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The Novel Role of SERPINB9 in Cytotoxic Protection of Human Mesenchymal Stem Cells

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Clinical trials using allogeneic mesenchymal stem cells (MSCs) are ongoing for the purpose of providing therapeutic benefit for a variety of human disorders. Pertinent to their clinical use are the accessibility to sufficient quantities of these cells allowing for repetitive administration, as well as a better understanding of the specific mechanisms by which allogeneic MSCs evade host immune responses that in turn influence their life span following administration. In this report, we sought to characterize and compare human peripheral blood MSCs (hPB-MSCs) with bone marrow-derived MSCs. hPB-MSCs met the established criteria to characterize this cellular lineage, including capacity for self-renewal, differentiation into tissues of mesodermal origin, and expression of phenotypic surface markers. In addition, hPB-MSCs suppressed alloreactive proliferation as well as the production of proinflammatory cytokines. Examination of the mechanisms by which allogeneic MSCs evade the host immune response, which is crucial for their therapeutic use, demonstrated that constitutive expression of serine protease inhibitor 9 (PI-9) on hPB-MSCs and bone marrow-derived MSCs is a major defense mechanism against granzyme B-mediated destruction by NK cells. Similarly, MSCs treated with small interfering RNA for PI-9 increased MSC cellular death, whereas expression of transgenic PI-9 following retroviral transduction protected MSCs. These data significantly advance our understanding of the immunomodulatory role for hPB-MSCs as well as the mechanisms by which they evade host immune responses. These findings contribute to the development of MSC-based therapies for diseases. The Journal of Immunology, 2011, 187: 000–000.

Due to the plasticity and immunomodulatory function of mesenchymal stem cells (MSCs), multiple preclinical and clinical trials in regenerative medicine, graft versus host disease, inflammatory diseases, and autoimmunity have used these cells (1–4). Allogeneic MSCs represent an “off-the-shelf” cell therapy, one allowing for an omission of the normal time span for cell isolation and expansion. Allogeneic MSCs, albeit hypoimmunogenic, are subject to rejection, which could compromise their therapeutic benefit (5–7).

Although bone marrow-derived MSCs (BM-MSCs) are well characterized in the literature, the procedure for bone marrow extraction is invasive, and the number of progenitor cells isolated may be inadequate for MSC proliferation. In particular, the process is quite cumbersome when the need arises for repetitive administration of MSCs. Consequently, alternative sources, such as cord blood, adipose tissue, and peripheral blood, have recently become an increasing focus of research (8–10). Although the concentration of hPB-MSCs is significantly less than that of BM-MSCs in their respective tissues, contributing to the difficulty in hPB-MSC expansion from peripheral blood, in pathological conditions in which stimuli for their release is present (e.g., patients with burns, cancer, and graft rejection) hPB-MSCs have been isolated (11, 12).

Moreover, it is of great value to understand the mechanisms by which allogeneic MSCs evade host immune responses and, conversely, how they are rejected in therapeutic settings (13, 14). MSCs that express class I MHCs are killed by GrB-producing CTLs and NK cells (14). In contrast, NK cells constitute a major component of the innate immune system and do not express TCRs for recognizing Ags bound to MHC molecules as opposed to CTLs (15). Therefore, NK cells have recently been shown to have regulatory effects on adaptive responses. As many NK cell functions share common features with functions of adaptive CD8+ T cells, such as cytotoxicity and cytokine production, NK cell-activating receptors can stimulate overlapping signaling pathways used by the T cell Ag receptor (16). Cell-mediated death can occur through either the slow Fas-Fas-L cascade or the rapid GrB-perforin–dependent cascade (17, 18). MSCs pulsed with peptides from viral Ags stimulated the secretion of IFN-γ, which resulted in the GrB-caspase-dependent–mediated apoptosis of MSCs (19). GrB homeostatic regulation is, in turn, mediated through interaction with inhibitors belonging to the serine protease inhibitor (serpin) superfamily. Proteinase inhibitor 9 (PI-9/SERPINB9) in
humans specifically inactivates GrB in an irreversible manner (20). Serine protease inhibitor 6 (Sp6) is the mouse homolog of PI-9 in humans and is required to protect CTLs from GrB-mediated death (21, 22). PI-9 is expressed in the cytoplasm and nuclei of CTLs, immunoprivileged cells (23), and embryonic stem cells (24); PI-9 overexpression allows for the evasion of GrB-mediated cytotoxicity (25, 26). Endogenous GrB inhibitors have been characterized in both mice and humans. We have recently shown the role of Sp6 in the survival of murine BM-MSCs (5). No data are yet available on the presence and protective role of PI-9 in human MSCs against the GrB machinery. In this article, we describe the isolation and characterization of MSCs from peripheral blood and also explore the protective function of PI-9 as a potential escape pathway of MSCs.

Materials and Methods

Human samples

Human peripheral blood samples were obtained from nine healthy individuals. We were able to isolate MSCs from only six individuals. NK cells were isolated with a kit (Milteny Biotec) according to the manufacturer’s guidelines. BM-MSCs and foreskin-derived fibroblasts were commercially purchased (Lonza-Poietics). YT cells were cultured as previously described (27, 28).

MSCs and fibroblast cell culture

A total of 80 ml peripheral blood was collected from healthy individuals, and PBMCs were isolated using Ficoll-Paque (GE Healthcare). PBMCs were cultured at a concentration of 15 × 10^6 per 25-cm² flasks in DMEM complete medium (DMEM from Lonza), containing 10% FBS (Gemini Bio-Products), 1% penicillin-streptomycin, 1% glutamine (both from Lonza), and supplemented with 6 ng/ml human basic fibroblast growth factor (b-FGF) (Peprotech). The above conditions were used for the standardized expansion of PB-MSCs; various parameters, such as the blood volume drawn (40–80 ml), the number of seeded PBMCs for initial culture (5–50 × 10^6 cells per 25-cm² flasks), cell confluence in primary culture (60–90%), and the concentration of b-FGF (2–10 ng/ml) were additionally assessed. Cells were incubated at 37°C and 5% CO_2 for 3 d, after which half the medium was changed. Once cell confluence reached above 80%, MSCs were trypsinized (0.25% Trypsin-EDTA 1×; Life Technologies) to a new passage. BM-MSCs and fibroblasts were cultured under the same conditions. All MSCs, as well as fibroblasts, used in this study were passaged four or five times before use.

MSC characterization and differentiation

hPB-MSCs and BM-MSCs were analyzed for surface Ag median fluorescence intensity (MFI) expression with a panel of positive and negative markers. Anti-human Abs against CD29, CD44, CD49e, CD73, CD90, CD105, CD166, CD105, CD45, CD34, and CD80 were purchased from BD Pharmingen. MSC differentiation into chondrocyte, adipocyte, and osteocyte lineages was induced with the relevant differentiation media (Lonza). Cells were cultured at 37°C (5% vol/vol CO_2) in eight-well culture plates (Thermo Scientific, Dharmacon Accell siRNA delivery media) and added to all wells. Anti-CD3/CD28 was added to all wells, except the negative control wells, and these cultures were incubated for 72 h at 37°C and 5% CO_2. Cell proliferation was detected by flow cytometry as a percent division of the original population. The supernatant from the wells was collected to assess the production of proinflammatory cytokines IFN-γ and TNF-α, using Luminex (Milliplex Map; Millipore). PBMC proliferation was assessed with FlowJo software (Tree Star).

Cell killing assay

Human NK cells were isolated with an NK cell isolation kit (Miltenyi Biotec) and were immediately used as effector cells against target MSCs and fibroblasts as a control. hPB-MSC, BM-MSC, and fibroblast killing was assessed using LIV/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen Detection Technologies) according to the manufacturer’s recommendations. Briefly, the two-color fluorescence cell viability assay detects live and dead cells, using two probes that measure recognized parameters for cell viability: intracellular esterase (calcein acetoxymethoxy [AM]) and plasma membrane integrity (ethidium homodimer), respectively (30). MSCs and fibroblasts were incubated with calcein AM prior to the killing assay, because this intense green fluorescent dye is well retained within live cells. Effector NK cells were coincubated with stained MSCs or fibroblasts at an increasing E:T ratio for 2 h (1:1, 4:1, 16:1) at 37°C and 5% CO_2 in RPMI complete medium, supplemented with IL-2 (10 ng/ml). At the end of the incubation, ethidium homodimer, which enters through the damaged membranes of apoptotic cells and undergoes a 40-fold enhancement of red fluorescence upon binding to nucleic acids, was added to the wells for 15 min at room temperature. Samples were measured using a dual-scanning microplate spectrophotometer (Versamark Microplate Reader) (excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 15 nm). Percent lysis was calculated with the same formula used for the GrB assay and presented as follows: % Cell Lysis = (Experimental Wells − Spontaneous Release)/(Maximum Release − Spontaneous Release) (30). Caspase-3 in MSCs, a GrB intracellular second messenger, was assessed using the Caspase-3 Colorimetric Assay (R&D Systems) according to the manufacturer’s recommendations. Briefly, lysed MSCs were tested for protease activity by the addition of a caspase-specific peptide conjugated to the color reporter molecule p-nitroanaline. Cleavage of the peptide by caspase releases the chromophore p-nitroanaline, quantified spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction.

Small interfering RNA knockdown of PI-9 in hPB-MSCs and BM-MSCs

Small interfering RNA (siRNA) against PI-9 was purchased from Dharmacon (SMARTpool-siRNA and Accell nontargeting pool as negative control from Thermo Scientific). hPB-MSCs and BM-MSCs were transfected as directed in the manufacturer’s protocol. Briefly, the MSCs were plated at 150,000/well in six-well plates. PI-9 and nonspecific scrambled siRNA were individually mixed with Accell siRNA delivery medium (Thermo Scientific, Dharmacon Accell siRNA delivery media) and added to MSCs to give a final concentration of 1 μmol/l per well. After 72 h incubation, MSCs were harvested, PI-9 expression was assessed by flow cytometry, and they were subjected to the killing assay as described above.

Transfection and infection of hPB-MSCs using the Phoenix amphotrophic packaging line

DNA was diluted in Tris–EDTA buffer to a final concentration of 1 μg/ml (MIRGI1; MIRGI1–PI-9). Lipofectamine transduction of cells was performed according to the manufacturer’s protocol (Invitrogen). Transduction of samples at a final concentration of 1 μg/ml was made in 50 μl DMEM (without FBS) and incubated at room temperature for 5 min. Lipofectamine reagent was diluted to 2 μl/50 μl media. Diluted DNA was mixed gently with lipofectamine reagent and then incubated for 20 min at room temperature to allow for DNA–lipofectamine complexes to develop. Following incubation, the DNA–lipofectamine mixture was added to the 60–70% confluent Phoenix amphotrophic cells (American Type Culture Collection) cultured in T150 flasks. Transduced Phoenix cells were left to incubate at 37°C. After 24 h, the medium was changed, and cells were incubated at 35°C to induce production of viral packaging proteins containing the plasmid. Conditioned medium was collected every 8 h from the Phoenix cells and transferred to human MSCs seeded in T75 flasks (at 70–80% confluence) and replaced with fresh media. Conditioned medium was removed every 8 h over a period of 3 d.

Serpinep9 Protects Human Peripheral Blood MSCs

which were incubated without MSCs. CFSE-labeled PBMCs were added to all wells. Anti-CD3/CD28 was added to all wells, except the negative control wells, and these cultures were incubated for 72 h at 37°C and 5% CO_2. Cell proliferation was detected by flow cytometry as a percent division from the original population. The supernatant from the wells was collected to assess the production of proinflammatory cytokines IFN-γ and TNF-α, using Luminex (Milliplex Map; Millipore). PBMC proliferation was assessed with FlowJo software (Tree Star).
Statistics

Data were expressed as the mean ± SD of at least three separate experiments performed in duplicate. Student t test was used for comparison of results.

Results

MSC characterization and differentiation

Cultured hPB-MSCs and BM-MSCs were characterized at passage 4 and/or 5 with a panel of flow cytometry Abs for stem cells. We did not find a significant difference in the positive MFI expression of hPB-MSCs and BM-MSCs for various MSC markers (Fig. 1A, 1B). Both hPB-MSCs and BM-MSCs showed comparable MFI expression levels for CD29 (200 ± 6 versus 190 ± 9), CD44 (480 ± 12 versus 466 ± 15), CD49e (160 ± 6 versus 138 ± 6), CD90 (926 ± 13 versus 948 ± 7), CD166 (41 ± 3 versus 40 ± 6), CD105 (46 ± 5 versus 44 ± 2), and CD73 (240 ± 9 versus 238 ± 3), for hPB-MSCs and BM-MSCs, respectively. Hematopoietic lineage markers CD45 and CD34 and costimulatory molecule CD80 were negative in both MSC types (Fig. 1A–C). Both MSC types shared similar fibroblastic characteristic morphology, as observed under light microscopy (×400 magnification) (Fig. 1D, 1E). In addition, hPB-MSCs and BM-MSCs differentiated individually into chondrocytes, osteocytes, and adipocytes upon stimulation with a commercially available differentiation medium for each cell lineage (Fig. 1D, 1E).

Proliferation assay and cytokine profile

To examine the immunomodulatory role of hPB-MSCs vis-à-vis that of BM-MSCs, increasing concentrations of both cell types were added to an anti-CD3/CD28 T cell proliferation assay and assessed by flow cytometry using CFSE staining. Compared with the positive control, hPB-MSCs and BM-MSCs similarly and significantly suppressed T cell proliferation in a dose-dependent manner (p < 0.02) (Fig. 2A, 2B). We also compared the role of hPB-MSCs with that of BM-MSCs in suppressing the production of two proinflammatory cytokines, IFN-γ and TNF-α. As shown with both cytokines, both MSC types exhibited an analogous and significant suppression in comparison with the positive control (p < 0.01) (Fig. 2C, 2D).

PI-9 constitutive expression in MSCs and its protective role against NK cell-mediated cytotoxicity

Immunostaining and FACS analysis revealed a constitutive expression of intracellular PI-9 in hPB-MSCs and BM-MSCs, and no expression in fibroblasts (Fig. 3A, 3B). To assess the protective role of PI-9 against NK cell-mediated cytotoxicity, freshly isolated NK cells were incubated with HLA-mismatched MSCs and fibroblasts for 2 h in a killing assay. The purity of NK cells was >95% (data not shown). As shown in Fig. 4A, a proportional increase in percent killing of hPB-MSCs and BM-MSCs was observed, with
FIGURE 2. hPB-MSCs and BM-MSCs similarly suppress T cell proliferation in anti-CD3/CD28 stimulation assays. Anti-CD3/CD28 stimulation assay on PBMCs was performed in the presence of increasing concentration of hPB-MSCs and BM-MSCs. Proliferating cells were stained with CFSE and analyzed by flow cytometry as percent division (A, B). The histograms show representative proliferation, and the bar graph is the percent division of proliferating T cells. hPB-MSCs and BM-MSCs similarly suppressed PBMC proliferation in a dose-dependent manner. *p < 0.02. C and D, Both MSC types significantly suppressed the production of inflammatory cytokines TNF-α and IFN-γ. Data represent the average of five experiments, with each parameter performed in six replicates. *p < 0.01. NEG CTRL, negative control (PBMCs in medium); POS CTRL, positive control (PBMCs cocultured with anti-CD3/CD28).
significant increase attained at an E:T ratio of 16:1 compared with 1:1 (37 ± 3.8 versus 2.4 ± 1.6 for hPB-MSCs and 40 ± 4.1 versus 1.8 ± 0.7 for BM-MSCs, respectively). In contrast, the fibroblast cells revealed a significantly higher percent killing at all E:T ratios studied in the killing assay (p < 0.02) (Fig. 4A).

To determine the role of GrB in the NK-mediated killing of MSCs and fibroblasts, we used compound 19, a potent and specific GrB inhibitor (GrB-i) in our killing assay (31). We added three concentrations (25 μM, 50 μM, and 75 μM) of GrB-i to freshly isolated NK cells for 45 min prior to the killing assay. A significant dose-dependent drop in percent killing occurred in all three cell types at GrB-i concentrations of 50 μM and 75 μM, although a decreasing trend was evident at 25 μM (Fig. 4B) (p < 0.02).

Because GrB induces the activation of caspase-3 in the target cells, we measured the activity of caspase-3 in MSCs and fibroblasts, using labeled peptide substrate. When MSCs and fibroblasts were subjected to NK cells, caspase-3 activity in the three target cell types was significantly higher than in the target cells alone (p < 0.02) (Fig. 4C). In contrast, a significantly higher caspase-3 activity was noted in fibroblasts compared with both MSC types subjected to NK cells (p < 0.02) (Fig. 4C). NK cells alone, like MSCs alone, revealed relatively no caspase activity (data not shown). These data imply that PI-9 protects MSCs from NK-mediated apoptosis by suppressing GrB activity.

**Downregulating PI9 using siRNA enhances MSC susceptibility to death by NK cells**

To assess the significance of PI-9 in protecting MSCs from GrB-mediated cytotoxicity, we used specific siRNA for PI-9 to downregulate PI-9 expression in MSCs. Compared with MSCs transfected with nonspecific scrambled siRNA, PI-9 siRNA transfection resulted in a significant reduction in the expression of PI-9 (Fig. 5A). hPB-MSCs and BM-MSCs were individually transfected with PI-9 siRNA and scrambled siRNA for 72 h. The expression of PI-9 was assessed by flow cytometry after 72 h of MSC treatment. Subsequently, hPB-MSCs and BM-MSCs were trypsinized and incubated with NK cells at three E:T ratios of 1:1, 4:1, and 16:1 in the killing assay, as mentioned above. Percent killing was compared with that in MSCs transfected with scrambled siRNA. Results revealed a significantly higher percent killing in the knockdown experiments than in MSCs transfected with scrambled siRNA in all three E:T ratios assessed (Fig. 5B).

**Overexpression of PI-9 protects hPB-MSCs from killing**

To assess the independent role of PI-9 in protecting hPB-MSCs from cytotoxic killing, we transduced our hPB-MSCs with the MIRG1–PI-9 plasmid, to increase PI-9 expression, and challenged them with YT cells, a human NK cell line. Using flow cytometry, we first assessed the transduced hPB-MSCs for the expression of GFP with MIRG1 (empty vector) and MIRG1–PI-9, compared with nontransduced hPB-MSCs. GFP expression in hPB-MSC–MIRG1–GFP and hPB-MSC–PI-9–GFP was compared with that in nontransduced MSC (hPB-MSCs) (Fig. 6A). Overall expression of GFP in these pools of hPB-MSC–MIRG1–GFP and hPB-MSC–PI-9–GFP was 97 ± 5% and 91 ± 6%, respectively (Fig. 6A). Subsequently, the three cell types were incubated with YT cells to assess the protective role from overexpression of PI-9. It is worth noting that NK cells and the YT cell line undergo similar mechanisms of cytotoxicity (32), are stimulated by common surface Ags (33), and express similar surface markers (34). Therefore, both cell types are used interchangeably in the literature. hPB-MSC, hPB-MSC–MIRG1, and hPB-MSC–MIRG1–PI-9 were
loaded with calcein AM and challenged for a standardized 8-h period with YT cells. When we subjected them to higher E:T ratios of YT cells, both hPB-MSC and hPB-MSC–MIRG1 cells showed increased lysis compared with hPB-MSC–MIRG1–PI-9 cells. MSC-MIRG1–PI-9 cells showed a significant inhibition of percent killing at the highest ratio (50:1) when compared with hPB-MSC and hPB-MSC–MIRG1 cells (\(p < 0.01\)) (Fig. 6B).

Therefore, overexpression of PI-9 can protect MSCs from cell-mediated lysis.

**Discussion**

Recent years have seen an unprecedented increase in the number of clinical trials in which MSCs are used to treat various immune-mediated diseases and metabolic and genetic disorders, as well as in tissue repair (35). Indeed, to date, >160 clinical trials using MSCs have been listed at www.clinicaltrials.gov.

Due to ease of administration, the ability to use controlled or optimized cells, eliminating the need for dysfunctional autologous senile MSCs or dysfunctional MSCs with genetic predisposition, as well as the ability to administer multiple doses, allogeneic MSCs have increasingly been considered for treating various diseases (36). In this regard, we have recently shown that allogeneic MSCs, but not autologous MSCs, reverse autoimmune diabetes in NOD mice (4, 37).

Because the isolation of abundant MSC numbers from peripheral blood would set the stage for an improved cell-based therapy, the use of bone marrow as a source requires further analysis. Currently, bone marrow represents the most abundant source of MSCs for examining their function experimentally as well as their therapeutic roles in clinical studies. hPB-MSCs are lower in concentration than are BM-MSCs in their respective tissues and are rarely detected in normal individuals (9, 10, 38, 39). CFU-fibroblastic have been detected in peripheral blood of humans, although they are few in number and difficult to maintain and culture, compared with bone marrow CFU-fibroblastic (11, 40). In instances in which stimulants such as G-CSF or GM-CSF have been used, or in pathological conditions in which intrinsic stimuli have been present (e.g., patients with cancer, burns, allograft rejection, or stroke), enhanced release and recovery of MSCs were observed. hPB-MSCs have been isolated and expanded with various levels of success (9, 12, 41). A total of 500 ml of blood was needed to obtain almost 10,000 mesenchymal precursor cells without growth factors, leading to an MSC culture capable of differentiating into the three mesodermal lineages (42).

Our data suggest that higher cell confluence, which increased the likelihood of generating MSCs in primary cultures, was obtained from samples with a greater amount of blood drawn and higher numbers of PBMCs isolated, as well as the addition of b-FGF at a concentration of 6 ng/ml. The supplementation of b-FGF to culture medium enhanced the growth and proliferation of MSCs obtained from low-yield areas, such as dental pulp and synovium-derived stem cells (43, 44). Interestingly, Bian et al. (45) recently showed that peripheral blood of patients with bone sarcomas had higher numbers of CD105+ MSCs than did the peripheral blood of healthy individuals. This increase was associated with increased growth factors, such as hepatocyte growth factor and vascular endothelial growth factor, in the plasma (45). Therefore, it is of value for future studies to better examine the synergistic effects of...
various growth factors in optimizing hPB-MSC isolation. In our standardization process, we also experienced difficulties in isolating MSCs from nine donors. We learned that there was notable donor variability in the numbers of PBMCs isolated from blood. In three donors of nine, the initial number of MSCs was below optimal to sustain a culture, and thus we were unable to isolate MSCs from them in repeated experiments. These individuals may need a stimulus, such as G-CSF or GM-CSF, to increase the

![FIGURE 5.](image)

hPB-MSCs and BM-MSCs show higher percent killing when PI-9 is knocked down. A, Both nonspecific scrambled siRNA and PI-9 siRNA were added to BM-MSCs for 72 h prior to the assessment of PI-9 expression by flow cytometry. Results revealed a significant reduction in PI-9 protein expression in the PI-9 siRNA-treated MSCs (right), compared with the scrambled siRNA-treated MSCs (red line represents the PI-9 expression and blue line the isotype control). B, Coincubation of NK cells with hPB-MSCs and BM-MSCs knocked down for PI-9 by PI-9 siRNA revealed a significant increase in percent killing with increasing E:T ratio (1:1; 4:1; 16:1), compared with their relevant transfected cells with scrambled siRNA. *p < 0.02.

![FIGURE 6.](image)

Overexpression of PI-9 affords protection against YT killing. hPB-MSCs were infected with either MIRG1 (empty vector) or MIRG1–PI-9. GFP expression in hPB-MSC–MIRG1–GFP and hPB-MSC–MIRG1–PI-9 cells was compared with that in uninfected MSCs (hPB-MSCs). Overall expression of GFP in these pools of hPB-MSC–MIRG1–GFP and hPB-MSC–MIRG1–PI-9 cells was 97 ± 5% and 91 ± 6%, respectively. A, Both hPB-MSC and hPB-MSC–MIRG1 cells showed increased lysis over that found in MSC–MIRG1–PI-9 cells (n = 3 experiments). B, MSC–MIRG1–PI-9 cells showed reduced percent killing when compared with MSC and MSC–MIRG1 cells. *p < 0.01.
shedding of bone marrow progenitors to the periphery. Future studies to better comprehend the source of variability in the number of progenitors among individuals and innovative technology to improve their isolation from blood will greatly contribute to the success of isolating hPB-MSCs. Although much work is needed to further optimize the likelihood of recovering MSCs from peripheral blood, our data indicate that successfully retrieved MSCs from peripheral blood shared similar surface markers with BM-MSCs, as well as differentiation potential and immunomodulatory characteristics (46, 47).

The immunomodulatory effect of hPB-MSCs was assessed using the anti-CD3/CD28 T cell stimulation assay. Our results revealed that hPB-MSCs showed a dose-dependent antiproliferative and anti-inflammatory effect. The data also show that the immunosuppressive ability of hPB-MSCs is similar to that of BM-MSCs and MSCs (48–50). In addition, as cytokines play key roles in the pathogenesis of various diseases, we assessed the anti-inflammatory effect of our hPB-MSCs on IFN-γ and TNF-α in these proliferation assays (51). The hPB-MSCs suppressed the production of these cytokines in a fashion comparable to the effect of BM-MSCs. These data indicate that immunomodulatory effects of hPB-MSCs were in concordance with those of BM-MSCs (13).

We further investigated the presence of PI-9 in MSCs as a strategic component for immune-evasion and a critical factor for the substantial improvement of cell-based therapy. Recent reports indicate that, despite being hypoimmunogenic, transplanted allogeneic MSCs are recognized by the host immune response and are eventually rejected (52). Several defense mechanisms have been reported as the means whereby MSCs avoid cytotoxicity, but none of these mechanisms fully explain how they evade the host immune response (53–55). Lack of MHC class II expression or costimulatory molecules could contribute to MSC hypoimmunogenicity. Nevertheless, Western blotting of cell lysates has shown that MSCs contain intracellular deposits of class II alloantigens (54, 55). Furthermore, MSCs stimulated by IFN-γ mimicking inflammatory milieu exhibit induction of class II MHC expression (56).

In this study, we report the constitutive intracellular expression of PI-9 in hPB-MSCs and BM-MSCs, but not in fibroblasts. PI-9 is a specific GrB inhibitor and is present in CTLs, NK cells, APCs, endothelial cells, and immunoprivileged sites (57). The overexpression of PI-9 contributes to protection of CTLs from GrB-mediated apoptosis (20). Our data show that PI-9 contributes to the protection of hPB-MSCs and BM-MSCs from NK cell-mediated cytotoxicity, whereas lack of PI-9 was associated with an increased percent killing of fibroblasts. In line with previous reports, our results corroborate the induction of lytic activity against MSCs by freshly isolated NK cells at high E:T ratios (58).

The specificity of GrB-mediated killing was demonstrated by blocking the lytic capacity of NK cells with our GrB-i. The significant decrease in percent lysis of both MSC types and fibroblasts in a dose-dependent manner with GrB-i underscores the essential role of GrB in the killing of MSCs, thereby emphasizing the fundamental protective role of PI-9 for MSC survival. Evidence of GrB activity in MSCs subjected to NK cells was associated with enhanced caspase-3 activity, compared with that in MSCs and fibroblast controls, which signifies a GrB–caspase-3–dependent killing mechanism. Furthermore, when PI-9 was knocked down by siRNA, percent killing was significantly higher in comparison with that in controls. The percent killing of MSCs was inversely proportional to the expression of PI-9 in MSCs. These data show that in the absence of the protective effect of PI-9, cells are rendered susceptible to GrB-induced apoptosis. Furthermore, these results agreed with our recent work on the protective role of Sp6, the mouse homolog to PI-9, in mouse MSCs (5). In our previous work, we generated CTL machinery by sensitizing BALB/c mice via a skin allograft from a C57BL/6 donor. This model mimics a scenario in which allogeneic wild-type MSCs become the target for CTL killing following administration. In this report, we have primarily used NK cells, which use similar GrB machinery. However, NK cells constitute a major component of the innate immune system, without engaging TCRs, as opposed to CTLs.

We transduced hPB-MSCs with the MirG1–PI-9 plasmid to enhance PI-9 production. Overexpression of PI-9 in NK-treated hPB-MSCs revealed an almost 50% reduction in cell lysis, compared with that in untreated MSCs. In contrast, lack of PI-9 expression and increased caspase-3 activity in fibroblasts evidently reinforce the protective role of PI-9 against NK cell-mediated killing. It is important to note that these data not only are important for the cycles of MSC therapy, but also will establish a potential novel ground for developing anti–PI-9 strategies in instances in which unwanted growth of tumor occurs in the course of MSC therapy. These new data advance our understanding of the immunomodulatory properties of hPB-MSCs and enable the modulation of a newly discovered pathway in hPB-MSCs to prolong their survival postadministration. These findings will reduce the need for repetitive injection, while increasing the efficacy of the cells, and will substantially contribute to the improvement of cell therapies using MSCs.

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Disclosures
The authors have no financial conflicts of interest.

References
typically identical to human mesenchymal bone marrow stem cells circulate in large amounts under the influence of acute large skin damage: new evidence for their use in regenerative medicine. Transplant. Proc. 38: 967–969.


