

ORIGINAL ARTICLE

Increased Number of Mesenchymal Stem Cell-like Cells in Peripheral Blood of Patients with Bone Sarcomas

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Background and Aims. The number of peripheral blood mesenchymal stem cells (PBMSCs) may increase under pathological conditions. We sought to compare the number of MSC-like cells in the peripheral blood of patients with bone sarcomas with healthy controls and to analyze related cytokines in the peripheral blood plasma.

Methods. Peripheral blood mononuclear cells (PBMNs) of patients with bone sarcomas and control subjects were isolated for culture and analyzed by flow cytometry for MSC phenotype. Cytokines in the plasma obtained after cell separation were analyzed using enzyme-linked immunosorbent assay (ELISA). Annexin-V and β -galactosidase staining were used to investigate whether the cells died from apoptosis or senescence.

Results. Flow cytometric analysis demonstrated an >9-fold increase in the number of cells with MSC-like phenotypes (CD34⁻, CD45⁻, CD105⁺) in patients with bone sarcomas compared with control subjects ($p < 0.05$). ELISA results showed that concentrations of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) in patients with bone sarcomas were statistically higher than those in the control subjects ($p < 0.05$), whereas there was no significant difference in plasma concentrations of leptin and stromal cell-derived factor 1 between the two groups. A significant, positive correlation between the percentages of PBMSC-like cells and concentrations of HGF in all samples ($R = 0.618$; $p = 0.011$). Annexin-V staining of MSC-like cells was positive, whereas β -galactosidase staining was negative.

Conclusions. Peripheral blood of patients with bone sarcomas has more cells with MSC phenotypes than blood of healthy persons. The increased number is accompanied by increased HGF and VEGF in the plasma. © 2009 IMSS. Published by Elsevier Inc.

Key Words: Bone sarcoma, Mesenchymal stem cells, Peripheral blood, Hepatocyte growth factor, Vascular endothelial growth factor.

Introduction

Currently, bone marrow represents the main source of mesenchymal stem cells (MSCs) for both experimental and clinical studies (1,2). The number of human bone marrow MSCs (BMMSCs) is in the range of 1 per $5 \times 10^3 - 10^4$ bone marrow mononuclear cells (MNCs),

whereas the number of peripheral blood (PBMSCs) is much less and usually are not detectable (3–6). However, the number of PBMSCs may increase under pathological conditions. For instance, a significant number of MSCs increase after acute or chronic skeletal muscle injury (7). Mansilla et al. (8) found a significant increase of circulating MSCs in burn patients, and the percentage of MSCs correlated with the size and severity of the burns. Fernández et al. (9) reported an increase of circulating MSCs in breast cancer patients. Moreover, it has been demonstrated that cultured MSCs were recruited to the wound sites after being infused into animals (10–14). Microenvironments of solid tumors are similar to those of wounded tissues (15,16),

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and several investigators have demonstrated that MSCs can be recruited to the sites of solid tumors. Studies using animal models of melanoma (17), breast cancer (18), glioma (19), and colon carcinoma (20) have indicated that systemically infused MSCs preferentially distributed within tumors and were involved in the formation of tumor stroma.

We hypothesize that the number of PBMSCs in patients with bone sarcomas is higher than in healthy individuals and that MSCs are involved in the development of bone sarcomas. Using flow cytometry, we quantified the number of circulating MSC-like cells in patients with bone sarcomas and control subjects. MNCs (including PBMSC-like cells) were isolated and cultured, and separated plasma was harvested. In patients with bone sarcomas, we found an increase of >9-fold of circulating MSC-like cells and a significantly higher level of plasma hepatocyte growth factor (HGF) than in the control subjects.

Materials and Methods

Patients

Patients with untreated bone sarcomas (osteosarcomas, chondrosarcomas, fibrosarcoma of the bone, etc.) without distant metastases were hospitalized in the Department of Orthopedic Surgery of the Ninth People's Hospital of Shanghai, China between May 2006 and December 2007. All patients who enrolled provided informed consent in accordance with our institution's regulatory requirements, and we conducted the study according to our institution's guidelines with ethical permission.

In all cases, diagnosis of bone sarcoma was established by clinical characteristics and by radiological findings and pathological examination. Study exclusion criteria included secondary metastatic bone tumor, other neoplasms, traumatic fracture, infection or inflammatory diseases, and metabolic bone diseases. Only patients without these conditions were included in the study. Participants included nine patients (six males, three females) with a median age of 30 years (range: 15–50 years). There were six cases of osteosarcomas, two cases of chondrosarcomas, and one case of fibrosarcoma). The study included seven healthy volunteers (six males, one female) with a median age of 31 years (range: 25–38 years). The two groups had no significant difference in composition regarding age or sex ($p > 0.10$). Approximately 20–30 mL of peripheral blood samples were collected from all study participants after fasting. The blood was treated with heparin at a concentration of 100 U/mL.

Separation of Peripheral Blood Mesenchymal Stem Cell-like Cells

We reserved 1 mL of peripheral blood for flow cytometric analysis. The remaining blood was loaded over an equal

volume of Lymphoprep (1.077 g/mL; Axis-Shield PoC AS, Oslo, Norway) and centrifuged at $800 \times g$ for 30 min at room temperature. Plasma was harvested and stored at -80°C until analysis. The MNC-enriched low-density fraction was collected, rinsed with serum-free α -minimal essential medium (α -MEM; GIBCO, Grand Island, NY) and plated at 0.5×10^6 nucleated cells/cm². MNCs were cultured in α -MEM supplemented by 20% fetal bovine serum (FBS; Hyclone, Tauranga, New Zealand; α -MEM/10% FBS) and in antibiotics (penicillin 100 U/mL, streptomycin 100 $\mu\text{g/mL}$; Hyclone, Logan, UT) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. Three days later, half of the culture medium was replaced and an equal volume of fresh medium was added. Afterward, the culture medium was changed every 3 days. Growth of MNCs was monitored by counting the numbers of colony-forming unit fibroblasts (CFU-Fs).

Flow Cytometric Analysis of Peripheral Blood Cells

MSC phenotype was defined as cells positive for CD29, CD44, CD90, and CD105 and negative for CD14, CD34, and CD45. PBMCs were subjected to flow cytometry analysis. Peripheral blood (100 μL) was stained for 30 min at room temperature in the dark with the following monoclonal antibodies: mouse anti-human CD105-Alexa Fluor 647 (AbD Serotec, Raleigh, NC) in combination with mouse anti-human CD34-PE (Invitrogen, Beijing, China) and mouse anti-human CD45-PerCP-CY5.5 (BD Biosciences, Shanghai, China). As controls of nonspecific staining, cells were labeled with conjugated nonspecific isotype antibodies. Thereafter, erythrocytes were lysed, and the remaining cells were washed with phosphate-buffered saline and fixed in 1% paraformaldehyde before being analyzed with a FACSCalibur flow cytometer (BD Biosciences). Data were acquired with CellQuest software (BD Biosciences). For each analysis, at least 100,000 cells with the appropriate ratio of forward scatter to side scatter were collected, and all MNCs were gated. PBMSC-like cells were defined as CD105⁺, CD34, and CD45⁻. The results are shown as mean values \pm SEM.

Detection of Cytokines in Peripheral Blood Plasma Using Enzyme-linked Immunosorbent Assay

Peripheral blood plasma samples were thawed at 4°C . Plasma levels of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), leptin, and stromal cell-derived factor-1 (SDF-1) were measured with the enzyme-linked immunosorbent assay (ELISA) method using commercially available kits (Your Sun Biological Technology Corporation, Ltd., Shanghai, China) according to the manufacturer's instructions. Sensitivities of all kits are 10 pg/mL. Undetectable values were assigned a value of half the sensitivity limit (5 pg/mL).

Apoptosis and Senescence Assay

PBMSC-like cells from patients with osteosarcoma were passaged into wells of 24-well plates when they started to decrease, and the annexin-V and β -galactosidase staining were used to determine whether the cells died from apoptosis or senescence.

Annexin-V Staining

Cells were washed twice with PBS and incubated at 37°C for 10 min with 1:100 diluted annexin-V-FITC (BD Biosciences) in the dark. Cells were observed under a fluorescence microscope using a filter set for FITC.

β -Galactosidase Assay

β -Galactosidase assay was performed using the previously described method (21). Cells were washed twice with PBS, fixed for 3 min (room temperature) in 0.05% glutaraldehyde, washed, and incubated at 37°C (no CO₂) for 3 h with fresh β -galactosidase staining solution containing 1 mg of 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) per ml, 40 mM citric acid (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂. β -Galactosidase-positive cells were then observed under the microscope.

Statistical Analysis

Data were analyzed for statistical significance using Student's *t*-test or two-sample Wilcoxon rank sum test; measurement data are expressed as mean \pm SEM. The relation between the percentage of PBMSC-like cells and HGF was determined by calculating Pearson's correlation coefficient. A probability value of <0.05 was considered significant. Statistical analyses were conducted using software SPSS (version 11.5, SPSS Inc., Chicago, IL, <http://www.spss.com>).

Results

Isolation and Culture of Peripheral Blood Mesenchymal Stem Cell-like Cells

Most PBMNCs isolated from the control (normal) subjects were in suspension 3 days after seeding. Only a small amount of cells adhered to the culture vessels with heterogeneous cell shapes. Most cells were round or rod-shaped, a few cells were spindle-shaped, and no CFU-Fs were formed during the 3-week study period. Proliferation of adherent cells was poor, and the number of adherent cells decreased with time and they could not be passaged (Figure 1A). For PBMNCs isolated from bone sarcoma patients, spindle-shaped adherent cells were formed and the number of adherent cells was significantly higher than

that of control subjects. CFU-F formation was observed in four patients with osteosarcoma and one patient with chondrosarcoma (~ 1 CFU-F/ 10^7 MNCs), and the colonies of the CFU-Fs enlarged with time (Figure 1B).

Quantification of Peripheral Blood Mesenchymal Stem Cell-like Cells

PBMNCs were quantified by flow cytometry. MSC-like cells in our study are defined as positive for CD105 and negative for CD34 and CD45. Average percentages of MSC-like cells in patients and control subjects were 0.0251 ± 0.0103 and 0.0028 ± 0.0016 , respectively, and the percentage of MSC-like cells in peripheral blood of patients with bone sarcomas was 9.12-fold higher than that of control subjects ($p < 0.05$; Figure 2).

Concentrations of HGF, VEGF, Leptin, and SDF-1 in Plasma

ELISA results indicated that the peripheral blood HGF concentrations in patients with bone sarcomas (average, 396.55 ± 90.93 pg/mL) were significantly higher ($p = 0.033$) than in control subjects (average, 162.16 ± 16.99 pg/mL), as were VEGF concentrations ($p < 0.01$) in bone sarcomas patients, which were 18.38 ± 8.91 pg/mL vs. undetectable (<5 pg/mL) in controls, respectively. Average peripheral blood concentrations of leptin and SDF-1 in patients with bone sarcomas and control subjects were similar: 687.62 ± 207.58 pg/mL vs. 667.59 ± 182.99 pg/mL ($p = 0.945$) and 598.35 ± 122.70 pg/mL vs. 715.10 ± 239.62 pg/mL ($p = 0.650$), respectively. We found a significant, positive correlation between the numbers of PBMSC-like cells and concentrations of HGF in all samples ($R = 0.618$; $p = 0.011$).

Apoptosis and Senescence Assay

After annexin-V-FITC staining, an obvious green fluorescence was observed in the PBMSC-like cells under a fluorescence microscope (Figure 3A), whereas β -galactosidase staining of PBMSC-like cells was negative in our experiment (Figure 3B).

Discussion

Recent research has suggested that MSCs have a close relationship with certain pathological processes, with the number of PBMSCs increasing under pathological conditions. Ramírez et al. (7) found an increased number of PBMSCs after chronic or acute skeletal muscle injury and suggested that the mobilized MSCs play a role in skeletal muscle regeneration; similarly, Mansilla et al. (8) discovered a significantly increased number of PBMSCs in burn patients and found that the percentage of MSCs correlated with the size and severity of the burn. These authors

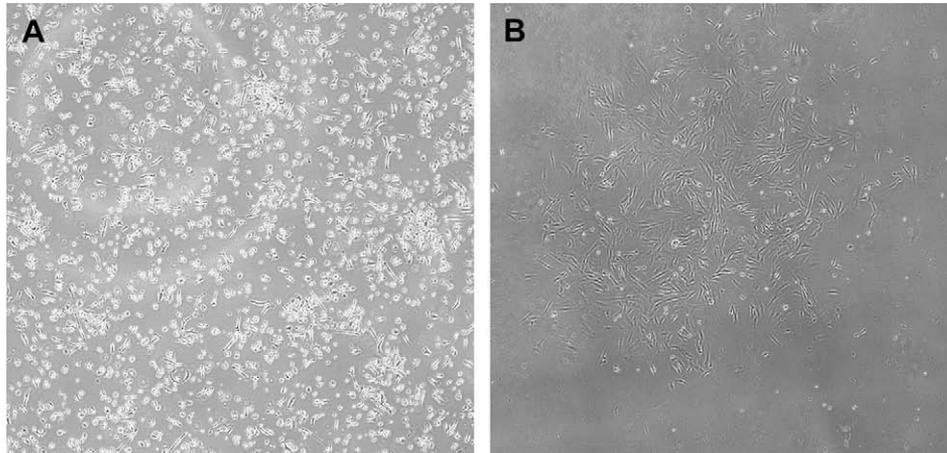


Figure 1. (A) Morphology of peripheral blood mononuclear cells (PBMCs) from control subjects: cells were round or rod-shaped. (B) Morphology of PBMCs from a patient with osteosarcoma: cells were spindle shaped, and a colony-forming unit fibroblast was formed.

suggested that PBMSCs may be involved in skin regeneration. When rats were exposed to chronic hypoxia, the number of PBMSCs increased (22). Fernández et al. (9) demonstrated an increased number of PBMSCs in patients with breast cancer. In addition, other authors demonstrated that after cultured MSCs were infused into animals with trauma or tumors, MSCs could be recruited to the lesion locations. For instance, when human BMMSCs were injected into a fetal lamb, they selectively migrated to trauma sites and were involved in tissue regeneration (11,12). It has been demonstrated that after myocardial infarction, intravenously injected MSCs were recruited to the injured heart, where they undergo differentiation and participate in post-infarct remodeling of the scars (23). Studies of fracture healing revealed that systemically infused exogenous MSCs homed to the fracture sites and were involved in bone regeneration (10,13,14).

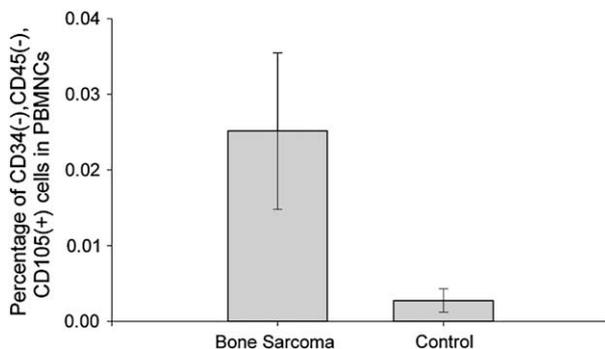


Figure 2. Average percentage of cells with surface antigens of mesenchymal stem cells (MSCs) in PBMCs of patients with bone sarcomas and of study control subjects were 0.0251 ± 0.0103 and 0.0028 ± 0.0016 , respectively. Percentage of cells with surface antigens of MSCs in PBMCs of patients with bone sarcomas was 9.12-fold of those of study control subjects, which was statistically significant ($p < 0.05$).

The microenvironment in solid tumors is similar to that in wounded tissues (15,16), and there is evidence that MSCs could be recruited to sites of solid tumors. Studies of melanoma (17), breast cancer (18), glioma (19), and colon carcinoma (20) demonstrated that systemically infused exogenous MSCs preferentially homed to tumors and were involved in the formation of tumor stroma. The present study has demonstrated that the numbers of PBMSC-like cells increased in patients with bone sarcomas, suggesting that the increased MSC-like cells may be involved in the development of bone sarcomas.

MSCs are positive for CD44, CD90, and CD105 and negative for markers of hematopoietic cells such as CD14, CD34, and CD45 (2,24); therefore, cells positive for CD105 and negative for CD34 and CD45 were enumerated by flow cytometry in our study. Results indicated that numbers of cells with the phenotype of MSCs in patients with bone sarcomas were significantly higher than those of the control subjects, suggesting that bone sarcoma may have stimulated MSC release into the peripheral circulation.

In our study, PBMCs were isolated from patients with bone sarcomas and control (normal) subjects and then cultured. No CFU-Fs were formed in the PBMCs from control subjects, but 5/9 patients with bone sarcoma formed CFU-Fs. These cells displayed a spindle shape, similar to that of BMMSCs. However, these cells decreased gradually after being passaged and were insufficient to perform multi-differentiation analysis. They were unable to be further compared with BMMSCs from the same patients. These PBMSC-like cells were spindle shaped and had limited proliferation potential, which is different from sarcoma cells from tumor sites of patients that displayed oval to polygonal shape and high proliferation capacity as reported previously (25,26). One possible reason for the difficulty of culturing PBMSC-like cells may be the low number of

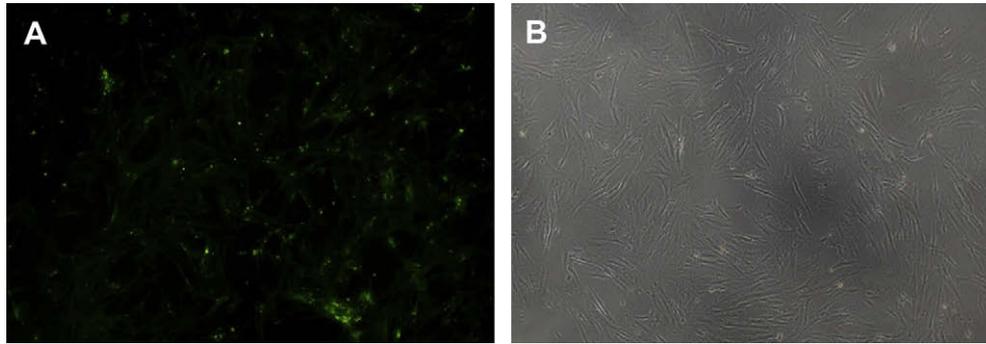


Figure 3. (A) Apoptosis assay: after annexin-V-FITC staining, obvious green fluorescence was observed in PBMSC-like cells under a fluorescence microscope. (B) Senescence assay: β -galactosidase staining of PBMSC-like cells was negative. Color version available online at www.arcmedres.com.

MSC-like cells in peripheral blood ($2.5 \text{ cells}/10^4 \text{ MNCs}$) and the limited peripheral blood collected from patients (20–30 ml). Growth of PBMSC-like cells is slow under low seeding density. One study of BMMSCs revealed that BMMSCs require a minimal seeding density to proliferate; if cultured below minimal densities, they undergo apoptosis (27). Our results of positive annexin-V staining and negative β -galactosidase staining of MSC-like cells further demonstrated that the cells died from apoptosis, not senescence (21,28). The other possibility is that the culture condition that PBMSC-like cells require may be different from those of BMMSCs. When BMMSCs are cultured *in vitro*, coexisting hematopoietic cells can secrete numerous regulatory molecules that form the basis of intercellular cross-talk networks, which then regulate survival and growth of MSCs in an autocrine or paracrine manner (29). Reading et al. (6) isolated MSCs from blood by magnetic-activated cell separation using magnetic beads coated with the STRO-1 antibody. Approximately 30 stromal colonies were generated per 10^7 MNCs . However, after about 1 week in culture, all colonies spontaneously died. Reading et al. (6) considered this loss of colonies to the cytotoxic effect of T cells present in the blood. Therefore, culture conditions of PBMSC-like cells may be different from those of BMMSCs. Whether some pro-proliferative cytokines should be supplemented should be further investigated.

It has been demonstrated that levels of several cytokines in the peripheral blood of patients with bone sarcomas are elevated, such as VEGF, basic fibroblast growth factor, interleukin-6 (IL-6), IL-8, IL-10, and macrophage colony-stimulating factor (30,31). Bone sarcoma cells can produce VEGF, platelet-derived growth factor, and HGF (32–34), factors that have been demonstrated to have chemotactic effects on MSCs (35–37). Thus, systemic mobilization of MSC-like cells in bone sarcoma patients may be mediated by cytokines released by tumor cells. ELISA results in our study indicated that VEGF levels in the peripheral blood were higher in patients with bone sarcomas than patients in the control group. VEGF has a chemotactic effect on

MSCs and can promote MSC proliferation and is involved in angiogenesis and cancer development (37,38). Level of HGF was significantly higher in patients with bone sarcoma than in control subjects. Chattopadhyay et al. demonstrated that human osteosarcoma cells can secrete HGF *in vitro* and that released HGF functions in an autocrine manner, resulting in mitogenesis (32). Other researchers have also revealed that HGF can enhance proliferation, migration, and invasion potentials of osteosarcoma cells (39,40). HGF can also promote migration of MSCs (35,41,42) and a significant and positive correlation between the percentages of PBMSC-like cells and concentrations of HGF were found. Taken together, these findings indicate that the increased number of PBMSC-like cells in bone sarcoma patients may be mobilized by the elevated levels of HGF and VEGF and may home to the sarcoma sites to contribute to sarcoma growth. The increased number of PBMSC-like cells and elevated plasma level of HGF and VEGF may be used as a diagnostic or prognostic indicator for bone sarcoma patients.

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