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Protection of Bone Marrow-Derived CD45$^+$/CD34$^-$/lin$^-$ Stromal Cells with Immunosuppressant Activity against Ischemia/Reperfusion Injury in Rats

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Abstract

Non-hematopoietic CD45$^+$ precursor cells are not known to differentiate into cardiomyocytes. We found that CD45$^+$/CD34$^-$/lin$^-$ stromal cells isolated from mouse bone marrow (BMSCs) potentially differentiated into cardiomyocyte-like cells *in vitro*. Therefore, we hypothesized that the CD45$^+$/CD34$^-$/lin$^-$ BMSCs might protect rat hearts against ischemia/reperfusion (IR) injury following xeno-transplantation. In the present study, BMSCs were isolated by immunoselection and their cellular phenotype and biochemical properties were characterized. The immunological inertness of BMSCs was examined by the allogeneic and xenogeneic mixed lymphocyte reaction (MLR). The potential role of BMSCs for cardioprotection was evaluated by intravenous introduction of 1×10^6 cells into rat IR hearts, induced by left coronary ligation for 45 min and released for 72 h. Changes in cardiac contractility and the degree of myocardial injury were assessed. Our findings indicated that BMSCs expressed the muscle-cell marker α-actinin after 5-azacytidine treatment. CD45$^+$/CD34$^-$/lin$^-$ stromal cells were characterized as mesenchymal progenitor cells based on the expression of Sca-1 and Rex-1. The MLR assay revealed an immunosuppression of BMSCs on mouse and rat lymphocytes. After xeno-transplantation, the BMSCs engrafted into the infarct area and attenuated IR injury. However, increases in intracardial TGF-β and IFN-γ contents of IR hearts were not affected by BMSC treatment. Interestingly, *ex vivo* evidence indicated that CXCR4, SDF-1 and TGFβ-1 receptors were up-regulated after the cells were exposed to tissue extracts prepared from rat post-IR hearts. In addition, IFN-γ treatment also markedly increased Sca-1 expression in BMSCs. Mechanistically, these results indicated that CXCR4/SDF-1 and TGF-β signals potentially enhanced the interaction of BMSCs with the damaged myocardium, and increased IFN-γ in post-ischemic hearts might cause BMSC to behave more like stem cells in cardioprotection. These data show that CD45$^+$/CD34$^-$/lin$^-$ BMSCs possess cardioprotective capacity. Evidently, the accurate production of soluble factors TGF-β and IFN-γ in parallel with increased expression of both TGF-β and Sca-1 receptors may favor BMSCs to achieve a more efficient protective capacity.

Key Words: bone marrow, CD45$^+$ stromal cells, ischemia/reperfusion, cardioprotection, soluble factor

Introduction

The myocardium has a high demand for oxygen supply. Interruption of blood supply for a certain period followed by reperfusion always causes irreversible myocardial damage which is described as

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ischemia/reperfusion (IR) injury. The changes in myocardial tissues following IR injury contribute to functional failure. Until now, cardiac transplantation is the only solution to cure severe terminal heart failure. Cellular cardiomyoplasty is a new therapeutic approach characterized by the delivery of appropriate healthy donor cells to the injured myocardium to replace the damaged cardiomyocytes. Various non-progenitor cells, such as adult cardiomyocytes, skeletal myoblasts, immortalized myoblasts, smooth muscle cells and fibroblasts, have been transplanted into the injured myocardium (18, 28, 41, 45, 46). Nevertheless, stem/progenitor cells have been the widely used choice to regenerate cardiac tissues damaged by myocardial infarction (7, 8, 17, 24, 36, 40, 43, 49).

It is well known that bone marrow stromal cells (BMSCs) contains heterogeneous adult stem cells, or progenitors, and can give rise to various mesenchymal and non-mesenchymal cell types (14, 15, 21, 38). An *in vitro* study has demonstrated that BMSCs can express phenotypic characteristics of cardiomyocytes after myogenic induction (33). Later, the efficiency of BMSCs on improvement of cardiac functions was reported (47). Furthermore, animal studies also showed that BMSCs can ameliorate cardiac functions after myocardial infarction (19, 32, 35, 42). Non-adherent bone marrow cells expressing $CXCR4^+/Sca\text{-}1^+/lin^-/CD45^-$ mononuclear cells in mice and $CXCR4^+/CD34^+/AC133^+/CD45^-$ mononuclear cells in humans have been reported to be capable of mobilizing into the peripheral blood and chemoattracting to the infarcted myocardium (24).

Recently, transplanted bone marrow (BM)-derived adherent cell population attenuated cardiac dysfunction in several animal models. To this end, most evidence indicates that BM-derived stem cells are $CD45^-/CD34^-/lin^-$ with $CD105^+$, $CD73^+$ (human) or $Scal\text{-}1^+$ (mice) and contaminate populations of bone marrow cells (12, 37). Furthermore, Jiang and colleagues (20) have reported that bone marrow-derived multipotent adult progenitor cells (MAPCs) showed minimal engraftment into the myocardium after being injected into the embryonic blastocyst. Yoon *et al*. (51) recently identified novel multipotent stem cells from bone marrow that attenuated cardiac dysfunction when transplanted into an animal model of myocardial infarction. The view of the majority is that $CD45^+$ adherent stromal cells do not have therapeutic effectiveness on cardioprotection. However, although BMSCs exhibit potential effects on cellular therapy or regeneration medicine, it is still not known whether a specific subpopulation of the BMSCs is actually responsible for these effects. Here, we provide evidence that adult mouse BMSCs harbor an adherent population of $CD45^+/CD34^-/lin^-$ non-hematopoietic mononuclear cells that are capable of differentiating into cardiomyocyte-like cells *in vitro* with characteristics of mesenchymal progenitor cells. After injecting BMSCs into rat IR hearts by xeno-transplantation, we found that murine BMSCs were capable of migration and engraftment into an area of infarction to achieve cardioprotection.

Materials and Methods

Animals

Inbred BALB/c and C57BL/6 mice (10~12 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan, ROC) and used as donors for bone marrow stromal cells or hematopoietic stem cells. Male and female Sprague Dawley rats (200-225 g) were purchased from BioLASCO Taiwan Co., Ltd and used for the myocardial infarction procedure. Upon arrival, mice or rats were caged in a colony room where a 12-h light-dark cycle was maintained through artificial illumination. Animals were provided with free access to food and water throughout the experiment unless otherwise noted, and a 2-week acclimatization period was provided prior to experimental manipulation. All animal protocols were approved by the Institutional Animal Care and Use Committee.

$CD45^+/CD34^-$ Bone Marrow Stromal Cell Isolation and Culture

Male BALB/c mice were used as the source of BMSCs. Briefly, bone marrow was harvested by flushing the femoral and tibial shafts with collecting buffer as described previously (9). Red blood cells were lyzed with osmotic lysis buffer and washed three times with collecting buffer and complete culture medium (CCM). The $CD45^+/CD34^-$ mononuclear cells were obtained by negative immuno-depletion of CD3-, CD11b-, B220-, NK1.1-, Gra-1- and Ter-119-positive cells using a negative selection magnetic bead solution kit (Collection Biotin Binder kit, Dynal Invitrogen, Carlsbad CA, USA) followed by Ficoll-Paque (Amersham Bioscience, Piscataway, NJ, USA) density gradient centrifugation (1.077 g/cm^3) and then plated in non-coated tissue culture flasks (Nunc, Roskilde, Denmark) in expansion medium. Cells were allowed to adhere overnight, and non-adherent cells were washed out with medium changes. Medium changes were carried out twice weekly thereafter. The expansion medium consisted of Iscove's modified Dulbecco's medium (IMDM, Gibco-BRL, Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (Gibco-BRL). Adherent cells that grew

with a homogeneous fibroblastic morphology and reached 50%~60% confluence were collected and re-plated in the same culture medium.

In Vitro *Cardiomyocyte Differentiation*

Cardiomyocyte differentiation was accomplished by culturing cells for two weeks in a standard medium supplemented with 5 μM 5-azacytidine (Sigma-Aldrich, St. Louis, MO, USA). The characterization of cardiomyocyte differentiation was confirmed by the presence of α-actinin.

Induction of Rat Myocardial Infarction and Administration of Mouse BMSCs

The rats were anesthetized by intrapertioneal ketamine (60 mg/kg) and sodium pentobarbital (35 mg/kg). The rats were ventilated with room atmospheric air at 60 breaths min^{-1} and a tidal volume of 8 ml/kg after intubation. The rectal temperature was maintained at 37°C with a servo-null heating pad. Left thoracotomy was performed under aseptic conditions. The left anterior descending artery close to its origin, about 3 mm away from the left coronary ostium, was looped with 7-O Prolene (Ethicon Inc., Somerville, NJ, USA) as previously described (31). The looped stitch was snared for 40 min and then released. Ischemia was confirmed by the appearance of regional cyanosis on the epicardium distal to the ligation or by akinesia or bulging in this area. In the stem cell-treated rats, 1×10^6 BMSCs prepared in phosphate buffered saline in a volume of 0.1 ml were administered intravenously *via* the external jugular vein within 5 min in the IR hearts after coronary ligation and, at the same time, in the control hearts. Then, the wound and thorax were closed under aseptic conditions, and each rat was returned to its own cage for recovery.

Determination of Myocardial Injury

After 72 h, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood was collected from the abdominal aorta before the administration of 1 ml of 500 IU heparin *via* the inferior vena cava. The hearts were then rapidly excised, weighed, and immersed in ice-cold Krebs-Henseleit (KH) buffer as previously described (31). Briefly, the aorta was cannulated for retrograde perfusion, and the heart was then mounted on a Langendorff apparatus (Radnoti, Monrovia, CA, USA). The coronary perfusion pressure at the aortic site was measured using a pressure transducer (BD Biosciences, Franklin Lakes, NJ, USA) connected to the perfusion line. The mechanical performance of left ventricle was continuously measured using a water-filled latex balloon inserted into the left ventricle for continuous measurement of the left ventricular developed pressure (LVDP). The coronary perfusion pressure and LVDP were digitized and continuously recorded with a computer.

Blood (1 ml) was centrifuged at 620 g, and the plasma was collected and then stored at -70°C for subsequent analysis. The activity of creatine kinase MB (CK-MB) was determined using an electrolyte analyzer (Dri-Chem 3500i, Fujifilm, Tokyo, Japan). After obtaining the contractile function, the middle slice of the heart was sampled and incubated for 20 min at 37°C in 1% 2,3,5-triphenyltertrazolium chloride in 0.1 M phosphate-buffered saline (Sigma) to distinguish between the infarction (pale) and the viable myocardial area (red), and the slices were scanned immediately as previously described (6, 31). The areas of the infarct and the whole section were quantified by computerized planimetry, and the infarct size was expressed as the percentage of the whole section area. The remaining tissue slices were stored at 80°C for further analyses.

Assay for CFCs

The hematopoietic activities of BMSCs were determined by plating 1×10^4 test cells or 500 c-kit^+ sca-1^+ hematopoietic stem cells (as a positive control) in methylcellulose M3434 medium supplemented with EPO, TPO, GM-CSF, M-CSF, G-CSF, IL-3 and SCF (StemCell Technologies). Numbers of colony-forming cells (CFU-GM, BFU-E, CFU-Mix) were scored at day 7-day 14. Colonies containing more than 30 cells were scored after 12 days of incubation at 37°C. Colony types were determined by *in situ* observation using an inverted microscope.

Marrow Reconstituted Transplantation

Recipient C57BL/6 ($H2^b$) mice received myeloablative total body irradiation (1000 cGy) from a 137Cs source followed by intravenous infusion of allogenic (BALB/c) or syngenic (B6) bone marrow HSC cells or BMSCs (BALB/c; $H\text{-}2^d$) as the donor source. Peripheral blood was obtained at 10 days to 2 weeks after BMT. At the end of the experiment, recipient mice were anesthetized with isofluorane, or anesthesia and killed by cervical dislocation. Blood and marrow tissues were collected for engraftment evaluation.

Allogeneic and Xenogeneic Mixed Lymphocyte Reaction (MLR)

Mouse BMSCs were used as the source of the

stimulator. Splenocytes from C57BL/6 mice or rats were used as the source of the responder. Responder splenocyte suspensions were dissociated into a single-cell suspension by grinding the spleen between the frosted ends of two glass slides. Cells were washed 3 times with CCM consisting of RPMI-1640 supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), Hepes (10 mM) (Gibco-BRL), and 10% fetal bovine serum (HyClone). Allogeneic or xenogeneic responder cells (4×10^5) were cultured with 0.5 mg/ml mitomycin-treated stimulator cells (8×10^5) in CCM for 3 days in 96-well flat-bottomed tissue culture plates in 5% CO_2 in air. The stimulated proliferation was evaluated by the reduction ratio of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by the mitochondrial dehydrogenase of viable cells to blue formzan products which were measured spectrophotometrically. At the end of the incubation period, 5 mg/ml MTT solution was added, and then crystal formzan was dissolved in acid-isopropanol. The optical density was measured in a microplate spectrophotometer (SPECTRAmax 340PC^{384}, Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Results are expressed as the mean stimulated minus the resting optical density (OD) ± S.E.M.

Flow Cytometry

For cell surface antigen phenotyping of BMSCs, cells were detached and stained with fluorescein- or phycoerythrin-coupled antibodies and analyzed using a flowcytometer (Partec CyFlow, Deutschland, Germany). The FITC-, biotin- and tricolor-conjugated rat or mouse monoclonal antibodies against the murine antigens CD11b, CD13, CD14, CD31, CD34, CD44, CD45, CD49d, CD90, CD105, CD106, CD117 (c-kit), CxCR4, Sca-1, H-2^d and I-A^d were purchased from Becton Dickinson or eBioscience (San Diego, USA). FITC-, biotin- and PECy 5-conjugated hamster, rat or mouse IgG1, IgG1a, IgG1k, IgG2ak and IgG2b isotype standard antibodies were used as control antibodies.

Preparation of Heart Tissue Extracts and Western Blot Analysis

The cultured cells or cardiac tissues were prepared as a homogenate of total protein. Protein samples were quantified by a commercial assay kit (Bio-rad, Hercules, CA, USA). For the *in vitro* co-culture assay, 25-50 μg normal or ischemia heart protein extracts was added to the BMSC cultures. Two days later, cells were collected, and the expression of the TGF-β receptor (CD105) was analyzed by flow cytometry. For the western blot assay, protein extracts were separated and electrophoretically transferred to nitrocellulose membranes (Amersham-Pharmacia Buckingham, England, UK) as previously described (31). After blocking with 5% skim milk, the membranes were incubated overnight at 4°C with different antisera including CXCR4, SDF-1 and β-actin (Santa Cruz). After washing, the membranes were incubated for 1 h at room temperature with either horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Vector, Burlingame, CA, USA) or anti-mouse IgG (Lenico, St. Louis, MO, USA) as appropriate; the membranes were then washed and the bound antibody was detected using a commercial ECL kit (Amersham-Pharmacia).

Alkaline and Acid Phosphatase Activity Detection

BMSCs were seeded on glass slides and fixed with 3.7% formaldehyde/PBS solution. After fixation, cell slides were treated with 1% Trition-X100 solution and then washed several times with PBS. For alkaline phosphatase activity, cell slides were incubated with BCIP/NBT Liquid Substrate System (Sigma) for 20 min at 25°C. For acid phosphatase activity, cell slides were incubated for 1 h at 25°C in a reaction medium containing 3% β-glycerophosphate, 0.1% lead nitrate and 1% sucrose in 0.05 M acetate buffer (pH 5.0). Later, the cell slides were treated with 1% ammonium sulfide for a few minutes at 25°C.

Immunofluorescent Staining

For the BMSC homing assay, 1×10^6 BMSCs were incubated with 1 μg/ml Hoechest 33342 (Molecule Probe, Carlsbad, CA, USA) at 37°C for 1 h. After incubation, the cells were washed with medium several times, prepared in phosphate buffered saline in a volume of 0.1 ml and administered intravenously *via* the external jugular vein within 5 min after coronary ligation in the IR animals. After 72 h of BMSC administration, whole heart tissue was removed and processed *via* a frozen section preparation procedure. The post-fixative cardiac slices were stored in 10% sucrose in 4% paraformaldehyde solution at 4°C, embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek, Torrence, CA, USA), and frozen at -20°C until used to prepare 5-μm sections on a cryostat (Microm, Heidelberg, Germany) as previously described (31). Briefly, after rehydration and washing with PBS, the tissue sections were processed for indirect immunofluorescence. After incubation with a blocking solution (5% skim milk in PBS) for 1 h at room temperature (RT), the sections were incubated overnight at 4°C with a rabbit anti-α-actinin antibody diluted 1,000-fold in the blocking solution, then for 1 h at RT with a FITC-conjugated donkey anti-mouse antibody (1:100 dilution in blocking solution) (Jackson Immuno Research, West Grove, PA, USA), and they

Table 1. Specific primers used for RT-PCR amplification

Gene	Sequence (5'—>3')	Product size (bp)	Annealing (Tm)	Cycles
Oct-4	F ggc gtt ctc ttt gga aag gtg ttc R ctc gaa cca cat cct tct ct	1,124	55	38
Nanog-1	F atg agt gtg ggt ctt cct ggt R tat ttc acc tgg agt cac a	880	52	38
Tert	F tgt acc aaa ttt gtg cca cca cgg R ttc ctg cag tga tag ctt gcc gta	944	58	38
Rex-1	F acg gag agc tcg aaa cta aag cg R tca gca ttt ctt ccc tgc ctt tgc	243	58	38
Sca-1	F aaa gag ctc agg gac tgg agt gtt R tac att gca gag gtc ttc ctg gca	280	58	38
FGF-4	F tac tgc aac gtg ggc atc gga tt R tag gcg ttg tag ttg ttg ggc aga	248	58	38
GAPDH	F acc aca gtc cat gcc atc ac R tcc acc acc ctg ttg ctg ta	500	56	38

were finally examined under an Olympus BX51 microscope (Tokyo, Japan) equipped with a fluorescent image analytic system (Diagnostic Instruments, Sterling Heights, MI, USA) at 400× magnification.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from BMSCs using the RNAZol B (Tel-Test INC., Friendswood, TX, USA) with RNase-free DNase treatment according to the manufacturer's instructions. RT-PCR was performed using the Advantage™ RT-PCR kit (Clontech, Mountain View, CA, USA) with specific primers. Reaction mixtures for PCR included 10 µl of cDNA, 2.5 µl of 10X buffer, 1 µl of 10 mM dNTP mix, 1 µl of 10 mM of each forward and reverse primer and 0.2 µl of Taq polymerase. The primer sequences are listed in Table 1. Polymerase chain reactions were performed for 28-32 cycles, with each cycle consisting of denaturing at 94°C for 30 s, annealing at 55-63°C for 30 s, elongation at 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified DNA fragments were electrophoresed in a 1.5% agarose gel. The gels were stained with ethidium bromide (10 µg/ml) and photographed on a UV transilluminator (VDS, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical Analysis

All data are presented as means ± S.E.M., and the differences between groups were assessed by the Student's *t* test or one-way ANOVA with a significance level of $P < 0.05$.

Results

The heterogenous BMSCs with strong CD45 expression but negative for CD34 expression were established from the bone marrow of the BALB/c mice. $CD45^+/CD34^-$ BMSCs proliferated in the culture medium, adhered to the bottom of the culture dish, and developed a spindle-shaped morphology for characterization. In order to determine the differentiation potential of BMSCs into cardiomyocytes, 5-azacytidine was used for *in vitro* differentiation. The phase-contrast photography and immunocytochemical staining with anti-α-actinin antibodies were performed on BMSCs to confirm the cardiomyocyte-like morphological changes in BMSCs after 5-azacytidine treatment. Fig. 1 shows that BMSCs exhibited a fibroblast-like morphology before the 5-azacytidine treatment (day 0). The morphology of the cells started to change on day 6 after the 5-azacytidine treatment. Approximately 15-20% of the BMSCs gradually increased in size, formed a ball-like appearance, or lengthened in one direction at day 12, but we were not able to detect myotube formation or a pulsating cells by the time the culture was terminated. The immunohistochemical staining of α-actinin expression is presented in Fig. 1C. However, although the staining pattern does not indicate functional localization of α-actinin observed in mature cardiomyocytes, this staining pattern is very similar to that previously reported (33, 42). Flow cytometric analysis revealed that BMSCs isolated by the described method were negative for the expression of CD13, CD14, CD31, CD34, CD38, scal-1 and $I\text{-}A^d$ (MHC II). In

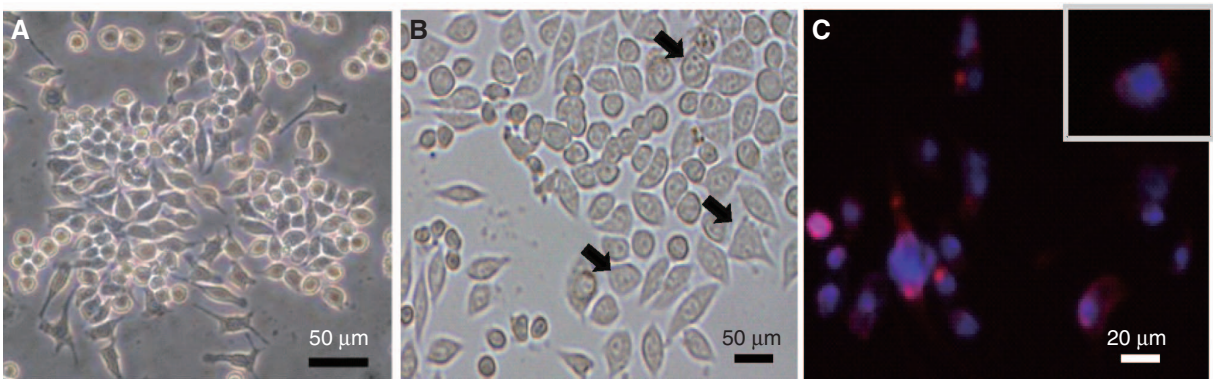

Fig 1. Phase-contrast photographs of BMSCs before and after 5-azacytidine treatment. (A) BMSCs show a fibroblast-like morphology before 5-azacytidine treatment (day 0). (B) One week after treatment, some cells gradually increased in size and formed a ball-like appearance (arrowheads). (C) Immunostaining of BMSCs with an anti-α-actinin antibody (red fluorescence) and DAPI nuclei dye (blue fluorescence) at 2 weeks after 5-azacytidine treatment. Scale bars: 20 μm (200× magnification) or 50 μm (400× magnification). BMSCs, bone marrow-derived stromal cells.

addition, cells were strongly positive for CD45, CD44 and MHC-I (H-2^d), and partially positive for CD49d, CD90, CD105, CD106 and CXCR4 expression (Fig. 2A). Analysis of alkaline or acid phosphatase activities indicated that BMSCs exhibited acid phosphatases activity but were negative for alkaline phosphatase (Fig. 2B). RT-PCR analysis indicated that the BMSCs did not express pluripotent transcription factor genes, such as Oct-4 (octamer binding protein), Nanog-1, or Tert (telomerase reverse transcription), but they did express Rex-1 and the mouse mesenchymal stem cell-related gene, Sca-1. In addition, BMSCs did not express the fibroblast growth factor gene FGF-4 (Fig. 2C).

Accumulating evidences have indicated that bone marrow consists of both hematopoietic and non-hematopoietic stem cell populations. Both types of stem cells have shown differently contributed effect on *in vitro* myogenic differentiation and *in vivo* cardioprotection. In this study, a non-hematopoietic stromal type of our $CD45^+/CD34^-/lin^-$ cells was isolated and a stable heterogenous cell line was established. Despite the evidence of existence of Sca-1 in RT-PCR profile, the result of flow cytometry indicated the absence of expression of hematopoietic phenotype. However, in order to rule out contamination with hematopoietic stem cells or progenitors in BMSC preparation, *in vitro* colony forming unit (CFU) assay and *in vivo* HSC or BMSC cell transplantation to reconstitute marrow after lethal irradiation were accomplished to examine the presence of substantial numbers of hematopoietic stem cells or progenitors in BMSC cell preparation. The result of CFU assay showed that there was no colony growing in day 14 after plating test BMSC cells in methylcellulose medium supplemented with hematopoietic cell growth factors, but there was colony growing in the group plating HSCs (Fig. 2D). Moreover, results of donor cell engrafted in transplanted BMSCs into lethal irradiated recipient mice showed that transplanted BMSCs alone could not reconstitute marrow hematopoietic activities in comparison with transplanted HSCs recipient mice (Fig. 2E). BMSC transplanted mice died on days 15-20 after transplantation (data not shown). This result suggested that BMSCs did not possess the capable of hematopoietic activities even though they possessed CD45 surface markers. Moreover, there was no existence of hematopoietic stem cells or progenitors in the BMSC culture as a proof with colony forming assay.

There is a growing body of evidence that BMSCs possess unique immune modulatory capabilities (22). It has been reported that adult BMSCs could be tolerated and further implanted into allogenotransplant and xenotransplant recipients (32). To determine if mouse BMSCs could induce a proliferative response in allogeneic and xenogeneic lymphocytes, we tested the antigenicity of marrow-derived $CD45^+CD34^-$ cells using allogeneic and xenogeneic mixed lymphocyte culture reactions prior to the rat myocardial infarction operation. Splenocytes (4×10^5) from C57BL/6 mice or SD rats were used as responder cells in primary cultures stimulated with mitomycin-C-treated BMSCs (1×10^5) from BALB/c mice. C57BL/6 splenocytes were used as stimulators in the control cultures. The addition of BMSCs to the mouse or rat splenocyte cultures suppressed lymphocyte proliferation (Fig. 3) that was not caused by cell death because EtBr/AO vital staining showed > 98% cell viability at the time of harvest (data not shown).

To assess the protective effect of BMSCs on the post-ischemic myocardium, BMSCs were delivered intravenously into the IR hearts after coronary ligation. Functional analysis has shown that BMSCs ameliorate

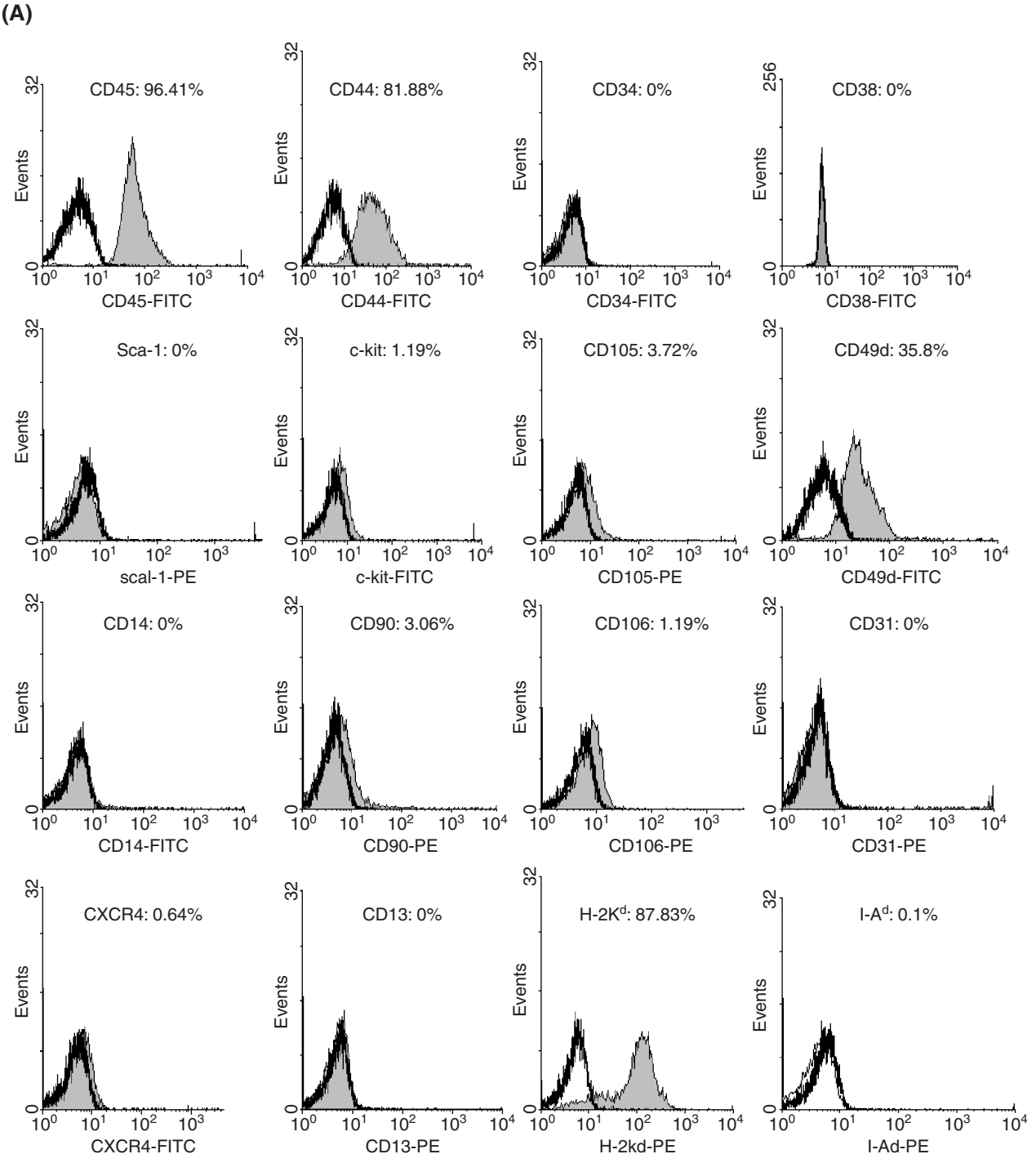

Fig. 2. Characteristics of BMSCs. Cells were strongly positive for CD45, CD44 and MHC-I (H-2^d) and were partially positive for CD49d, CD90, CD105, CD106 and CXCR4 but negative for CD13, CD14, CD31, CD34, sca-1, c-kit and MHC-II (I-A^d). The open histogram indicates the isotope control; the shaded histogram indicates the reactivity of the cell samples analyzed (A). Cells stained positive for acid phosphate (bottom panel of B) but not for alkaline phosphatase (upper panel of B). RT-PCR analysis showed that the stromal cells expressed Sca-1 and Rex-1 but not oct-4, Nanog-1, Tert or FGF-4 (lane 3, C). MEF, a fetal fibroblast primary cell line, was used as a negative control (lane 1). ES-R1, a mouse embryonic stem cell line, was used as a positive control (lane 2). The colony forming unit assay was set up while culture HSC or BMSC into methylcellulose medium included mixtures of GM-CSF, M-CSF and M-CSF. Colony forming assay proof that BMSCs did not possess hematopoietic activities (panel D). The donor cell engraftment in irradiated recipients. C57BL/6 mice (H-2^b) received a lethal dosage of TBI (1000 cGy) 24 h before transplantation. 1×10^6 BALB/c HSCs, B6 HSCs or BMSCs were intravenously injected by tail vein. Blood from recipients was collected and donor cell engraftment was analyzed by flow cytometry as used a donor specific H-2 antibody. Results showed that BMSCs did not possess the capable of marrow reconstitution (panel E).

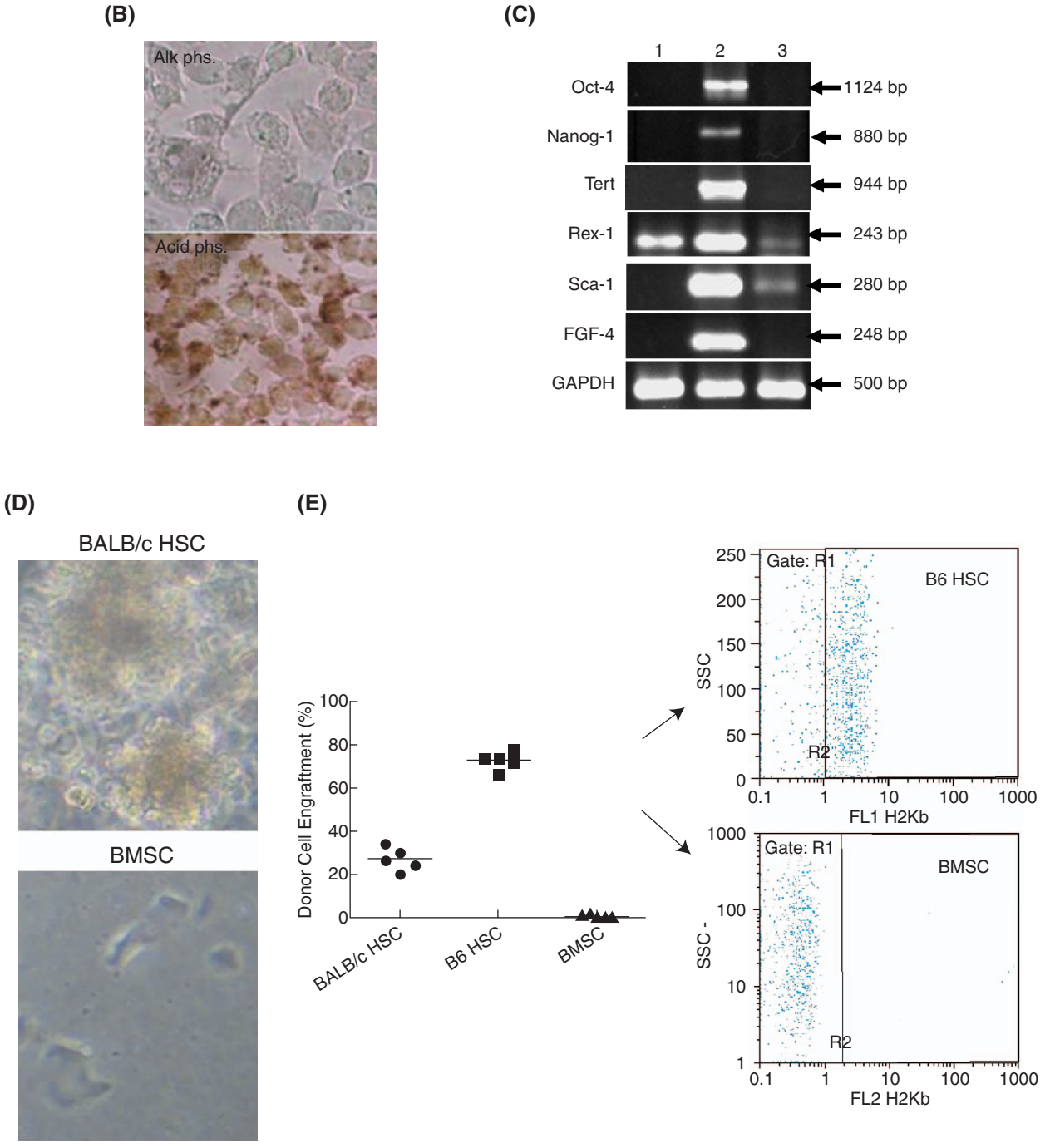

Fig. 2. (Continued)

cardiac injury and improve the cardiac contractile function. Fig. 4 shows that IR increased cardiac damage *via* the presence of infarction and plasma CK-MB elevation which is associated with the poor LVDP seen in the IR hearts. Administration of BMSCs alone showed no effect on the control hearts but significantly reduced the infarct size and the plasma level of CK-MB in IR rats. BMSCs also improved the LVDP of the IR hearts. In order to determine whether xenogeneic BMSCs can reside in the injured heart and contribute to the attenuation of the functional deterioration in IR hearts, we further examined the homing effect of BMSCs in IR hearts. Fig. 5A shows the representative blots obtained for the BALB/c mouse MHC class I protein, H-2^d, which was only expressed in the BMSC-treated IR hearts. Interestingly, pre-labeling of BMSCs with Hoechst 33342 (as blue nuclear stain) was detected in the cardiac section of

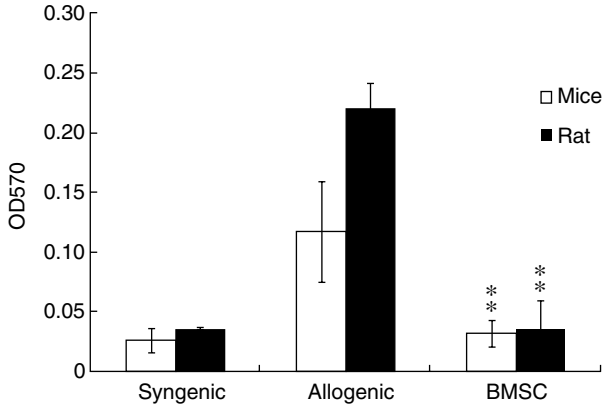

Fig. 3. Inhibition of allogeneic and xenogeneic lymphocyte responses by mouse bone marrow-derived stromal cells (BMSCs). BMSCs showed an inhibition of allogeneic (□) and xenogeneic (■) lymphocyte responses, while control groups exhibited no inhibitory responses. The groups of syngeneic and allogeneic/xenogeneic animals served as negative and positive controls, respectively. Data are expressed as the mean of the OD ± SD of triplicates of three independent separate experiments. $^{**}P < 0.01$ compared to the allogeneic control group.

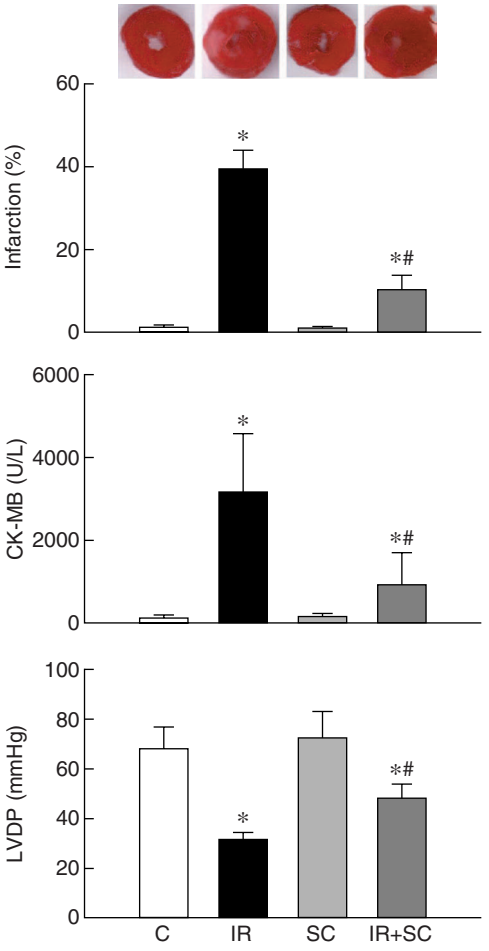

Fig. 4. BMSCs ameliorate cardiac injury and poor contractility in post-ischemic heart. The upper panel shows the representative sections used for TTC staining, and the following bar graph shows the infarct size. The middle and lower bar graphs show the changes in plasma levels of CK-MB and LVDP in the hearts. N = 7 in each group. $^{*}P < 0.05$ compared to the control group. $^{\#}P < 0.05$ compared to the SC group.

the infarction area in the IR hearts at 48 h after treatment, but the number of Hoechst 33342-labeled cells in the IR heart was reduced to the status that could not be calculated at 72 h after treatment.

Recently, accumulated evidences have indicated that the SDF-1/CXCR4 receptor axis plays a pivotal role in cardioprotection *via* the recruitment of circulating progenitor cells to the injured heart (1, 4, 39, 44). We therefore examined whether this signal was involved in the BM-derived $CD45^+/CD34^-/lin^-$-mediated cardioprotection in terms of xeno-transplantation into rat IR hearts. We attempted to sort the Hoechst 33342 positive cells from the heart of IR+SC treated group; however, the numbers of cells were too low to be analyzed after the enzymatic digestion procedure. Therefore, protein crude extracts from the IR rat hearts were prepared and added to the BMSC culture and the SDF-1/CXCR4 expression was examined. Fig. 5B shows that control BMSCs mildly expressed α-chemokine SDF-1 proteins; however, flow cytometric analysis showed that there was no expression of CXCR4, a cognate receptor for SDF-1, on the BMSCs. Interestingly, expression of both SDF-1 and CXCR4 was significantly increased in the BMSC-treated tissue extracts prepared from the IR hearts. This finding clearly indicates that BMSCs are capable of homing to the IR hearts through SDF-1/CXCR4 signaling.

We further identified the possible soluble factors involved in the cardioprotection of BMSCs because it has been shown that various cytokines, growth factors or survival factors surrounding the damaged tissue provide an environmental niche that potentially attract tissue-specific progenitor cells into damaged tissues (11). Two soluble factors, namely transforming growth factor (TGF)-β and interferon-γ (IFN-γ), were found to be highly expressed in ischemic heart tissues (Figs. 6 and 7). Fig. 6A shows that the level of TGF-β was markedly increased in the IR groups, but BMSC treatment had no effect on the level of TGF-β. Low TGF-β was found in the control hearts, and this level was not affected by BMSC treatment. Interestingly, cultured BMSCs treated with IR heart extracts displayed increased expression of CD105 (as the TGF-β1 receptor) on BMSCs (Fig. 6B). In addition, while the expression of IFN-γ was increased in the IR heart, BMSC treatment of the IR hearts again showed no effect on the elevation of IFN-γ expression (Fig. 7A).

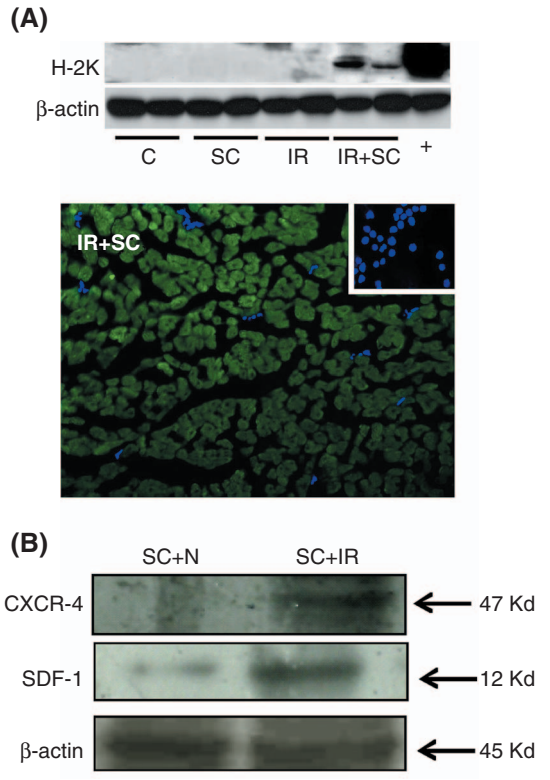

Fig. 5. BMSC homing to the IR heart through the SDF-1/CXCR4 receptor axis. The upper panel of 5A shows the representative blots of mouse MHC class I protein in hearts. +: positive sample obtained from mouse hearts. The lower picture of panel 5A shows BMSCs pre-stained with Hoechst 33342 before injection (inset picture at the right corner) and 48 h after treatment of the IR heart. The protein expression levels of CXCR4 and SDF-1 in bone marrow stromal cells increased when ischemic heart extracts were added to the culture.

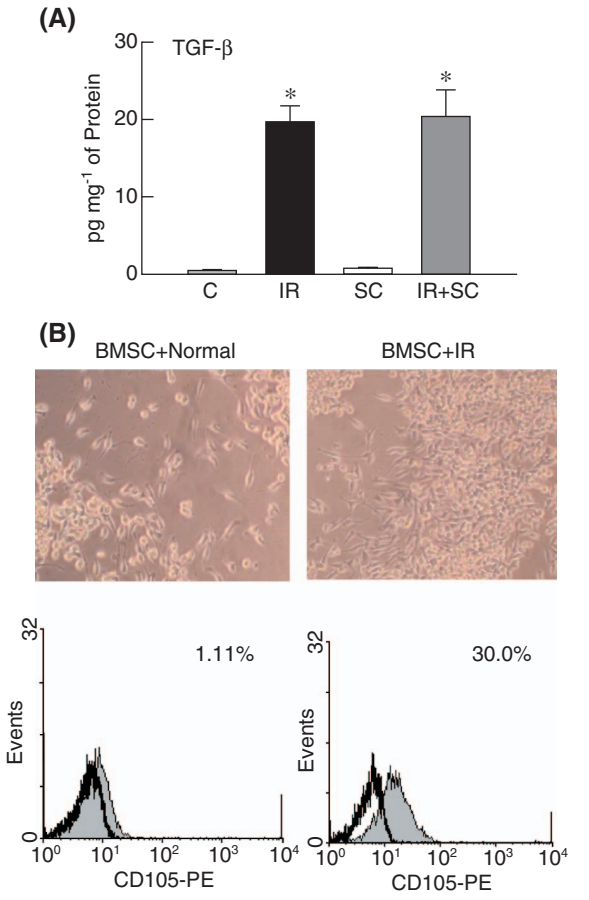

Fig. 6. The production level of TGF-β1 in rat hearts was increased in the IR and IR+SC groups (panel A). The proliferation rate and expression level of the TGF-β1 receptor (CD105) on BMSCs increased when ischemic heart extracts were added to the culture (panel B). $*P < 0.05$ compared to the control group.

However, the expression of stem cell antigen-1 (Sca-1) was significantly increased on BMSCs when the cells were treated with IFN-γ *in vitro* (Fig. 7B) indicating that IFN-γ treatment may change the phenotype of BMSCs into more stem cell-like. Hence, the IR-induced proinflammatory factors may contribute migration and residence of BMSCs at infarction area of the heart.

Discussion

There is a growing body of evidence that BMSCs are effective in improving cardiac performance of IR hearts (19, 32, 36, 42, 48). BMSCs contain multipotent adult stem/progenitor cells that are capable of differentiation into both mesenchymal and non-mesenchymal lineages. The majority of *in vivo* studies has used the non-selective, plastic-adherent approach to BMSC preparation. For easy separation from non-adherent hematopoietic cells, traditional isolation of mesenchymal stromal cells from bone marrow has been based on simple density gradient separation of mononuclear cells and subsequent collection of mesenchymal cells that are adherent to the plastic culture plates. The colonies derived by plastic adherence are heterogeneous with respect to size, morphology and the degree of contamination with more mature mesenchymal cells and non-mesenchymal cells. It has been thought that hematopoietic stem cells are $CD34^+$, whereas marrow stromal stem cells are $CD34^-$. Recently, it has reported that hematopoietic stem cells may originally derive from a $CD34^-$ subpopulation and that the CD34 marker is expressed after activation and mobilization. In addition, BMSCs contain $CD45^+$ and $CD45^-$ non-hematopoietic cell populations. However, most research results indicate that murine $CD45^-$ cells are highly enriched in mesenchymal stem cells (26), and it is still not known whether a specific subpopulation of BMSCs is actually responsible for these effects.

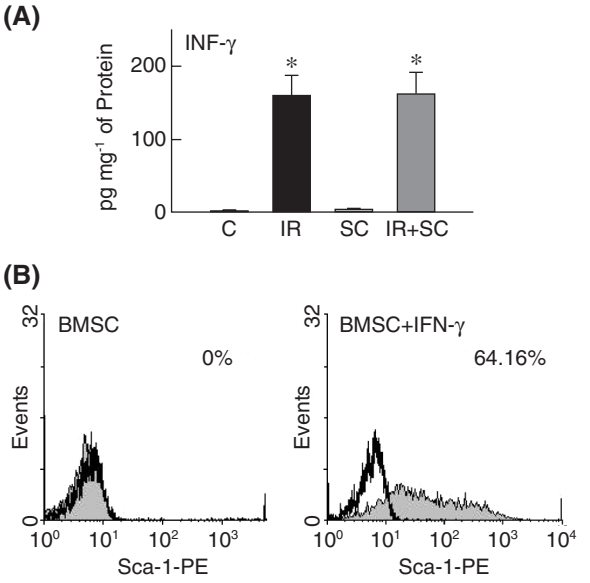

Fig. 7. The production level of IFN-γ in rat hearts was increased in the IR and IR+SC groups (panel A). The expression level of the Sca-1 receptor on BMSCs was significantly increased when IFN-γ (5 U/ml) was added to the culture (panel B). $*P < 0.05$ compared to the control group.

Here, $CD45^+/CD34^-/lin^-$ adherent mononuclear cells were isolated by immunoselection. Both phenotypic analysis and RT-PCR demonstrated that these marrow-derived $CD45^+/CD34^-/lin^-$ stromal cells did not express the embryonic stem cell-related genes, Oct-4 and Nanog-1, but the cells did possess mesenchymal stem/progenitor characteristics with Rex-1 and Sca-1 gene expression. However, FACS analysis demonstrated that they were neither endothelial cells nor were they macrophages or and fibroblasts. In order to determine whether BMSCs expressed cardiac properties, 5-azacytidine was added to the BMSC cultures to examine their capability for cardiomyocyte differentiation. After 2 weeks of induction, BMSCs did express the cardiomyocyte-related marker α-actinin. We did not observe myotube formation or pulsating cells. This finding is consistent to previous reports (33, 42); however, the results can be explained by the lack of enough differentiating factors in the culture medium.

There is increasing evidence that mesenchymal stem cells are immune-privileged cells. It has been reported that intrapertioneally transplanted human mesenchymal stem cells could engraft in fetal sheep and fetal mouse (10, 30). The results of MLR showed that $CD45^+/CD34^-/lin^-$ BMSCs also possessed immunosuppressive activity, another phenotype of mesenchymal stromal cells. According to this immunological characterization, we examined the functional contribution of marrow-derived $CD45^+/CD34^-/lin^-$ stromal cells to cardiac protection *in vivo*. We xenotransplanted murine BMSCs into the rat heart with myocardial infarction by injecting the cells into the jugular vein after coronary artery ligation. Functional assessment indicated that transplanted BMSCs attenuated the myocardial infarct area and CK-MB deposition and improved the left ventricular function of the IR heart. Our data suggest that $CD45^+/CD34^-/lin^-$ BMSC-treated hearts had a tendency to dilate less than control hearts, as demonstrated by the increased LVDP. In addition, BMSCs labeled with the DNA dye, Hoechest 33342, provided the supportive evidence that the BMSCs grafted into the injured heart and resided in the heart for 72 h. Although the mechanism of the improvement remains unclear, it has been suggested that the functional improvement may be attributable to improved contractile properties *via* the attenuation of remodeling and LV dilatation (32).

In a mechanistic study, it is desirable to identify factors that possibly contribute to the therapeutic effects of $CD45^+/CD34^-/lin^-$ BMSC on myocardial infarction. Both well-defined mediators and direct cell-to-cell contacts might contribute to the migration of BMSCs into the injured area of the heart. It is well accepted that the various chemoattractants and survival factors of the surrounding tissues provide an environment that potentially attracts various stem cells, progenitors or stromal cells to reside in injured tissues to provide a signal input that induces the differentiation of BMSCs into cardiomyocytes, or to attenuate the inflammatory response around the damaged tissues. It has been reported that the secreted chemokine SDF-1 and its receptor CXCR4 play a pivotal role in organogenesis, angiogenesis, and stem cell trafficking (2, 13, 25, 34). Moreover, SDF-1-/CXCR4 have been implicated in cardiogenesis. Signaling downstream of CXCR4 can trigger a chemotactic response that results in migration toward an increasing SDF-1 gradient. Our results suggest that factors in IR heart extracts can increase the expression levels of SDF-1 in the cytosol and of CXCR4 on the surface of BMSCs.

Two pro-inflammatory cytokines, TGF-β and IFN-γ, were investigated in detail in the present study. TGF-β1 plays a key role in the development of embryonic heart (3). It has been reported that TGF-β stimulates cardiomyogenic differentiation of BMSCs and embryonic stem cells *in vitro*. Furthermore, CD117-positive BMSCs treated with TGF-β show an increased expression of cardiac markers (29). Our results showed an increased expression level of TGF-β in the heart tissues of the IR group. Although BMSC treatment did not change the level of TGF-β production, the *in vitro* study showed that addition of tissue extracts from the IR heart into BMSC cultures significantly increased CD105 marker expression on their surface. CD105 markers are highly expressed

on stem cells. Several studies indicate that TGF-β signaling might play an important role in the cardiomyogenic differentiation of stem cells (5, 27). We have measured several hematopoietic and non-hematopoietic cytokine production including TGF-β. BMSCs secrete a high level of TFG-β (1250.6 pg/ml), but a low amount in IL-6 (34.9 pg/ml) production. The cytokines IL-2, IL-4, IL-5, IL-10, IL-12 and IFN-γ were not produced in culture supernatants of 5×10^6 BMSCs (data not shown). The endogenous production of TGF-β from BMSCs may be used to explain why BMSC-treated IR rats still retained a high level of TFG-β production in heart tissue extracts even when the heart function had recovered.

High IFN-γ levels can be found in many inflammatory tissues including the heart. Ischemic hearts show increased IFN-γ production. The BMSC transplantation did not attenuate the expression level of IFN-γ. Interestingly, *in vitro* addition of heart extract proteins from IR hearts to the BMSC cultures increased Sca-1 (stem cell antigen-1) expression on the surface of BMSCs. It has been reported that bone marrow-derived mesenchymal stem cells express abundant Sca-1 and differentiate into cardiomyocytes *in vivo* (16). The administration of Sca-1$^+$/CD31$^-$ cardiac progenitors improves the functional performance of ischemic hearts (50). Recently, it has been reported that Sca-1 signaling might play an important role in efficient cardiovascular regeneration (46). Recently, Meldrum group (23) has reported that the expression of TNFR1 and TNFR2, a receptor for TNF, on bone marrow-derived mesenchymal stem cells play important roles in MSC-mediated cardiac protection following myocardial ischemia. In addition, receptor expression levels to IFN-γ and TGF-β on BMSCs were significantly enhanced after contact with the IR heart extracts. Therefore, receptor to IFN-γ and TGF-β may be involved in BMSC-mediated cardiac protection. However, further studies are required to confirm this.

In summary, although the levels of TGF-β and IFN-γ production in IR hearts remained unchanged after BMSC treatment, the presence of TGF-β and IFN-γ may provide a microenvironment that stimulates the induction of expression of stem cell-related markers initiating BMSC differentiation. As the data demonstrated, the levels of CXCR4, SDF1, INF-γ receptor and TGF-β receptor were all increased in BMSCs after treatment with tissue extract of IR hearts. Moreover, high myocardial levels of INF-γ and TGF-β could be seen in the rat IR hearts. These molecules correspond to each other well to enhance cell-to-cell contact in myocardium. However, further studies are required to refine the hypothesis since maximal levels of INF-γ and TGF-β seen in the IR hearts were not affected by BMSC treatment that may imply that the effect of cell recruitment in rat hearts had possibly reached a maximal level. Our results suggest that hearts engrafted with CD45$^+$/CD34$^-$/lin$^-$ BMSCs can provide cardioprotection after infarction. Both soluble factors and direct cell-to-cell contacts might contribute to recovery of cardiac functions.

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