National Cancer Center Hospital; Tokyo Women's Medical University Hospital; Institute of Medical Science, University of Tokyo; Nippon Medical School Hospital; Kanagawa Cancer Center; Yokohama City University Medical Center; Nagano Red Cross Hospital; Shinshu University Hospital; Toyama Prefectural Central Hospital; Kurobe City Hospital; Kanazawa University Hospital; Shizuoka General Hospital; Japanese Red Cross Shizuoka Hospital; Hamamatsu University Hospital; Hamamatsu Medical Center; Anjo Kosei Hospital; Fujita Health University Hospital; Japanese Red Cross Nagoya Daiichi Hospital; Japanese Red Cross Nagoya Daini Hospital; Meitetsu Hospital; Nagoya University Hospital; Nara Medical University Hospital; Tenri Hospital; Takanohara Central Hospital; Kyoto University Hospital; Kyoto-Katsura Hospital; Osaka Red Cross Hospital; Osaka Medical Center for Cancer and Cardiovascular Diseases; Takatsuki Red Cross Hospital; Seichokai Fuchu Hospital; Kinki University Hospital; Wakayama Medical University Hospital; Hyogo College of Medicine; Institute of Biomedical Research and Innovation; Kurashiki Central Hospital; Okayama Medical Center; Hiroshima Red Cross Hospital & Atomic-bomb Survivors Hospital; Shimane Prefectural Central Hospital; Yamaguchi University Hospital; Ehime University Hospital; Ehime Prefectural Central Hospital; Kochi Medical School Hospital; Kitakyushu Municipal Medical Center; University of Occupational and Environmental Health; Kyushu Cancer Center; Kyushu Medical Center; Kyushu University Hospital; Kurume University Hospital; Ryukyu University Hospital.

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Letter to the Editor Open Access

The Preventative Effects of Recombinant Thrombomodulin on Transplantation-Associated Coagulopathy after Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

We investigated the preventive effects of recombinant thrombomodulin (rTM) on Transplantation Associated Coagulopathy (TAC) and TAC-related biomarkers in 271 patients after hematopoietic stem cell transplantation (HSCT). There were no significant differences between the rTM (+) or (-) groups and patient background, types of disease or HSCT regimens. When we examined patients with confirmed complications, all frequencies of aGVHD, VOD and/or TMA, as well as uncomplicated cases were significantly lower in the rTM (+) group. HMGB1 and MCP-1 showed a clear induction after transplantation, which peaked for HMGB1 at day 0 and for MCP-1 at day 7. Although MCP-1 levels did not exhibit significant differences between the two groups, HMGB1 levels in the rTM (+) group showed a significant reduction after day 4 compared with the rTM (-) group. The levels of PAI-1, sE-selectin and sVCAM-1 showed a significant increase in the groups that did not receive rTM. In contrast, the groups that received rTM did not show significant changes and significant differences were found between the two rTM-treated groups. Our multi-institutional study findings suggest that this agent is beneficial as part of preventive therapies for established TAC after HSCT.

Keywords: rTM; TAC; Stem cell transplantation; sE-selectin; HMGB1

Letter

Hematopoietic Stem Cell Transplantation (HSCT) involves specific serious transplant-related complications [1,2], and recovery from these complications is vital for achieving a successful HSCT outcome. Therefore, taking steps to mitigate coagulation- and Graft-Versus Host Disease (GVHD)-related complications following HSCT is very important. Several interactions between coagulation-related blood components and the fibrinolytic systems are involved in the progression of vascular angiopathy. Notably, plasminogen activator inhibitor (PAI)-1 plays an important role in the pathophysiology of many vascular abnormalities [3]. Recombinant thrombomodulin (rTM) is composed of the active extracellular domain of TM. Like membrane-bound TM, rTM binds to thrombin to inactive coagulation. The thrombin–rTM complex activates protein C to produce active protein C (APC), which in the presence of protein S inactivates factors VIIIa and Va, thereby inhibiting further thrombin formation [4]. Therefore, rTM might be useful for transplantation-associated coagulopathy (TAC) after HSCT. Indeed, there are some reports of the efficacies of therapies for TAC, such as veno-occlusive disease (VOD) and thrombotic microangiopathy (TMA) [5,6]. However, the preventive effects of rTM for TAC or TAC-related biomarkers following HSCT are poorly understood. Here, we investigated the preventive effects of rTM for TAC and TAC-related biomarker levels after HSCT. To our knowledge, this report of a multi-institutional joint study is the first to document the potential ability of rTM to prevent TAC after HSCT.

The study cohort included 271 patients who underwent SCT between June 2011 and February 2014 at one of 24 institutions in Japan. All patients received allogeneic SCT (Table 1). The 161 male and 110 female allogeneic SCT patients ranged in age from 7 to 71 years (median: 45 years). Patient diagnoses consisted of 102 acute myeloid leukemia cases, 63 acute lymphoblastic leukemia cases, 38 myelodysplastic syndrome cases and 68 other diagnoses. Conditioning was applied as follows: total body irradiation for 175 patients and non-total body irradiation for 96 patients. The donor sources for transplantation were 139 bone marrow cells, 57 peripheral blood stem cells and 75 cord blood cells (Table 1). Written informed consent was obtained from all patients who were registered by faxing documents to Kansai Medical University prior to SCT. The rTM, consisting of daily doses of 380 units/kg (Asahi Kasei Pharma, Tokyo, Japan), was administered as a preventive therapy for TAC. This protocol was

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	rTM (-) (n=156)	rTM (+) (n=115)	P-value
Age, years, median (range)	44 (7-77)	47 (16-71)	0.3988
Sex, Male/Female	92/64	69/46	0.8651
Disease			
AML	61	41	0.6959
ALL	39	24	0.5290
MDS	21	17	0.7883
Other	35	33	0.3650
Conditioning regimen including TBI			
Yes	102	73	0.8823
No	54	42	-
Donor type			
Related	52	43	0.7493
Unrelated	104	72	-
Stem Cell Source			
Bone marrow	79	60	0.8875
Peripheral blood	39	18	0.1292
Umbilical cord blood	38	37	0.2868
Complication after HSCTC			
Known	113	96	0.4440
Yes, aGVHD	75	35	0.0149*
Yes, VOD and/or TMA	40	18	0.0424*
No	22	45	0.0025**
Unknown	43	19	0.0877
Survival at Day 60			
Known	108	89	0.5548
Yes	72	61	0.5136
No	36	24	0.7295
Unknown	48	22	0.0940
Quality of life			
Known	70	59	0.5336
Good	43	38	0.4756
Not so good	27	21	0.8652
Unknown	41	24	0.4177

rTM: Recombinant Thrombomodulin; AML: Acute Myeloid Leukemia; ALL: Acute Lymphocytic Leukemia; MDS: Myelodysplastic Syndrome; TBI: Total Body Irradiation; HSCT: Hematopoietic Stem Cell Transplantation; aGVHD: Acute Graft-versus-host Disease; VOD: Veno-occlusive Disease; TMA: Thrombotic Microangiopathy.
*: $P< 0.05$; **: $P< 0.01$

Table 1: Patient characteristics.

completed from days 4 to 14 after HSCT. An anticoagulation regimen of 5000 U heparin 24 h per day was used prior to rTM administration. Heparin or no anticoagulation therapy was also administered to the control groups.

Blood samples from patients were collected into tubes containing either sodium citrate or no anticoagulant; in the latter group, blood was allowed to clot at room temperature. Then, serum or citrated plasma was isolated by centrifugation for 20 min at 1000×g and 4°C. The serum was divided into aliquots and frozen at −30°C until use. As a positive control, recombinant proteins were used in each assay, as well as the standard solutions that were provided with the commercial kits. Interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein (MCP)-1, RANTES, sE-selectin and PAI-1 ELISA kits were purchased from BioSource International Inc (Camarillo, CA, USA). High mobility group box 1 (HMGB1) was measured using the HMGB1 ELISA Kit II (Shino-test Corp., Kanagawa, Japan). All ELISA kits were used according to the manufacturers' instructions. Data are expressed as the means ± SD and were analyzed by two-factor ANOVA for repeated measures as appropriate. Between-group comparisons were made using the Newman–Keuls and Scheffe's tests. Differences in characteristics between patient groups who used rTM or not were assessed using χ^2 tests of association for categorical values. All statistical analyses were performed using StatFlex (ver. 6) software and $P<0.05$ was used as a threshold for statistically significant differences.

There were no significant differences between the rTM (+) and (–) groups for patient background, type of disease or HSCT regimen (Table 1). When we examined patients who had confirmed complications, the frequencies of aGVHD and VOD and/or TMA were significantly lower in the rTM (+) groups (Table 1). In addition, the frequency of uncomplicated cases was significantly higher in the rTM (+) groups (Table 1). However, there were no significant differences with survival at day 60 and quality of life between two groups.

HMGB1 and MCP-1 levels clearly increased after transplantation, with HMGB1 peaking at day 0 and MCP-1 peaking at day 7 (Figure 1). Although MCP-1 levels did not show a significant difference between the two groups, HMGB1 levels in the rTM (+) group showed a significant reduction after day 4 compared with the rTM (–) group. Significant differences in IL-6, TNF-α and RANTES levels were not observed between the two groups. The levels of PAI-1, sE-selectin and sVCAM-1 showed a significant increase in the groups in which rTM was not used. In contrast, the groups in which rTM was used did not show significant changes, and significant differences were found between the two rTM-treated groups (Figure 1).

It is thought that the risk for TAC includes many factors, such as age of the patient, basal disease or the degree of disease remission before HSCT [7,8]. Additionally, previous reports have documented the effect of conditioning regimens that included TBI or not [9]. In our study, the increase of HMGB1 levels after HSCT was a very interesting finding, and suggested that HMGB1 plays an important role in the development of TAC following the HSCT conditioning treatment regimen [10]. Cutler et al. [11] reported that increased vascular endothelial cell dysfunction is a predictive indicator of VOD. Although the etiology of VOD remains unclear, we believe one of the causes of VOD is increased levels of proinflammatory cytokine, such as HMGB1 [12,13]. Therefore, the direct anti-inflammatory effect mediated via the rTM lectin domain is thought to play an important role in preventing VOD. Our findings suggest the possibility that rTM can help prevent TAC after allogeneic HSCT [10]. Recently, Ikezoe et al. [14] reported that rTM was useful to improve clinical outcomes of transplant recipients with coagulopathy. They exhibited that the treatment for coagulopathy by rTM significantly improved clinical ourcomes of patients at day 100 and dramatically prolonged patient's overall survival, while that was a single-institutional study.

We found that increased levels of PAI-1, sE-selectin and sVCAM-1 could be observed in the group not treated with rTM after HSCT. By contrast, levels of these molecules did not show significant changes in groups that received TM after HSCT. PAI-1 is synthesized in the liver and by endothelial cells, vascular smooth muscle cells and macrophages. PAI-1 expression can be regulated by many factors, including cytokines, oxidative stress and cellular signaling molecules. All patients with transplantation-related complications, especially patients with thrombotic complications, appear to have significant increases in the mean and maximum levels of PAI-1 during the observational period after HSCT [15,16]. A number of markers for endothelial injury and adhesion molecules are upregulated in patients with thrombotic complications in HSCT, including E-selectin, tissue

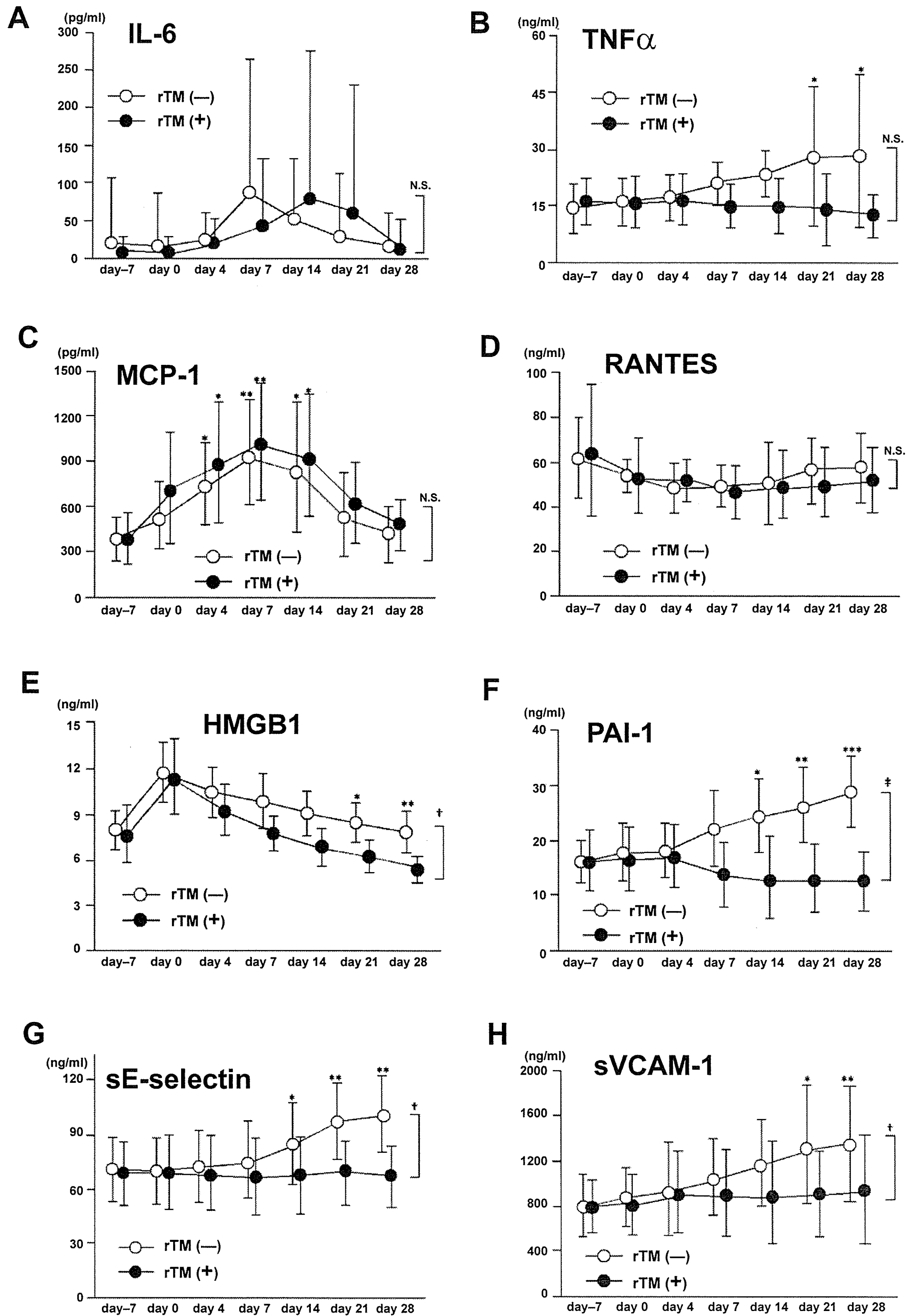

Figure 1: The levels of selected biomarkers before and after HSCT. Concentrations of (A) IL-6, (B) TNF-α, (C) MCP-1, (D) RANTES, (E) HMGB1, (F) PAI-1, (G) sE-selectin and (H) sVCAM-1 in patients with or without rTM treatment are shown. Data are shown as means ± SD. For comparisons versus day 0: *P<0.05; **P<0.01; ***P<0.001. For ANOVA analysis between two groups with or without rTM treatment: †P<0.05; ‡P<0.01; NS: Not Significant.