Distinct Mobilization of Circulating CD271⁺ Mesenchymal Progenitors from Hematopoietic Progenitors During Aging and After Myocardial Infarction

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**Key Words.** Mesenchymal stem cells • Mobilization • Cell surface markers • Aging • Cardiac

**ABSTRACT**

The specific cell surface markers on mesenchymal stem/progenitor cells (MSCs) have been poorly defined in vivo, but in one recent study, an MSC subpopulation was directly isolated from a CD271⁺-positive fraction of human bone marrow cells. The aim of this study was to identify circulating CD271⁺ MSCs in human peripheral blood and investigate whether the cells are mobilized after acute myocardial infarction (MI). A flow cytometric analysis identified CD45 low/−/CD34⁺/CD271⁺ cells in adult human peripheral blood. The numbers of circulating CD45 low/−/CD34⁺/CD133⁺ cells (hematopoietic lineage progenitors) were significantly lower in elderly subjects without coronary artery disease than in healthy young subjects, whereas the numbers of CD45 low/−/CD34⁺/CD271⁺ cells were comparable between elderly subjects and younger subjects. The CD45 low/−/CD34⁺/CD271⁺ and CD133⁺ cell counts were both higher in patients with acute MI than in patients with stable coronary artery disease. In our investigation of the time course changes after acute MI, the CD45 low/−/CD34⁺/CD271⁺ cell counts gradually increased up to day 7. Over the same period, the CD45 low/−/CD34⁺/CD271⁺ cell counts peaked at day 3 and then declined up to day 7. Importantly, the CD271⁺ cell counts at day 3 were positively correlated with the peak concentrations of creatine kinase after acute MI. Results of the present study suggest that the CD271⁺ MSCs are mobilized differently from the CD133⁺ hematopoietic progenitors and may play a specific role in the tissue repair process during age-related changes and after acute myocardial infarction.

**INTRODUCTION**

Bone marrow (BM) has been the cell source most frequently used for the clinical cell therapies recently applied for cardiovascular disease [1–3]. Adult BM contains both hematopoietic stem/progenitor cells (HSCs) and subsets of nonhematopoietic progenitor cells, commonly referred to as mesenchymal stem/progenitor cells (MSCs) or multipotent stromal cells [4]. MSCs are typically isolated by a plastic adherence method in vitro. Accumulating evidence from animal studies shows that the exogenous administration of cultured MSCs ameliorates organ damage after injury or disease episodes, such as myocardial infarction (MI) or stroke [5–9]. On this basis, MSCs are now recognized as tissue-repairing cells. Studies have yet to determine, however, whether the MSCs circulate in human peripheral circulation for the maintenance of intrinsic regenerative mechanisms. This question is likely to remain unanswered until the specific cell surface markers on MSCs in vivo are more precisely defined.

Our group and others have recently shown that a purified MSC subpopulation can be directly isolated from human BM by magnetically activated cell sorting against CD271 (also known as p75 low-affinity nerve growth factor receptor) [7, 10, 11]. CD271 antibodies were initially shown to stain the stromal compartment of bone marrow [12]. This bone marrow fraction was shown to be highly clonogenic and to possess a marked potential for the production of both osteoblasts and adipocytes. Intriguingly, the freshly isolated CD271⁺ fraction was approximately 50% positive for CD34, which was downregulated to 0%–1% after culture [10]. More recently, anti-CD34 and anti-CD271 monoclonal antibodies were found to purify a subset of mesenchymal cells with high proliferative, clonogenic, and multipotent differentiation abilities from human adipose tissue [13].
Here, we report the first flow cytometric analysis to identify CD45<sup>low/medium</sup> CD34<sup>-/+</sup> CD271<sup>+</sup> cells (MSCs) within mononuclear cells in human peripheral and cord blood. MI leads to left ventricular remodeling and, subsequently, to chronic heart failure. The chemotaxtractants circulating at increased levels after acute MI mobilize CD34-positive cells from the BM, and this may encourage cardiac repair and help to prevent remodeling [14, 15]. Earlier studies showed that CD34-positive cells released into peripheral blood after acute MI consist of HSCs and endothelial progenitor cells [16, 17]. Our present study demonstrates that the circulating MSCs are also recruited into the peripheral circulation after acute MI in humans.

**Materials and Methods**

Isolation, Culture, and Differentiation of Human MSCs from CD271<sup>+</sup> BM Cells

Human MSCs derived from CD271<sup>+</sup> BM cells were isolated and cultured as described previously [7]. In brief, BM aspirates were taken from the iliac crest of healthy adult donors, and mononuclear cells were isolated with the use of density gradient centrifugation. CD271<sup>+</sup> cells were obtained from freshly isolated mononuclear cells by a procedure for magnetic-activated cell sorting using antibodies conjugated to dextran-coated iron beads according to the manufacturer’s instructions (CD271; Miltenyi Biotech, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). The entire isolate was cultured in a complete culture medium consisting of α-minimum essential medium (MEM), 10%–17% fetal bovine serum, 100 units per milliliter penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine.

The smooth muscle cells were differentiated by changing the medium to α-MEM containing 10% fetal calf serum, growing the cells to 80% confluence in the complete culture medium, and then supplementing the medium with 10 ng/ml recombinant transforming growth factor-β. For endothelial cell differentiation, the medium was changed to complete endothelial growth medium-2 containing 2 ng/ml recombinant vascular endothelial growth factor. The media were replaced every 3–4 days for 7 days. The cells were then fixed and examined by immunofluorescence to detect vascular cell markers. The primary antibodies were anti-vimentin (1:400; Dako, Glostrup, Denmark, http://www.dako.com), anti-α-smooth muscle actin (1:200; Dako), and anti-platelet endothelial cell adhesion molecule-1 (anti-PECAM-1) (1:100; Dako). For animal study, a serum-free conditioned medium (CdM) from CD271<sup>+</sup> MSCs was prepared and concentrated using our published method [7].

Mouse MI Model and Administration of Conditioned Medium from CD271<sup>+</sup> MSCs

Procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. The animal protocol for this study was approved by the institutional animal care and use committee of the University of Vermont.

To create the acute MI model, C57Bl/6J male mice at 8–9 weeks of age were ventilated mechanically under anesthesia, their chests were opened, and then their left anterior descending coronary arteries were ligated, as described previously [6]. Two hundred microliters of serum-free MEM (control, n = 4) or 30× serum-free CdM (n = 5) was injected into the left ventricle lumen through the apex under randomization at 24 hours after MI. Six days after injection, the hearts were excised and used for histological study. Sections from the hearts were stained with hematoxylin-eosin, and immunohistochemistry was performed for PECAM-1. Vessel numbers in the border areas were manually counted in a randomly selected high-power field (n = 3 in each animal).

Sample Collection from Cord Blood, Young and Older Subjects, and Patients with Coronary Artery Disease

Cord blood samples from 20 normal deliveries were provided from Kanagawa Cord Blood Bank (Yokohama, Japan) according to the institutional research guidelines. Peripheral blood was collected from 56 adult individuals: 15 patients with acute MI (age, 63.9 ± 3.2 years, 87% male) admitted within 24 hours of the onset of symptoms, 7 patients with stable coronary artery disease (sCAD; age, 69.7 ± 1.9 years, 71% male), 6 young healthy subjects (mean, 30.5 ± 2.4 years, 33% male), and 28 elderly subjects (age, ≥75 years) without any evidence of coronary artery disease (mean, 81.9 ± 0.9 years, 43% male). The diagnoses of acute MI and stable coronary artery disease were based on clinical symptoms, electrocardiographic changes, blood examinations, and coronary angiograms. Acute MI was defined by the following criteria: chest pain for longer than 20 minutes, ST segment elevation on electrocardiography, and a twofold or greater increase in creatine kinase from the normal range, as defined under the American College of Cardiology/American Heart Association guidelines [18]. All the acute MI patients underwent successful percutaneous coronary intervention on admission. Major exclusion criteria in the present study were chronic renal failure on hemodialysis, apparent infectious disease, hematologic disorder, and extensive myocardial infarction (peak creatine kinase [CK] >8,000 IU/l) with complications. We also harvested small amounts of bone marrow from a patient with chronic coronary artery disease during coronary bypass surgery. The patients in the study gave their written informed consent for participation. The study protocol conformed to the ethical guidelines set by the 1975 Declaration of Helsinki, as reflected in an a priori approval by the human research committee of our university.

Flow Cytometric Analysis

Circulating progenitor cells were determined by flow cytometer, as described previously [19]. Mononuclear cells with CD45<sup>low/medium</sup> CD34<sup>-/+/+</sup> CD271<sup>+</sup> and CD45<sup>low/medium</sup> CD34<sup>-/+/+</sup> CD133<sup>+</sup> in blood were defined as putative circulating mesenchymal and hematopoietic stem/progenitor cells, respectively. Cells were stained with peridinin chlorophyll protein-conjugated anti-CD45 monoclonal antibody (BD Biosciences, San Diego, http://www.bdbiosciences.com), fluorescein isothiocyanate-conjugated anti-CD34 monoclonal antibody (Beckman Coulter, Fullerton, CA, http://www.beckmancouler.com), and R-phycocerythrin-conjugated anti-CD271 monoclonal antibody (BD Biosciences) or allophycocyanin-conjugated anti-CD133 monoclonal antibody (Miltenyi Biotech). Samples were subjected to a two-dimensional side scatter-fluorescence dot plot analysis (FACSCalibur; BD Biosciences). After appropriate gating with low cytoplasmic granularity and with low expression of CD45, the numbers of CD34<sup>-</sup>, CD34<sup>+</sup> CD271<sup>+</sup>, and CD34<sup>-</sup> CD133<sup>+</sup> cells were quantified and expressed as the number of cells per 10<sup>7</sup> total events or the percentage of CD271<sup>+</sup> and CD133<sup>+</sup> among the total cell count for CD45<sup>-/+/</sup>CD34<sup>-</sup>. The number of cells per milliliter of blood was calculated. The flow cytometric analysis was performed by technologists blinded to the present protocol.
Statistical Analyses

All values are expressed as mean ± SEM unless otherwise indicated. Categorical variables were compared by the Fisher’s exact test. Comparisons of parameters between two groups were made by the unpaired Student’s t test. Multiple group comparison was performed by one-way analysis of variance followed by pairwise contrasts using the Dunnett’s test. The correlation coefficients between the two parameters were determined by Pearson’s simple linear regression analysis. p < .05 was considered significant.

RESULTS

Proangiogenic Potential of MSCs from CD271⁺ Cells in Bone Marrow

As reported earlier [10], the subpopulation of CD45爝/CD34⁺/CD271⁺ cells in bone marrow was copositive for CD271 in a patient with ischemic heart disease (Fig. 1A). The CD271⁺ cells magnetically isolated from human BM cells adhered to plastic culture dishes and propagated in a manner similar to nonselected MSCs. After culturing, the prospectively isolated MSCs had a fibroblastic spindle-like shape typical of MSCs and were immunopositive for vimentin (Fig. 1B). An earlier study by our group showed that MSCs derived from CD271 cells had an antigen profile similar to that of MSCs isolated by the plastic adherence method [7]. When exposed to the appropriate differentiation media, the cells differentiated into vascular smooth muscle-like cells that stained with α-smooth muscle actin and endothelial-like cells that stained with PECAM-1 (Fig. 1B).

We previously showed that the MSCs derived from the CD271⁺ cells secreted several angiogenic factors, such as vascular endothelial growth factor, hepatocyte growth factor, and stromal-derived factor-1 [7]. To test in vivo effects of the secreted factors, we injected concentrated serum-free CdM from

Figure 1. Human MSCs from bone marrow CD271⁺ cells. (A): Flow cytometric analysis for CD45爝/CD34⁺/CD271⁺ cells in bone marrow from a patient with chronic coronary artery disease (male, 59 years). (B): Top, phase contrast images for cultured MSCs isolated by plastic adherence (left) and prospectively isolated from bone marrow CD271⁺ cells (right). Bottom, red fluorescence in each panel indicated vimentin for mesenchymal cells, α-smooth muscle actin for vascular smooth muscle-like cells, and PECAM-1 for endothelial-like cells derived from CD271⁺ MSCs. Nuclei were stained with 4',6-diamidino-2-phenylindole and are shown in blue. Scale bars = 20 μm. (C): Mouse myocardial infarction model treated with conditioned medium from CD271⁺ MSCs. Top images, HE-stained sections; scale bars = 100 μm. Bottom images, PECAM-1-stained sections; scale bars = 50 μm. Brown indicates endothelial cells. Graph shows comparison of vessel density in infarcted myocardium between the controls and the mice treated with conditioned medium. *, p < .05. Abbreviations: Con, control; HE, hematoxylin-eosin; MSC, mesenchymal stem/progenitor cell; PECAM, platelet endothelial cell adhesion molecule; SMA, smooth muscle actin.
CD271+ MSCs into hearts after infarction in mice. In hematoxylin and eosin-stained sections, greater wall thickness and cellular density were observed in the border zone of infarction in mice treated with the CdM compared with the mice treated with the vehicle (Fig. 1C). Vessel density in the border area was significantly higher in the CdM-treated hearts than in the controls (p < .05). Thus, these results demonstrated a proangiogenic potential in CD271+ MSCs.

Identification of Circulating MSCs in Human Peripheral and Cord Blood

Mononuclear cells with CD45low/CD34+ CD271+ were identified in human peripheral and cord blood by flow cytometry. The CD45low/CD34+CD271+ cells and CD45low/CD34+CD133+ cells were quantified as putative circulating mesenchymal and hematopoietic stem/progenitor cells, respectively.

Cord blood contained a 10-fold higher number of CD45low/CD34+CD271+ cells than in adult peripheral blood (12,608.5 ± 1,535.9 vs. 1,203.3 ± 334.8 cells per milliliter, p < .001). The proportion of the CD133+ subpopulation was significantly higher in the cord-blood CD34+ cells than in the adult peripheral-blood CD34+ cells (79.9 ± 1.4% vs. 68.3 ± 4.8%, p < .01), whereas the proportion of the CD271+ subpopulation was significantly lower in the cord blood than in the adult peripheral blood (0.30 ± 0.04% vs. 0.68 ± 0.18%, p < .01).

The hematopoietic stem/progenitor cell function in BM declines with age [20]. In adult peripheral blood, the cell counts for circulating CD45low/CD34+ cells (p = .01) and the CD133+ fraction (p = .02) were significantly lower in the older subjects than in the young subjects (Fig. 2). On the other hand, the proportion of the CD271+ fraction was significantly greater in the older subjects than in the young subjects (p = .01), whereas the CD45low/CD34+CD271+ cell counts were somewhat higher in the older subjects than in the younger subjects, although not to a significantly different extent (p = .08). Similarly, the CD271+ cell counts in the older and younger subjects were not significantly different from those in the patients with sCAD. When the data for the young subjects, old subjects, and patients with sCAD were combined, age was positively correlated with the percentage of CD271+ fraction in the CD45low/CD34+ cells (r = .45, p = .004).

In contrast to the HSCs, mobilization of the circulating MSCs appears to be maintained throughout the aging process. These
results suggest that MSCs are continuously mobilized into peripheral circulation to maintain intrinsic reparative mechanisms in a physiological condition.

Mobilization of Circulating MSCs After Acute Myocardial Infarction

Next, we investigated time course changes in mobilization of the circulating stem/progenitor cells after acute MI compared with the cells in patients with sCAD. As shown in Table 1, there were no significant differences in baseline clinical characteristics between the patients with sCAD and patients with acute MI. The time course change of leukocyte counts after acute MI was as follows: 12,200 ± 1,011 cells per microliter on admission, 8,354 ± 524 cells per microliter at day 3, and 6,700 ± 406 cells per microliter at day 7.

The total CD45low/CD34+ cell counts on admission in patients with acute MI were somewhat higher than those in patients with sCAD, and they gradually increased up to day 7 after acute MI, although not to a statistically significant extent (Fig. 3). The percentage of CD133+ cell counts was higher in MI patients than in sCAD patients (p < .05, acute MI day 3 vs. sCAD). The CD133+ cell counts also gradually increased up to day 7, following a pattern of behavior similar to that of the total CD45low/CD34+ cells.

Our present findings revealed recruitment of the circulating MSCs into peripheral circulation after acute events (Fig. 4). The proportion of the CD271+ fraction in CD45low/CD34+ cells in MI patients at day 3 was significantly higher than that in patients with sCAD and that in MI patients at days 0 and 7 (p < .05, respectively). The CD271+ cell counts were somewhat higher than those in patients with sCAD on admission, rose to peak levels by day 3 (p < .05 vs. sCAD), and then declined up to day 7. The results indicate that the MSCs are transiently mobilized from BM after acute MI independently of the hematopoietic progenitor mobilization.

Next, we examined the potential relationship between the numbers of circulating progenitors and myocardial damage in acute MI. A simple regression analysis revealed that the CD271+ cell counts on day 3 were positively associated with peak CK concentrations after acute MI (r = 0.67, p = .006, Fig. 5A). The total CD34+ cell counts on day 3 were not related to the concentration (Fig. 5B). The CD133+ cell counts at each time point showed no correlation with the peak CK levels. Hence, circulating MSCs may mobilize when the acute myocardial damage worsens.

| Clinical characteristics | sCAD (n = 7) | AMI (n = 15) | p |
|--------------------------|-------------|-------------|---|
| Age (yr)                 | 69.7 ± 1.9  | 63.9 ± 3.2  | .26|
| Male (%)                 | 71.4        | 86.7        | .56|
| Diabetes mellitus (%)    | 57.1        | 53.3        | 1.0|
| Dyslipidemia (%)         | 71.4        | 46.7        | .38|
| Hypertension (%)         | 71.4        | 60          | 1.0|
| Chronic kidney disease (%) | 71.4      | 40          | .36|
| Peak CK (IU/l)           | 2,517 ± 351 | 351 ± 63.9  | .006|

Abbreviations: AMI, acute myocardial infarction; CK, creatine kinase; sCAD, stable coronary artery disease.

Figure 3. Quantitative analysis by flow cytometry for hematopoietic stem/progenitor cells (HSCs) in human peripheral blood after acute myocardial infarction. Shown are time course changes in levels of circulating CD45low/CD34+ cells and HSCs. Measurements are given of CD45low/CD34+ and CD45low/CD34+CD133+ cell counts (per milliliter) and proportion of CD133+ fraction in CD45low/CD34+ cells (%). Patients with sCAD, n = 7; patients with AMI, n = 15. *p < .05. Abbreviations: AMI, acute myocardial infarction; d, day; sCAD, stable coronary artery disease.

DISCUSSION

CD271 functions in pan-neurotrophin signaling during development and is expressed by germline stem cells [21]. In the postnatal state, CD271 antigen remains one of the most selective markers for enriching progenitor cells for MSCs from human bone marrow [7, 10, 11]. Previous studies from other groups defined CD45-CD34-CD105+ or CD90+ cells as circulating MSCs [22, 23], but CD105 and CD90 are not specific antigen markers for MSCs. To the best of our knowledge, this is the first study to track MSCs in human peripheral and cord blood on the basis of CD271 expression.
Data from many laboratories indicate that BM stem/progenitor cells participate in the repair of most, if not all, major organ systems. BM progenitors have been shown to actively replace not only differentiated cells but also stem-like cells from damaged tissues in the process of repair [24–26]. In other instances, tissue repair by BM progenitors occurs by virtue of the ability of BM progenitors to secrete a wide variety of cytokines and growth factors that modulate the injury microenvironment and improve healing [6, 7, 9, 27–29].

Acute MI induces a generalized inflammatory response manifested by increased chemokine levels in plasma, and then HSCs and endothelial progenitors mobilize in response [14–17]. In other instances, tissue repair by BM progenitors occurs by virtue of the ability of BM progenitors to secrete a wide variety of cytokines and growth factors that modulate the injury microenvironment and improve healing [6, 7, 9, 27–29].

Acute MI induces a generalized inflammatory response manifested by increased chemokine levels in plasma, and then HSCs and endothelial progenitors mobilize in response [14–17]. According to our present findings, the recruitment of MSCs into the peripheral circulation after acute MI took place earlier than the HSC mobilization. The cell counts peaked at 3 days after the infarction, and the peak numbers were correlated with the peak CK concentrations. The findings from our present clinical study are clearly of no value in clarifying the potential mechanisms by which the mobilized MSCs contribute to cardiac repair. We can surmise, however, that the MSCs are likely to participate in the intrinsic reparative process after acute MI in response to the degree of myocardial damage. Our in vitro study showed that cultured human MSCs from bone marrow CD271+ cells differentiated into vascular cell-like cells and, besides exhibiting differentiation potential, the MSCs secrete a cocktail of several angiogenic cytokines [7]. In fact, the secreted factors from CD271+ MSCs increased vessel numbers in mice after acute MI. Moreover, our group demonstrated, in earlier experiments with a porcine MI model, that cultured MSCs injected via the coronary vein promoted neovascularization and preserved cardiac function via endothelial differentiation and secretion of angiogenic factors [29]. On this basis, we speculate that the mobilized MSCs and hematopoietic/endothelial progenitors both migrate into the heart and induce vasculogenesis/angiogenesis after ischemic injury. As a fibroblastic feature, the MSCs may also contribute to granulation formation during the healing process after acute MI.

The number of circulating HSCs was lower in the elderly than in the young healthy subjects, whereas the number of circulating MSCs was positively correlated with age. Circulating MSCs may have a physiologic role in the maintenance of tissue integrity during aging or a pathologic role in the development of age-associated sequelae.

Although peripheral blood appears to be an attractive alternative cell source for MSCs, there are considerable difficulties in isolating and expanding the MSCs from blood [30]. We had no success in our attempts to culture and propagate peripheral blood CD271+ cells over a long time period under a conventional MSC condition. The poor MSC yield may have been partly attributable to the markedly low cell count in the blood. Further

**Figure 4.** Quantitative analysis by flow cytometry for mesenchymal stem/progenitor cells (MSCs) in human peripheral blood after acute myocardial infarction. Shown is distinct mobilization of circulating MSCs from hematopoietic stem/progenitor cells. Measurements are given of CD45low/CD34+/CD271+ cell counts (per milliliter) and proportion of CD271+ fraction in CD45low/CD34+ cells (%). Patients with sCAD, n = 7; patients with AMI, n = 15. *p < .05. Abbreviations: AMI, acute myocardial infarction; d, day; sCAD, stable coronary artery disease.

**Figure 5.** Correlation between the numbers of circulating progenitors and peak CK concentrations after acute myocardial infarction. (A): Positive correlation between mesenchymal stem/progenitor cell (CD45low/CD34+/CD271+) counts at day 3 and peak CK concentrations (r = 0.67, p = .006). (B): No correlation of total CD45low/CD34+ cells at day 3 with CK levels (p = .15). No correlation of hematopoietic stem/progenitor cell counts at each time point with the peak CK levels (data not shown). Abbreviation: CK, creatine kinase.
studies to optimize the culture condition will clearly be necessary for the MSC expansion.

**CONCLUSION**

We identified CD45<sup>-</sup>/lowCD34<sup>+</sup>/CD271<sup>+</sup> MSCs in adult peripheral blood in humans, albeit at low frequency. The differential mobilization of the circulating MSCs from HSCs suggests that the cells may play a specific role in intrinsic repair processes during aging and after acute myocardial infarction. Enhancing mobilization of circulating MSCs may become a new therapeutic strategy for such conditions.

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**AUTHOR CONTRIBUTIONS**

Y.I.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; S.Y., T.S., C.N.P., and S.K.: collection and assembly of data, final approval of manuscript; K.I. and M.M.: provision of study material or patients, final approval of manuscript; Y.K. and Y.T.: financial support, administrative support, final approval of manuscript; J.L.S.: provision of study material, manuscript writing, final approval of manuscript; H.S.: conception and design, financial support, administrative support, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

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**Mobilization of Mesenchymal Stem Cells**