Effect of mesenchymal stem cell transplantation on the engraftment of human hematopoietic stem cells and leukemic cells in mice model

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Effect of mesenchymal stem cell transplantation on the engraftment of human hematopoietic stem cells and leukemic cells in mice model

Directed by Professor **Yoo Hong Min**

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Seung Tae Lee

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This certifies that the Doctoral Dissertation of Seung Tae Lee is approved.

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Seung Tae Lee

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ABSTRACT

Effect of mesenchymal stem cell transplantation on the engraftment of human hematopoietic stem cells and leukemic cells in mice model

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Objective: The effects of human bone marrow derived culture expanded mesenchymal stem cells (MSCs) on the engraftment when cotransplanted with unrela- ted human umbilical cord blood (UCB) $CD34^+$ cells or acute myelogenous leukemia (AML) cells into NOD/SCID mice were investigated. The homing capability of MSCs to hematopoietic and nonhematopoetic tissues after systemic infusion was also assessed.

Materials and Methods: Forty-two NOD/SCID mice were administered sublethal irradiation followed by transplantation of various cell doses of UCB $CD34^+$ cells with (n = 35; 3 different cell doses of MSCs) or without (n=7) human BM-derived culture-expanded MSCs. Another 12 NOD/SCID mice were also sublethally irradiated followed by transplantation of AML cells with (n= 6) or without (n=6) MSCs. In 10 of these mice, MSCs were genetically modified with a adenoviral vector encoding the enhanced green fluorescent protein (eGFP) gene for tracking purposes.

Results: Cotransplantation of UCB $CD34^+$ cells and MSCs resulted in a signifi-cant increase in BM engraftment after 6 weeks, and this engraftment promoting effect of MSCs was proportional to the dose of MSCs and obvious when low doses of UCB $CD34^+$ cells were given. There were no significant differences in engraft-ment between mice transplanted with AML cells with and without MSCs. The

existence of MSCs in the major organs (liver, lung, brain, muscle, kidney, bone marrow) of 10 mice given eGFP-transduced MSCs were analyzed. RT-PCR (reverse transcriptase polymerase chain reaction) for eGFP revealed that all the organs analyzed (n=45) had m-RNA of eGFP. Liver specimens from 2 mice and BM stromal cells from another mouse showed the existence of eGFP, revealed by immunohistochemistry for eGFP. We could also observe eGFP fluorescence in the same liver specimen by confocal laser scanning microscope.

Conclusions: These data demonstrate that MSCs promote engraftment of UCB $CD34^+$cells in BM, but exert no effect on engraftment of AML cells, and are capable of homing to the major organs including BM following systemic infusion in NOD/SCID mice.

Key Words: mesenchymal stem cell, $CD34^+$, leukemia, stem cell transplantation

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I. INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) has been proved to be one of the most effective therapeutic strategies for some hematologic diseases of neoplastic and nonneoplastic origins, and its clinical applications are expanding to some solid cancers and autoimmune diseases. But graft failure and delayed engraftment remain significant obstacles to a successful outcome especially in the clinical settings when only limited numbers of hematopoietic stem cells (HSCs) are available such as UCB transplantation.

HSCs have the unique capability of homing to the bone marrow to settle in the marrow microenvironment[1] which plays a crucial role in the maintenance of hematopoiesis[2]. Therefore, the spatial organization of stem cells in the marrow, mediated by the bone marrow microenvironment and extracellular matrix, may be crucial for hematopoietic regeneration after HSCT[3]. But damage to the recipient's marrow microenvironment by chemoradiotherapy before HSCT is unavoidable, which might contribute to the delayed engraftment or graft failure along with limited numbers of HSCs transplanted. So it might be desirable to repair or replace a damaged bone marrow stromal compartment possibly by transplanting donor stromal

cells[4~7]. Given the fact that donor-derived stromal cells do not engraft following conventional HSCT due to the limited number of stromal cells present in a typical allograft even having a competitive advantage over the damaged recipient's stroma, donor stromal cell engraftment could be achieved by transplanting a large number of MSCs that had been expanded in culture[8]. Previous studies in mice and fetal sheep have demonstrated the capacity of marrow stromal cells to engraft if administered in sufficient quantity, providing additional support for this premise[9,10]. Recent researches have demonstrated that cotransplantation of human MSCs along with human HSCs enhance human hematopoietic engraftment in animal models[11~14] and in human[15,16]. Moreover, MSCs possess in vitro immunosuppressive properties that appear not to be the major histocompatibility complex restricted[17,18]. In these respects, cotransplantation of allogeneic MSCs with allogeneic HSCs can provide some beneficial effects such as the enhancement of engraftment and suppression of graft-versus-host activity[19]. However, a possible negative influence of MSCs' immunosuppressive properties on graft-versus-leukemia effect in allograft setting has not been mentioned. In vitro stromal cell dependent growth of leukemic cells also suggests the possibility of beneficial effect on leukemia survival following MSCs transplantation.

The therapeutic potential of bone marrow derived MSCs has recently been brought into the spotlight of many fields of research. Not only are these cells able to migrate to injured or defective tissues of different origin, but their progenies are also able to acquire a different phenotype in accordance with tissues to which they home[20]. But the ultimate fate of systemically delivered MSCs has not been clearly defined, with many published reports showing different results. A study in nonhuman primate suggested a broad and sustained engraftment[21]. But another study in rodents showed a broad initial distribution followed by a limited capacity for sustained engraft- ment[22]. In fetal sheep, human MSCs underwent a wide tissue distribution and could differentiate into multiple mesenchymal tissues following peritoneal implantation[23].

In this study, I investigated whether coinfusion of ex-vivo culture expanded human MSCs and unrelated UCB $CD34^+$ cells into NOD/SCID mice could enhance the hematopoietic engraftment and there is any correlation between transplanted cell doses of MSCs and patterns of hematopoietic engraftment. I next determined whether there were any leukemic cell engraftment promoting effects of MSCs when cotransplanted with leukemic cells into NOD/SCID mice. Finally, I assessed the homing capability of MSCs to hematopoietic and nonhematopoetic tissues after systemic infusion using eGFP marked MSCs. I found that cotransplantation of MSCs promoted the hematopietic engraftment in NOD/SCID mice, resulting in higher levels of human hematopoietic cells in a dose dependent manner, especially when UCB dose was limiting. These results suggest the possibility of using MSCs for UCB transplantation in an attempt to overcome the engraftment delay associated with UCB transplantation. I also found that MSCs distributed widely to a variety of nonhematopoietic tissues following systemic infusion.

II. MATERIALS AND METHODS

1. Collection and isolation of $CD34^+$ cells from human UCB

Human UCB samples were obtained from full-term deliveries after informed consent of the mother according to a protocol approved by the hospital's ethics committee. hUCB were collected in bags containing heparin and processed within 24 hours. Samples were diluted 1:2 in phosphate buffered saline (PBS) without Mg_2^+/ Ca_2^+ (Gibco, Grand Island, NY, USA). Low density mononuclear cells (MNCs) were collected after centrifugation on Ficoll-Paque density gradient (1.077 g/ml; Amersham Bioscience, Piscataway, NJ, USA) and washed in PBS. $CD34^+$ cells were isolated using the MiniMACS cell isolation kit (Miltenyi Biotec Gmbh, Bergish Gladbach, Germany) according to the manufacturer's instruction. After isolation, purity of $CD34^+$ cells was determined using flow-cytometry (Fig. 1) and cells were frozen in 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) with 10% dimethyl sulfoxide (DMSO) by rate-controlled freezer and stored in liquid nitrogen until use.

2. Isolation and culture-expansion of human bone marrow derived MSCs

For cotransplantation and homing studies, 20 to 25 mL of bone marrow cells were obtained from iliac crest aspirates from healthy donors giving their bone marrows for allogeneic transplantation purposes after informed consent. Aspirate was mixed with two volumes of Dulbecco's phosphate buffered saline (DPBS; Gibco, Grand Island, NY, USA). Pellets were layered onto 25 mL of Percoll (1.073 g/mL; Amersham Bioscience) at a density of 1 to 2×10^7 cells/mL. After centrifugation at 900 *g* for 30 minutes at 20℃, recovered mononuclear cells were resuspended in DPBS and centrifuged at 460 *g* for 10 minutes at 20℃. Cells were resuspended at 1×10^6 cells/mL in Dulbecco's modified Eagle medium, low glucose (DMEM-LG;

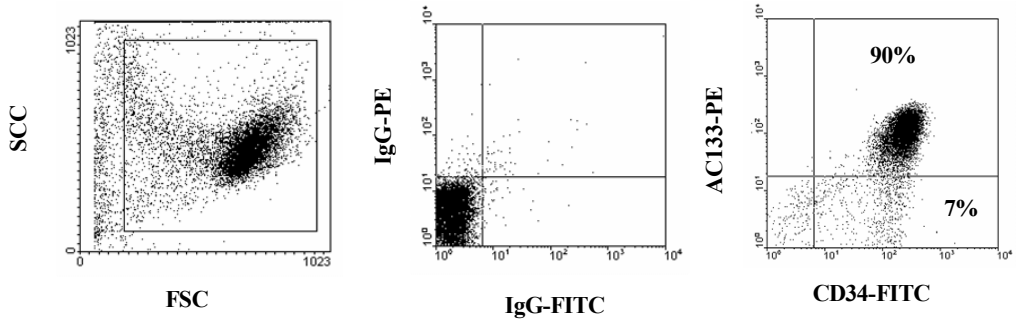

Fig. 1. Representative flow-cytometry profiles of $CD34^+$ cells isolated from full-term UCB showing 97% purity.

GibcoBRL) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 30 mL of cell suspension was plated in a 175 cm^2 flask (Falcon, Franklin Lakes, NJ, USA). MSCs were cultured in humidified incuba- tors with 5% CO_2 and initially allowed to adhere for 72 hours, followed by media change every 3 or 4 days. When cultures reached more than 90% confluence, adherent cells were detached with 0.05% trypsin-EDTA (Gibco) and replated at a density of 1×10^6 per 175 cm^2 flask until processing for infusion. After the first detachment, surface expressions of SH-2, SH-3 were determined on culture-expanded MSCs.

3. Generation of adenoviral vector

Human adenovirus serotype 5 (Ad5) DNA constructs comprise a full-length copy of the adenovirus genome of 637.5 kb, from which the early regions E1A and E1B genes for replication have been replaced by cDNA for GFP, which was subcloned from pEGFP-C1 (Clontech; Palo Alto, CA, USA) and modified to maximize expression in eukaryotic cells[24]. The reporter gene expression was driven by a cytomegalovirus (CMV) promoter. The vector was prepared as described and purified by double cesium gradient purification[25]. Recombinant viruses were grown in human embryonic kidney (HEK) 293 cells that complement the E1 early viral promoters. Viruses [10^{10} plaque-forming units (pfu)/ml] were suspended in phosphate-buffered saline (PBS) with 3% sucrose and frozen at -70℃ until use.

4. Adenoviral transduction of MSCs

When 1st passage MSCs cultures reached about 70% confluence (a median 14 days after starting culture), adenovirus-eGFP were inoculated into MSCs at a multiplicity of infection (MOI; defined as pfu/cell) of 5 at 37℃ in a CO_2 incubator. After 12~15 hours when most of the cells have been infected, the old medium was removed and cells were washed twice with PBS and rinsed once with fresh medium to remove uninfected particles. The efficiency of eGFP transduction following adenovial transduction was assessed using flow cytometry and fluorescence microscope equipped with a combination of filters suitable for visualization of eGFP (standard fluorescein isothiocyanate, FITC: barrier filter, 520 nm; excitation filter, 450-490 nm; dichroic mirror 510 nm; Nikon Combination B-2A). After adnovirus-eGFP inoculation, MSCs were expanded with 3 more passages to acquire enough number of MSCs for animal experiment.

5. Cotransplantation of MSCs and UCB $CD34^+$ cells or leukemic cells in NOD/SCID mice

Female NOD/LtSz*scid/scid* (NOD/SCID) mice (6~8 weeks old, Jackson Laboratory, Bar Harbor, ME, USA) were housed in the animal facility of the Yonsei Clinical Research Center (Seoul, Korea) under specific pathogen free conditions, using laminar airflow units. The mice received sublethal irradiation of 3.0 Gy from a linear accelerator and sterile diet and acidified water supplemented with 100 mg/L ciprofloxacin (Bayer AG, Leverkusen, Germany) were provided during the experimental period. Various combinations of UCB $CD34^+$ cells or primary acute myelogenous leukemia (AML) cells and MSCs were given to the mice via lateral tail vein within 24 hours after irradiation, and the mice were sacrificed 6 weeks after transplantation. For leukemic cells transplantation, cryopreserved primary AML cells from two patients were used.

6. Analysis of human cell engraftment

Mice were killed by cervical dislocation and cells from the blood, spleen, thymus, and femur and tibia bones were collected and treated with an RBC lysis solution (0.155 mol/L NH_4Cl + 0.01 mol/L $KHCO_3$). Single cell suspensions from spleen and thymus were obtained by mincing and filtration through a cell strainer (70 m; Becton Dickinson Labware, Franklin Lakes, NJ, USA). Aliquots of harvested cells were then incubated with 5% human serum (Gibco) and anti-mouse Fc receptor monocloncal antibody (2.4G2; SyStemix, Palo Alto, CA, USA) to decrease nonspecific antibody binding. Cells were then stained with mouse anti-human monoclonal antibodies for flow cytometric analysis such as AC133-PE (Miltenyi Biotec); CD45-PE, CD34- FITC, CD33-FITC, CD19- FITC, CD41-PE, CD3-FITC (Becton Dickinson, San Jose, CA, USA); Glycophorin-A-FITC (Pharmingen; San Diego, CA, USA). As controls, goat anti-mouse IgG_1-FITC and rat anti-mouse IgG_1-PE were used. The percentage of leukemic cells was determined by double staining of the samples with mouse anti- human CD45-PE (Becton Dickinson) and anti-murine CD45-FITC (Ly5; Pharmingen). Data acquisition was performed by FACS Calibur (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson); in all cases, at least 20,000 events were acquired. In addition, human CD45 immunohistochemistry on the bone marrow section of the NOD/SCID mice transplanted with AML cells was performed to document the existence of AML cells in the bone marrow.

7. Detection of human MSCs in NOD/SCID mice

For homing experiment, 10 mice were given eGFP-marked MSCs along with UCB $CD34^+$ cells or AML cells. Third passage MSCs were used for experiments. After detached from culture flask, MSCs were washed twice with PBS and resuspended in Hank's balanced salt solution (HBSS; Gibco) with 0.1% bovine seum albumin (BSA; Gibco) before infusion to exclude any possibility of systemic delivery of adenovirus-

eGFP. The mice were killed 6 weeks after the transplantation, and liver, kidney, lung, muscle, bone marrow and brain were obtained. To detect the existence of eGFP in tissue, RT-PCR, immunohistochemistry, and confocal laser scanning microscopic examination were performed.

A. RT-PCR (Reverse transcriptase-polymerase chain reaction)

Samples were homogenized using rotor-stator homogenizer (ultra-turrax cell homogenizer; IKA-WIERKE, Germany) and total RNA was isolated from tissue lysates using RNeasy columns (Qiagen, Valencia, CA, USA). Complementary DNA was prepared from samples of 1μg RNA each using Moloney murine leukemia virus (M-MuLV) BRL reverse transcriptase (Gibco, Gaithersburg, MD, USA) at 37℃ for 1 hour and used as a template for PCR with specific primers for GFP (Pr385 5'-tgaaccgcat cgagctgaag gg-3'; Pr386 5'-tccagcagga ccatgtgatc gc-3'). Reaction conditions were 3 minutes at 94℃ followed by 40 cycles of 94℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 1 minute. The reaction products were resolved by electrophoresis on a 1.0% agarose gel and visualized with ethidium bromide. eGFP vector and eGFP-marked MSCs were used as positive controls and samples from mice transplanted with untreated MSCs were used as negative controls. GAPDH primers were used as control primers.

B. Immunohistochemistry

Immunohistochemistry for eGFP was performed as described previously[26]. Briefly, tissue specimens obtained by dissection were fixed in 4% buffered formaldehyde for 6 hours at room temperature, and embedded in Histo-Comp® (Vogel, Giessen, Germany) using an automatic embedding equipment (Tissue Tek, Miles Scientific Inc., USA). Embedding solvents and times were as follows: 70% ethanol for 1 hour, three times 96% ethanol for 1 hour, 100% ethanol for 1 hour, three times xylene for 1 hour at room temperature, four times Histo-Comp for 1 hour at 50℃, embedding in Histo-Comp® at 56℃; 5μm thick sections were cut and mounted on

glass slides pretreated with poly-l-lysine. Sections were deparaffinized in xylene, rehydrated in a descending series of ethanol and mounted in anti-fading medium (Mowiol, Hoechst, Germany). Deparaffinized sections were pretreated with 0.6% hydrogen peroxide in methanol for 15 minutes to inactivate endogenous peroxidase and rinsed in tap water. After that, slides were incubated with 1.5% normal goat serum for 30 minutes at room temperature to minimize non-specific background staining. The primary antibody (rabbit anti-GFP polyclonal antibody; Abcam, Cambridgeshire, UK) was diluted in PBS to a final concentration of 1:1,000. Slides were incubated with the primary antibody overnight at 4℃. On the next day, slides were washed in PBS and covered with anti-rabbit HRP EnVision™ (DAKO corporation, Carpinteria, CA, USA) for 30 minutes at room temperature. After that, sections were washed in PBS and slides developed for 10 minutes at room temperature in 0.1% 3,3-diaminobenzidine (DAB) hydrochloride containing 0.03% hydrogen peroxide. The reaction was stopped in distilled water for 5 minutes. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, and moun- ted in DPX mounting medium (Fluka, Buchs, Switzerland).

C. Confocal laser scanning microscopy

Deparaffinized sections were evaluated with a confocal laser scanning microscope (TCS-NT, Leica, Heidelberg, Germany) using PL FLOUTAR 200× oil immersion objective. An argon/krypton laser with filter setting for FITC fluorescence was used for single channel detection.

8. Statistical Analysis

Statistical analyses of engraftment were done using SPSS version 10.0 software (SPSS, Chicago, IL, USA). Differences in engraftment percentage were calculated using the Student's T test. $p<0.05$ was considered statistically significant.

III. RESULTS

1. MSC cultures and expression patterns of eGFP in MSCs after adenoviral transduction

A morphologically homogenous population of fibroblast-like MSCs was obtained in primary cultures, which reached greater than 90% confluence in a median of 16 days (range 13-20 days; Fig. 2). Four individual donor bone marrow cells were used and third passage MSCs were used for individual experiments. It took a median of 7 days to proceed to the next passage. All MSCs preparations were morphologically identical and uniformly expressed SH2 and SH3 markers (Data not shown). The efficiency of eGPF transduction following adenoviral gene marking was more than 90% assessed by flow cytometry. And this high transduction efficiency was maintained after third pas- sage of culture, analyzed by flow cytometry and fluorescence microscopy (Fig. 3).

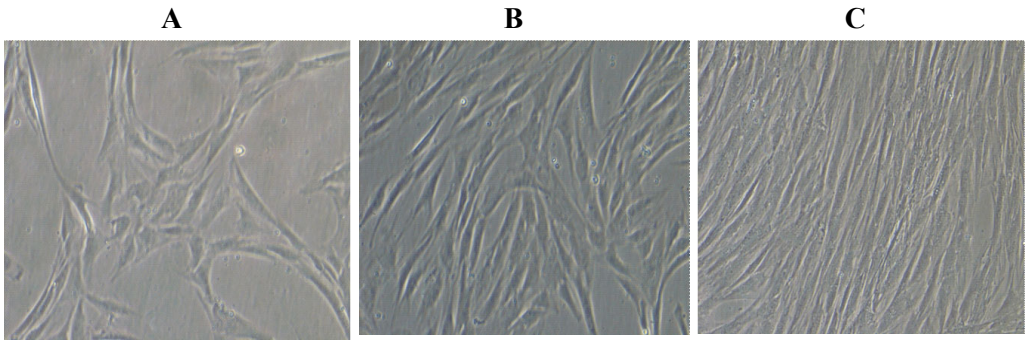

Fig. 2. Phase contrast photomicrograph of cultured human MSCs (magnification × 100). (**A**) Single fusiform adherent cells early (7 days) in the primary culture. (**B**) Late (12 days) in the primary culture, (**C**) Confluent patches of MSCs immediately before first passage (16 days).

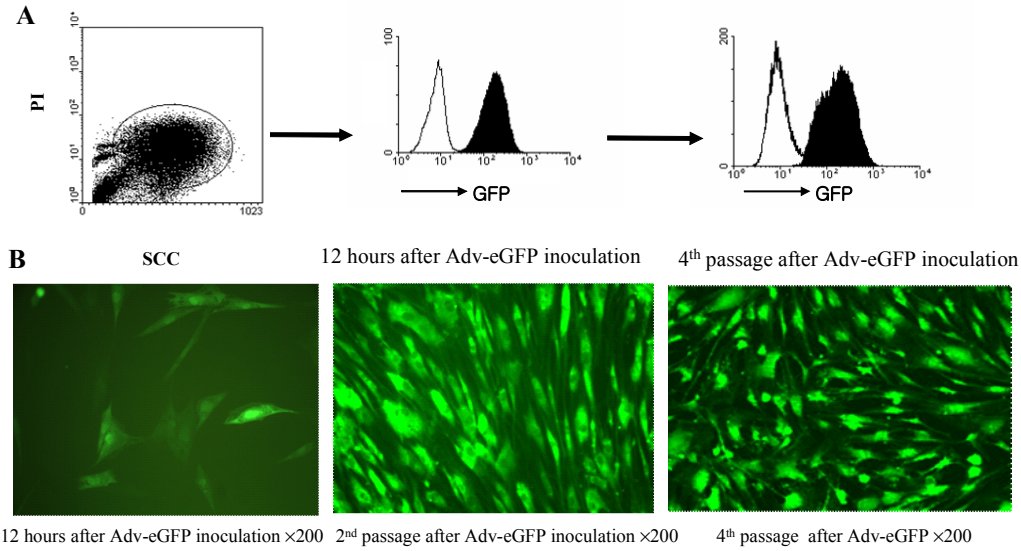

Fig. 3. Maintenance of eGFP expression in vitro from eGFP-transduced MSCs. Culture of eGFP-transduced MSCs was maintained and assayed for eGFP expression after 2nd and 4th passages. Flow cytometry histograms show more than 90% of eGFP expression. And this high transduction efficiency was maintained after 4th passage of culture, analyzed by flow cytometry (**A**) and fluorescence microscopy (**B**).

2. Engraftment of human hematopoietic cells in NOD/SCID mice after cotransplantation of UCB $CD34^+$ cells and MSCs

To determine whether human MSCs support engraftment of human hematopoietic stem cells in vivo and if there would be any correlation between transplanted cell doses and the levels of engraftment, we infused 4 different cell doses (0.1×10^6, 0.3×10^6, 0.5×10^6, 1.0×10^6 cells/mouse) of UCB $CD34^+$ cells with or without 3 different cell doses (0.1×10^6, 0.5×10^6, 1.0×10^6 cells/mouse) of unrelated MSCs into NOD/SCID mice after sublethal irradiation. A total of 42 NOD/SCID mice were used. Among them, 7 were infused only UCB $CD34^+$ cells, 34 were both UCB $CD34^+$ cells and MSCs, and the last one only MSCs for control. The distribution of each cohort according to test cell doses and patterns of hematopoietic engraftment are depicted in Table 1. Fig. 4 shows representative flow-cytometry profiles. Cotransplantation of UCB $CD34^+$ cells and MSCs resulted in significantly higher engraftment levels in the bone marrow of NOD/SCID mice than transplantation of UCB $CD34^+$ cells alone (Fig. 5A, $p<0.05$). Analysis of subpopulations of human cells in the bone marrow of NOD/SCID mice revealed that the engraftment promoting effect of MSCs was also evident in $CD45^+/CD3^+$, $CD45^+$/glycophorin-A^+, $CD45^+/CD41^+$ and $CD45^+/CD34^+$ cell populations (Fig. 5A, $p<0.05$). The engraftment-promoting effect was also found in the peripheral blood; higher percentages of human $CD45^+$ cells were found in the peripheral blood of NOD/SCID mice when MSCs were contransplanted (Fig. 5B). In the spleen of NOD/SCID mice, a similar pattern of enhanced engraftment was observed, but didn't reach a statistical significance. Thymus showed similar engraftment patterns between the cohorts with and without cotranplantation of MSCs (Fig. 5C, D).

The bone marrow hematopoietic engraftment promoting effect of MSCs was proportional to the transplanted cell doses of MSCs; the mice cotransplanted with 0.5×10^6 MSCs showed higher levels of human $CD45^+$ cell engraftment in the bone marrow compared with the those of the mice given 0.1×10^6 MSCs, and this pattern

was also observed between the cohorts given 0.5×10^6 MSCs and 1.0×10^6 MSCs. This relatively dose dependent engraftment promoting effect of MSCs was obvious after transplantation of relatively low doses of UCM $CD34^+$ cells ($0.1\text{-}0.3\times10^6$; Fig. 6). Thus it seemed that cotransplantation of MSCs with lower cell doses of hematopoietic stem cells would be particularly beneficial in a transplanted MSCs dose-dependent manner, but this engraftment promoting effect might disappear along with increase of transplanted HSCs doses.

Table 1. Effect of cotransplantation of MSCs on engraftment of UCB $CD34^+$ cells in bone marrow, blood, spleen and thymus of NOD/SCID mice 6 weeks after transplantation

	BM*	PB*	Spleen*	Thymus*
Transplanted cell dose of UCB $CD34^+$ cell = 0.1×10^6/mouse				
UCB only (n=1)	4.5	6.6	3.3	1.2
UCB + MSCs (n=10)	7.1±5.08	12.5±7.32	7.7±6.33	3.96±2.53
MSCs 0.1 ± 10^6 (n=4)	2.8±0.95	13.5±10.6	5.0±3.6	2.8±1.5
MSCs 0.5 ± 10^6 (n=3)	9.78±1.1	12.8±4.11	14.2±6.9	1.0±0.66
MSCs 1.0 ± 10^6 (n=3)	9.9±7.4	10.0±5.98	4.7±4.58	6.2±2.75
Transplanted cell dose of UCB $CD34^+$ cell = 0.3×10^6/mouse				
UCB only (n=2)	3.0±0.64	11.6±6.75	14.3±1.78	4.2±3.37
UCB + MSCs (n=8)	13.5±12.24	15.0±418.33	11.5±6.80	8.21±14.13
MSCs 0.1 ± 10^6 (n=4)	4.9±0.84	7.0±5.84	13.1±7.7	2.7±2.1
MSCs 0.5 ± 10^6 (n=3)	16.0±3.1	12.3±6.30	9.9±7.9	4.4±6.0
MSCs 1.0 ± 10^6 (n=1)	40.25	58.5	9.9	42.0
Transplanted cell dose of UCB $CD34^+$ cell = 0.5×10^6/mouse				
UCB only (n=2)	7.9±4.50	9.8±6.05	8.7±5.78	7.7±7.89
UCB + MSCs (n=9)	41.9±33.28	20.4±13.28	11.1±7.84	11.4±8.65
MSCs 0.1 ± 10^6 (n=2)	7.2±1.23	6.6±3.79	15.1±8.54	9.8±12.3
MSCs 0.5 ± 10^6 (n=3)	42.3±28.7	17.8±8.99	8.1±5.79	7.9±9.15
MSCs 1.0 ± 10^6 (n=4)	59.0±34.78	29.2±13.21	11.3±9.88	14.8±7.95
Transplanted cell dose of UCB $CD34^+$ cell = 1.0×10^6/mouse				
UCB only (n=2)	54.8±0.59	6.9±4.59	5.2±5.57	14.6±12.02
UCB + MSCs (n=7)	78.5±30.59	16.0±7.41	18.8±24.4	12.0±15.79
MSCs 0.1 ± 10^6 (n=1)	86.6	5.8	67.9	11.3
MSCs 0.5 ± 10^6 (n=2)	88.1±10.86	21.3±0.17	10.8±10.96	5.4±0.22
MSCs 1.0 ± 10^6 (n=4)	71.7±41.53	15.8±7.47	10.6±14.66	15.5±21.29
Transplantation of only MSCs				
MSCs=1.0×10^6/mouse (n=1)	0.92	0.5	0.1	0.1

*% of human $CD45^+$ cells

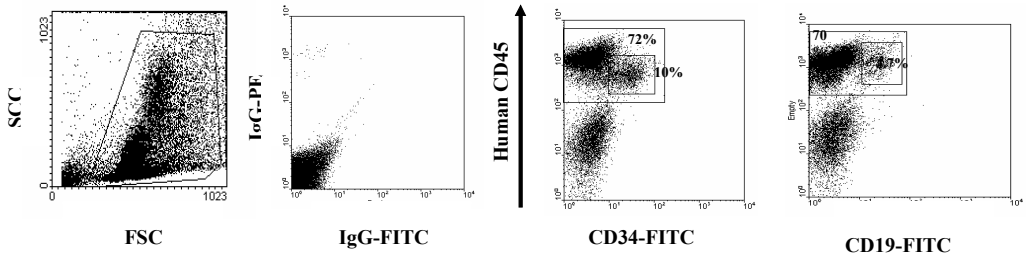

Fig. 4. Representative flow-cytometry profiles of bone marrow from NOD/SCID mice after cotransplantation of UCB $CD34^+$ cells and MSCs.

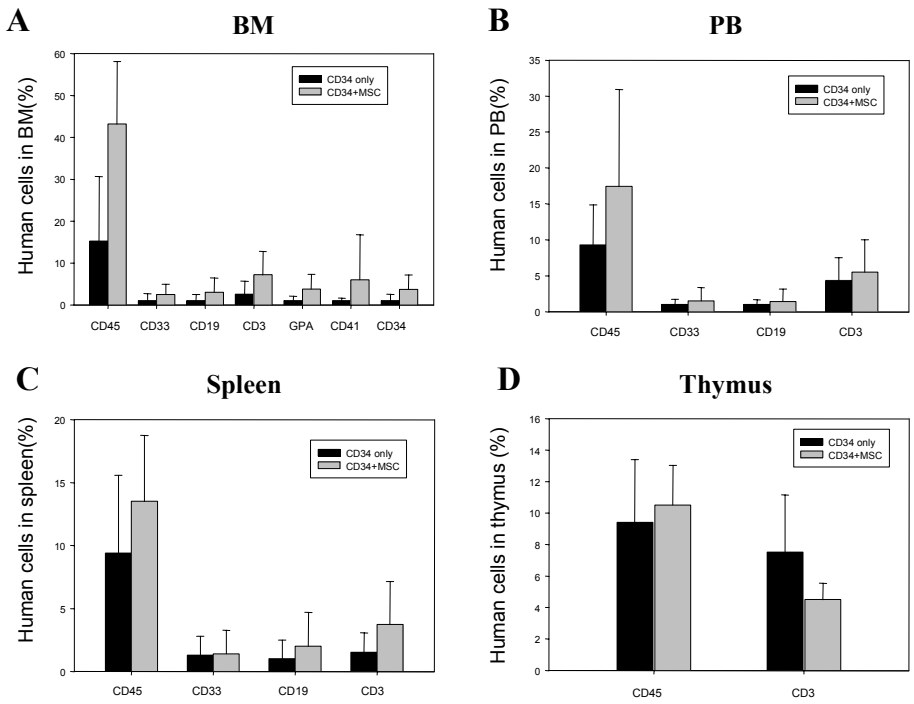

Fig. 5. Engraftment of human hematopoietic cells in NOD/SCID mice 6 weeks after cotransplantation of UCB $CD34^+$ cells and MSCs. MSCs cotrans- plantation resulted in significantly higher engraftment levels in the bone marrow of NOD/SCID mice compared with those transplanted with UCB $CD34^+$ cells alone. The engraftment promoting effect was also evident in T-lymphoid, erythroid, megakaryocytic, and $CD34^+$ cell lineages (**A**). The engraftment promoting effect was also found in the peripheral blood (**B**), but no significant effects were observed in the spleen and thymus (**C, D**). BM; bone marrow, PB; peripheral blood, GPA; glycophorin A, *p<0.05

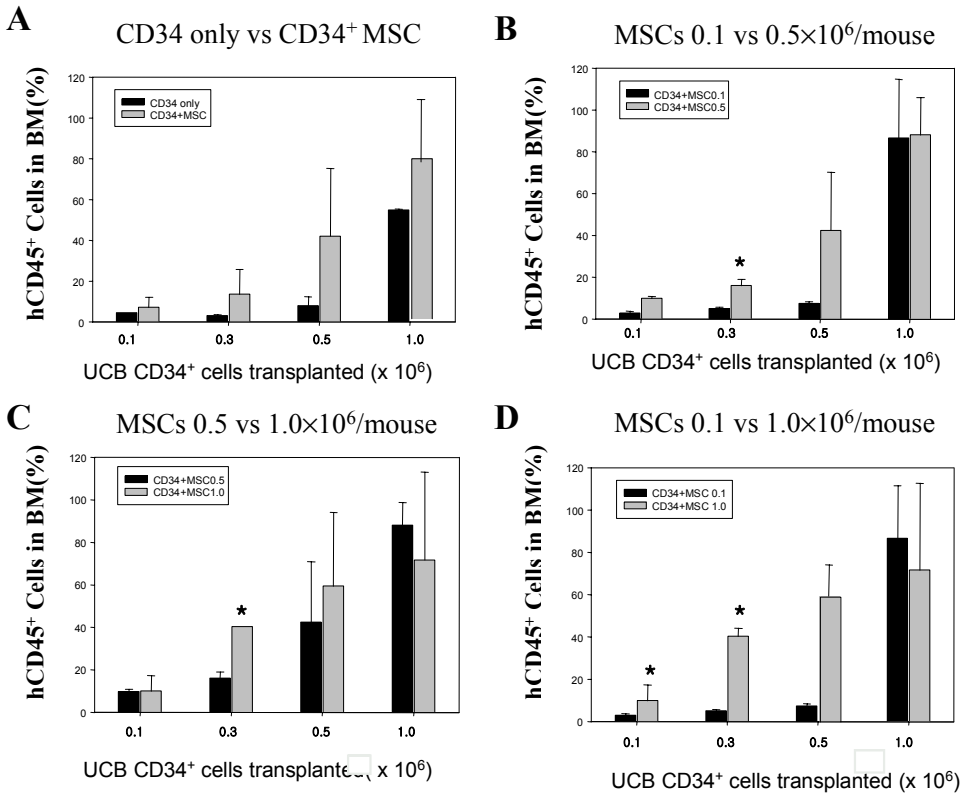

Fig. 6. Bone marrow engraftment patterns in NOD/SCID mice 6 weeks after cotransplantation of UCB $CD34^+$ cells and MSCs according to the transplanted cell doses of MSCs. The mice transplanted with lower doses of UCM $CD34^+$ cells ($0.1\sim0.3\times10^6$) showed higher levels of engraftment proportional to infused MSCs doses. *$p<0.05$

3. Engraftment of human leukemic cells in NOD/SCID mice 6 weeks after cotransplantation of AML cells and MSCs

Primary AML cells from two patients were used. Cell suspensions of both patients contained >85% leukemic blasts as was verified by morphologic and phenotypic analysis. Patients profiles are as follows; 21 year old female patient with relapsed AML (M1) after allogeneic bone marrow transplantation, the other was a 64 year old male with secondary AML to whom no treatment had been given. A total of 12 NOD/SCID mice were used, and each AML cells from the two patients were

given to 6 mice respectively. Six mice were infused AML cells only and the rest both AML cells and MSCs. Transplanted AML cell dose was 3×10^7/mouse, and 2 different doses of MSCs (0.5×10^6, 1.0×10^6cells/mouse) were transplanted. The patterns of leukemic cell engraftment after 6 weeks of transplantation are presented in Table 2 and Fig. 7. Patterns of leukemic cell engraftment analyzed as human $CD45^+$/ murnie $CD45^-$ cell population in bone marrow, peripheral blood, spleen and thymus were similar in both cohorts irrespective of MSCs cotransplantation. Neither the different origin of leukemic cells nor different doses of MSCs transplanted made any difference in leukemic cell engraftment.

Table 2. Effect of cotransplantation of MSCs on engraftment of human AML cells in bone marrow, blood, spleen and thymus of NOD/SCID mice 6 weeks after transplantation

	BM*	PB*	Spleen*	Thymus*
AML cells only (n=6)	13.5±10.68	19.6±20.02	4.9±3.01	6.0±9.69
AML cells + MSCs (n=6)	15.2±10.20	16.8±12.76	2.6±1.98	11.4±12.87
MSCs 0.5×10^6 (n=3)	16.8±12.26	17.7±13.39	3.4±2.18	12.8±14.88
MSCs 1.0×10^6 (n=3)	12.8±9.80	15.3±16.89	1.4±1.27	9.1±10.72

*% of human $CD45^+$ cells

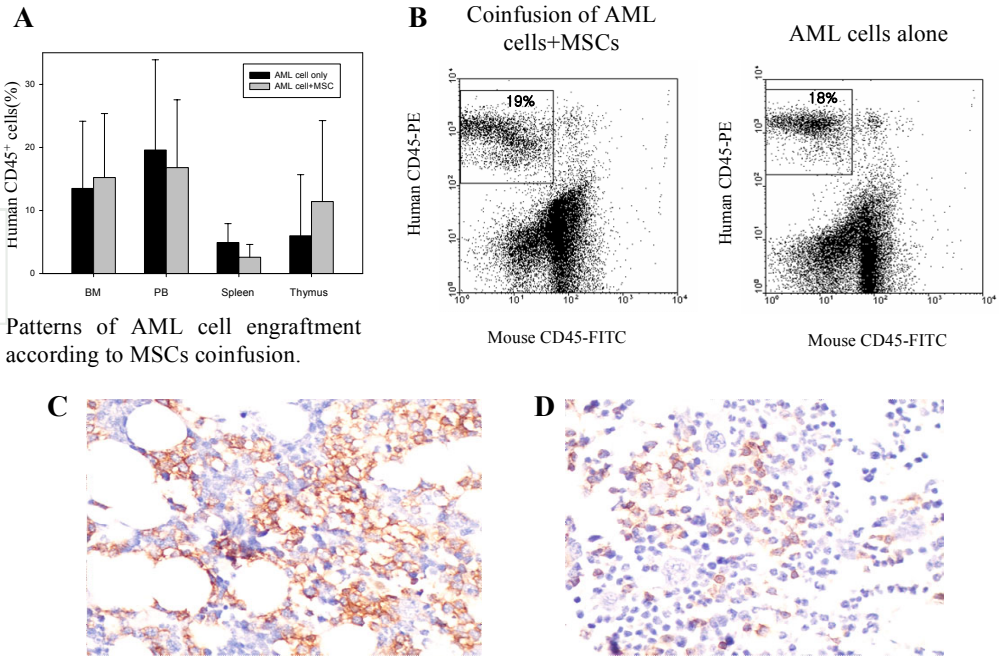

Fig. 7. Cotransplantation of AML cells and MSCs has no effect on leukemic cell engraftment. **A**. No significant difference was found according to the MSCs cotransplantation. **B**. Representative flow cytometry profiles of the bone marrow of the NOD/SCID mice. **C** & **D**. LCA (leukocyte common antigen) immunohistochemical staining clearly demonstrates the distribution of leukemic cells in the bone marrow of NOD/SCID mice (**C**. AML cells alone. **D**. AML cells + MSCs)

4. Homing and engraftment of MSCs - Detection of eGFP in organs of the NOD/SCID mice

Ten NOD/SCID mice were transplanted with eGFP marked MSCs, all at the same cell doses of 0.5 10^6cells/mouse after sublethal irradiation. Mice were killed 6 weeks after the transplantation and a total of 45 organ samples were obtained, which were comprised of liver, kidney, lung, muscle, brain, and bone marrow. All the 45 samples analyzed by RT-PCR showed the expression of eGFP messenger RNA, while samples from negative control (mice transplanted with untreatd MSCs) showed no expression of eGFP (Fig. 8). Immunohistochemistry using an antibody to

eGFP revealed eGFP expressing cells in 2 liver specimens. The eGFP expressing cells were scattered mostly around the portal tracts of liver, and these cells had cytologic features of hepatocytes (Fig. 9). Some auto-flourescent cells were also observed in the same liver specimens using confocal laser scanning microscope (Fig. 9). Immunohistochemistry of lumbar vertebrae specimen from another mouse also showed eGFP expressing cells. When compared with hematoxylin-eosin stained specimen, they could be distinguishable from the endothelial cells and hematopoietic cells (Fig. 9). Considering their morphology and locations within the bone marrow, these eGFP expressing cells could be assumed as bone marrow stromal cells.

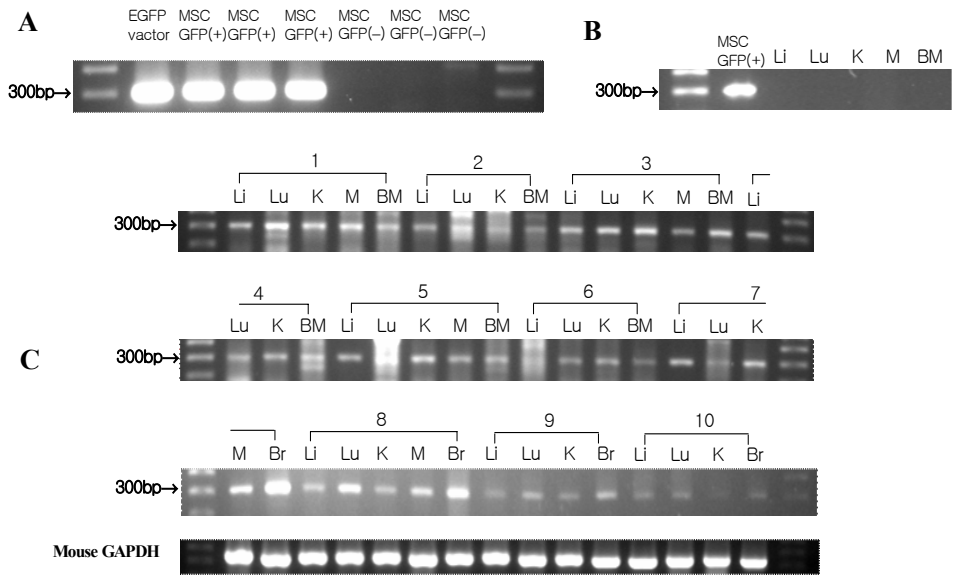

Fig. 8. eGPF expression analyzed by RT-PCR in NOD/SCID mice 6 weeks after eGPF transduced MSCs transplantation (0.5×10^6/mouse). **A**. Positive control using an eGFP vector and the eGFP transduced MSCs. **B**. Negative control using a mouse transplanted with untreated MSCs. **C**. Organ samples from 10 mice after eGFP transduced MSCs transplantation. Li: Liver, Lu: Lung, K: Kidney, M: Muscle, BM: Bone marrow, Br: Brain.

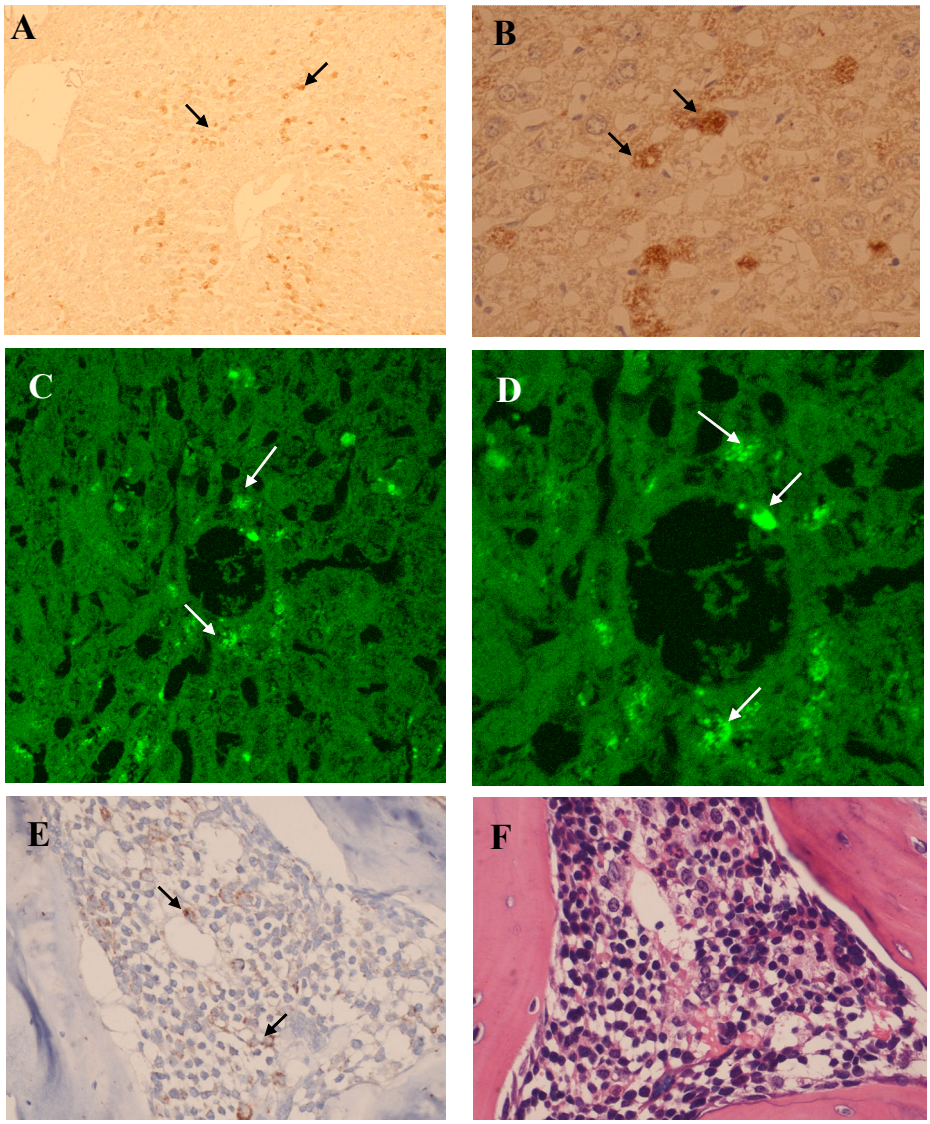

Fig. 9. Immunohistochemical staining and Confocal microscopic findings of organ specimens from NOD/SCID mice 6 weeks after transplantation of eGFP transduced MSCs. **A** & **B.** Liver stained with Peroxidase-DAB chromogen; ×100 & ×400. The arrows indicate hepatocyte-like cells which show positive reaction for eGFP. **C** & **D**. Confocal microscopic findings of Liver; 200 & ×400. The arrows indicate autofluorescent cells. **E**. Bone marrow stained with Peroxidase-DAB chromogen; ×400. The arrows indicate stromal cells with positive reaction for eGFP. **F**. Hematoxylin-eosin stain of bone marrow; ×400.

IV. DISCUSSION

Human MSCs give rise to marrow stromal cells that produce the stromal matrix, which constitutes the bone marrow microenvironment[27]. In vitro studies have demonstrated the capacity of human MSCs to secrete numerous hematopoietic cytokines and to support hematopoiesis[27] Cotransplantation of autologous MSCs with autologous HSCs has been previously reported to enhance HSCs engraftment in patients with breast cancer[15]. The engraftment of fully allogeneic MSCs has been reported in both canine and non-human primate models[28,29]. These animal models have demonstrated the potential for MSCs to enhance engraftment of allogeneic HSCs. In these regards, we inferred that MSCs could support the survival of hematopoietic stem cells and tested this hypothesis using an in vivo model of human hematopoietic stem cell engraftment and survival.

The results of this study demonstrate that cotransplantation of UCB $CD34^+$ cells and cultured MSCs resulted in significantly higher engraftment levels 6 weeks after transplantation than those observed after transplantation of UCB $CD34^+$ cells alone. This engraftment promoting effect of MSCs was obvious when low doses of UCB $CD34^+$ cells were given, whereas at relatively high doses of UCB $CD34^+$ cells, no further additive effect of MSCs on engraftment was observed. This result coincides with previous studies in mice and fetal sheep which have clearly demonstrated the capacity for stromal cell elements to enhance hematopoietic engraftment when cotransplanted with HSCs[11,12,28,30,31]. Furthermore, this effect of MSCs on hematopoietic engraftment seemed to be increased as transplanted MSCs numbers increase. But we could not test the optimal dose of MSCs for cotransplantation of HSCs because it was not possible to increase the dose of MSCs due to large MSCs size and eventual large graft volumes considering tiny size of the mice in this study. Therefore, to clarify the optimal dose of MSCs for cotransplantation of HSC and to test the maximal tolerable dose of transplanted MSCs, which could be applied to

human studies, large animal experiments might be needed. The engraftment promoting effect was not lineage restricted and also evident in T lymphoid, erythroid, megakaryocytic, and stem cells. From this data and the previous report suggesting that unrelated MSCs are not immunogenic and not elicit any allogeneic immune response[30], it is conceivable that therapeutic MSCs could be obtained from donors unrelated to the UCB donor in support of UCB transplantation. Noort et al[14] reported that human fetal lung-derived MSCs promoted human UCB engraftment in NOD/SCID mice. Similar to our findings, human engraftment was enhanced only when transplanted UCB $CD34^+$ cell numbers were limited. In contrast to adult bone marrow derived MSCs, the clinical use of human fetal lung derived MSCs may be more difficult due to the limited availability of such tissues and ethical concerns with procurement. Angelopoulou et al[31] recently reported that cotransplantation of human MSCs enhanced myelopoiesis and megakaryopoiesis in NOD/SCID mice transplanted with the limited doses of peripheral blood $CD34^+$ cells. Our results extend these observations to UCB cells and suggest an important supportive role for MSCs in the pre-clinical models of hematopoietic transplantation.

The mechanism of human hematopoietic support in NOD/SCID mice by human MSCs has not been clarified. Noort et al[14] did not find any impact of fetal lung MSCs on the homing of a coinfused $CD34^+$ cell. It is possible that cotransplanted MSCs provide human cytokines and hematopoietic niches to support human cells by direct contact. In this report, the wide distribution of human MSCs in NOD/SCID mice after intravenous infusion was observed. Evidence of bone marrow engraftment of MSCs was also found in dogs and nonhuman primates after intravenous infusion[8]. So, it is conceivable that engrafted MSCs to bone marrow might repair and reconstitute marrow microenvironment, supporting donor hematopoiesis. But the possible role of systemically operating humoral factors secreted by MSCs after cotransplantation cannot be excluded at this time.

Similar to normal hematopoietic cells, AML cells can proliferate and differentiate

in vitro in the presence of normal bone marrow stromal cells[32]. Interactions with bone marrow stroma are important in regulating growth, maturation, and survival of normal hematopoietic progenitors, and may be effective in maintaining survival and clonogenic potential of AML blasts even after chemotherapy exposure[33]. In addition, given the in vitro immunosuppressive properties of MSCs to inhibit primary mixed lymphocyte reaction (MLR) and prolong skin graft survival in baboons given third-party MSCs[34], cotransplantation of MSCs could prevent or mitigate graft-versus-host disease (GVHD) following allogeneic HSCT. However, it is also conceivable if MSCs have a suppressive effect on GVHD in allograft setting, it can also exert similar effect on graft-versus-leukemia activity. In this regard, the safety profiles of MSCs cotransplantation for patients with leukemia must be validated. Therefore, I was interested in the consequences of coinfusing MSC with primary AML cells into NOD/SCID mice. Leukemic cell engraftment patterns of two different AML samples were very similar, so I put each data together. There was no difference in the levels of leukemic engraftment 6 weeks after transplantation between mice cotransplanted with primary AML cells and MSCs and mice transplanted with AML cells alone. But I have to admit a drawback in this data because I used an immunosuppressed animal model which is not suitable for investigating alloimmune phenomenon such as graft-versus-leukemia effect. Although the number of mice studied here is small, the results suggest that MSCs don't exert any positive influence on leukemic cell engraftment by the same way working on normal hematopoietic cells when cotransplanted with AML cells into NOD/SCID mice.

Finally, the homing capability of MSCs was analyzed after systemic infusion of eGFP-marked MSCs. After analyzing 45 samples (bone marrow cells, liver, kidney, heart, lung, muscle and brain) obtained from 10 mice by RT-PCR, I found eGFP m-RNA in all of the samples analyzed. These data demonstrate that following systemic infusion into NOD/SCID mice, MSCs can be detected in a wide variety of nonhematopoietic tissues along with the bone marrow after 6 weeks of infusion. In

contrast, Noort et al[14] did not find such wide distribution of MSCs cultured from fetal lungs even after greater numbers of MSCs infusion compared with this study. They could detect MSCs only in the lung, explaining not only by trapping of cells in the lungs after intravenous injection, but other uncertain mechanisms such as different source of MSCs. Devine et al[8] initially could not detect baboon bone marrow derived MSCs in nonhematopoietic tissues outside the bone and bone marrow, suggesting that baboon MSCs home preferentially to the bone marrow. But later they could demonstrate the wide nonhematopoietic distribution of MSCs using more sensitive detection technique[21]. In contrast, I could easily detect eGFP using conventional PCR technique. This difference might be due to the higher transduction efficiency of eGFP obtained by adenoviral vector in this study. At first the adeno-associated virus (AAV) was tried to transduce eGFP into MSCs, but less than 30% of transduction efficiencies (data not shown) were obtained. So I chose the adenovirus as an eGFP gene delivery vehicle and could obtain more than 90% of transduction efficiency, which persisted for more than 3 passages of culture. Previous study demonstrated that transgene expression in MSC transduced by adenovirus can persist for more than 3 weeks without marked decrease in gene expression level in accordance with this data[35]. They also showed that adenoviral mediated gene transfer had no influence on differentiating capability of MSCs even with a higher MOI than this study[36]. I tried various MOIs between 2 and 10 referring to my previous experiments and found that viability of cells decreased significantly at more than MOI 5. Combination of MSCs and adenoviral vector could be an excellent ex vivo gene therapy strategy considering high gene transduction efficieny and long term in vivo survival without losing transgene expression. To document the existence of eGFP in mice tissues, confocal laser scanning microscopy and immunohistochemical staining for eGFP were performed. Despite an ubiquitous distribution of eGFP mRNA in mice organs detected by RT-PCR, only a limited number of liver and bone marrow specimens showed positive reaction for eGFP by immunohistochemistry, and autoflourescent cells were

also observed in the same liver specimens by confocal laser scanning microscopy. This discrepancy could be explained by the low amount of eGFP in tissues under the detection threshold for immunohistochemistry but high enough to be detectable by PCR technique. I did not test further whether eGFP expressing cells detected by immunohistochemistry were of human origin and whether they had hepatocyte phenotypes because the present study was not designed to confirm the ability of the infused MSCs to differentiate into other cell types after colonization. It could be considered the possibility of transdifferentiation of MSCs which had colonized in liver, although no definite evidence about the transdifferentiation capability of adult stem cells has been presented so far. Almeida-Porada et al[36] recently reported that human metanephric mesenchymal cells produced human blood as well as hepatocyte-like cells, suggesting that mesenchymal cells may represent a broad population of putative stem cells in multiple adult organs. In this regard, better understanding of the mechanism that attracts MSCs to specific organs and promotes differentiation may enhance the prospects of systemic delivery of MSCs becoming attractive therapeutic strategies for repair and regeneration of damaged tissues.

In summary, MSCs promote engraftment of UCB $CD34^+$ cells in the bone marrow, but exert no influence on engraftment of AML cells. After systemic infusion, MSCs can colonize in various organs including bone marrow in NOD/SCID mice. These findings suggest that cotransplantation of MSCs could be used safely to support UCB transplantation and ultimately broaden its clinical application. I also suggest the potential applicability of MSCs for repair and regeneration of damaged tissues in view of its wide distribution after systemic infusion. But the distribution kinetics after systemic delivery and transdifferentiation potential of MSCs still need to be clarified.

V. CONCLUSION

In this study, I investigated whether coinfusion of ex-vivo culture expanded human bone marrow derived MSCs and unrelated UCB $CD34^+$ cells into NOD/SCID mice could enhance the hematopoietic engraftment and correlation between transplanted cell doses of MSCs and patterns of hematopoietic engraftment. We next determined whether there were any leukemic cell engraftment promoting effects of MSCs when cotransplanted with leukemic cells into NOD/SCID mice. Finally, we assessed the homing capability of MSCs to hematopoietic and nonhematopoetic tissues after systemic infusion of eGFP marked MSCs.

1. Cotransplantation of UCB $CD34^+$ cells and cultured MSCs resulted in significantly higher engraftment levels 6 weeks after transplantation than those observed after transplantation of $CD34^+$ UCB cells alone. The engraftment promoting effect was not lineage restricted and also evident in T lymphoid, erythroid, megakaryocytic, and stem cells.

2. The engraftment promoting effect of MSCs was obvious at low doses of UCB $CD34^+$ cells, whereas at relatively high doses of UCB $CD34^+$ cells, no further additive effect of MSCs on engraftment was observed. Furthermore, this effect of MSC son hematopoietic engraftment was increased as transplanted MSCs numbers increase.

3. There was no difference in the levels of leukemic engraftment 6 weeks after transplantation between mice transplanted with primary AML cells along with MSCs and mice transplanted with AML cells alone.

4. After inoculation of adenovirus-eGFP into MSCs, more than 90% of eGFP transduction efficiency was obtained and it persisted for more than 4 passages of culture.

5. Six weeks after transplantation of eGFP marked MSCs into 10 NOD/SCID mice, organ samples including the bone marrow, liver, kidney, heart, lung, muscle and brain were analyzed for the existence of transplanted MSCs by RT-PCR,

immunohistochemistry, and confocal laser scanning microscopy. All the samples analyzed by RT-PCR showed the expression of eGFP m-RNA. Two liver and a bone marrow specimens showed positive reaction for eGFP by immunohistochemistry, and autoflourescent cells were observed in the same specimens by confocal microscopy.

In summary, MSCs promote engraftment of UCB $CD34^+$ cells in the bone marrow, but exert no influence on engraftment of AML cells. After systemic infusion, MSCs can colonize in various organs including bone marrow in NOD/SCID mice. These findings suggest that cotransplantation of MSCs could be used safely to support UCB transplantation. It also can be suggested the potential applicability of MSC for repair and regeneration of damaged tissues in view of its wide distribution after systemic infusion. But the distribution kinetics after systemic delivery and transdifferentiation potential of MSCs still need to be clarified.

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ABSTRACT (IN KOREAN)

중배엽간세포 이식이 마우스모델에서 사람 조혈모세포와 백혈병세포의 생착에 미치는 영향

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이 승 태

서론: 본 연구에서는 사람의 중배엽간세포와 조혈모세포 및 백혈병 세포의 동시 이식시 생착에 미치는 영향을 마우스 실험을 통하여 확인하고, 또한 투여된 중배엽간세포의 마우스 장기내 분포를 확인하고자 하였다.

재료 및 방법: NOD/SCID 마우스(n=42)에 3.0 Gy의 전신방사선 조사 후 24시간 내에 제대혈 CD34양성세포를 단독(n=7) 혹은 중배엽간세포와 동시에(n=35) 꼬리 정맥을 통하여 주입하였으며, 급성골수성백혈병 환자 2례의 골수세포를 단독(n=6) 혹은 중배엽간세포와 동시에(n=6) 주입하였다. 이중 10례에서는 아데노바이러스를 이용하여 eGFP 유전자 형질도입된 중배엽간세포를 주입하였다.

결과: 이식 6주후 조혈모세포의 생착율은 중배엽간세포를 동시이식한 군에서 유의하게 높았으며, 이러한 생착촉진 효과는 투여된 CD34 양성세포의 세포수가 적을수록 현저하였고, 투여된 중배엽간세포의 세포수에 비례하는 양상을 보였다. 백혈병세포의 생착양상은 중배엽간세포 동시투여군과 백혈병세포 단독 투여군에서 차이가 없었다. eGFP유전자 형질도입된 중배엽간세포를 투여받은 10례의 마우스의 장기(간, 폐, 뇌, 근육, 신장, 골수)를 역시 이식 6주후 RT-PCR로 분석한 결과, 검사한 모든 장기에서 eGFP의 mRNA존재를 확인할 수 있었으며, 이중 2례에서는 간, 다른 1례에서는 골수에서 면역조직화학염색 및 공초점 현미

경상 eGFP의 존재가 확인되었다.

결론: 중배엽간세포를 조혈모세포와 동시 이식했을 경우 조혈모세포 이식 세포수가 적은 경우에 현저한 생착 촉진효과를 보인 반면, 백혈병세포의 생착에 미치는 영향은 없었다. 또한 중배엽간세포는 이식 후 중요 장기에 광범위하게 생착됨을 확인할 수 있었다.

핵심되는 말: 중배엽간세포, CD34, 백혈병, 조혈모세포이식