Molecular Cardiology

CD34-Positive Cells Exhibit Increased Potency and Safety for Therapeutic Neovascularization After Myocardial Infarction Compared With Total Mononuclear Cells

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Background—We compared the therapeutic potential of purified mobilized human CD34+ cells with that of mobilized total mononuclear cells (tMNCs) for the preservation/recovery of myocardial tissue integrity and function after myocardial infarction (MI).

Methods and Results—CD34+ cells were purified from peripheral blood tMNCs of healthy volunteers by magnetic cell sorting after a 5-day administration of granulocyte colony-stimulating factor. Phosphate-buffered saline (PBS), 5×10^5 CD34+ cells/kg, 5×10^6 tMNCs/kg (low-dose MNCs [loMNCs]), or a higher dose of tMNCs (hiMNCs) containing 5×10^6 CD34+ cells/kg was transplanted intramyocardially 10 minutes after the induction of MI in athymic nude rats. Hematoxylin and eosin staining revealed that moderate to severe hemorrhagic MI on day 3 was more frequent in the hiMNC group than in the PBS and CD34+ cell groups. Immunostaining for human-specific CD45 revealed abundant distribution of hematopoietic/inflammatory cells derived from transplanted cells in the ischemic myocardium of the hiMNC group. Capillary density on day 28 was significantly greater in the CD34+ cell group (721.1±19.9 per 1 mm²) than in the PBS, loMNC, and hiMNC groups (384.7±11.0, 372.5±14.1, and 497.5±24.0 per 1 mm²) (P<0.01). Percent fibrosis area on day 28 was less in the CD34+ cell group (15.6±0.9%) than in the PBS, loMNC, and hiMNC groups (26.3±1.2%, 27.5±1.8%, and 22.2±1.8%) (P<0.05). Echocardiographic fractional shortening on day 28 was significantly higher in the CD34+ cell group (30.3±0.9%) than in the PBS, loMNC, and hiMNC groups (22.7±1.5%, 23.4±1.1%, and 24.9±1.7%) (P<0.05). Echocardiographic regional wall motion score was better preserved in the CD34+ cell group (21.8±0.5) than in the PBS, loMNC, and hiMNC groups (25.4±0.4, 24.9±0.4, and 24.1±0.6) (P<0.05).

Conclusions—CD34+ cells exhibit superior efficacy for preserving myocardial integrity and function after MI than unselected circulating MNCs. (Circulation. 2006;114:3154-3162)

Key Words: angiogenesis ■ endothelium ■ ischemia ■ progenitor cells ■ stem cells

Since endothelial progenitor cells (EPCs) were identified as circulating CD34 antigen–positive mononuclear cells,1 the therapeutic potential of purified EPCs or total (unpurified) mononuclear cells (tMNCs) containing both EPC and non-EPC fractions has been evaluated in many preclinical and clinical studies. Transplantation of purified EPCs augments ischemic neovascularization in mice with hind-limb ischemia,2,3 rats with acute myocardial ischemia,4,5 and swine with ischemic neovascularization in mice with hind-limb ischemia.2,3 Rats with acute myocardial ischemia,4,5 and swine with chronic myocardial ischemia.6 Recent pilot clinical trials also have suggested the therapeutic potential of EPC transplantation in patients with coronary artery disease.7,8 On the other hand, tMNC transplantation has been further reported. Trials using tMNCs have demonstrated their therapeutic efficiency to enhance ischemic neovascularization in animal studies9,10 and human clinical trials.11,12 Although tMNCs consist mainly (>99%) of non-EPCs and contribute to limited vasculogenic volume by EPCs, transplantation of the non-EPC fraction stimulates secretion of angiogenic cytokines in ischemic tissue.10 However, the fate of the non-EPC fraction after transplantation into ischemic sites is not well known. The non-EPC fraction of hematopoietic cells might cause excess inflammation in the ischemic...
tissue. The possibility for non-EPCs to differentiate into undesired lineage cells such as osteoblasts, chondroblasts, fibroblasts, adipocytes, or ectopic myocytes also remains to be clarified.

To the best of our knowledge, no report has compared the therapeutic potential and safety of EPC transplantation with those of tMNCs administration. Accordingly, we performed transplantation of human EPCs compared with tMNCs in a model of rat myocardial infarction (MI) and investigated the effects of these 2 potential cellular therapies for ischemic neovascularization, inhibition of left ventricular (LV) remodeling, and preservation of LV function after acute MI.

**Methods**

These experiments were performed as a part of a pre-IND (investigational new drug) study, which supported a clinical trial of autologous CD34\(^+\) cell transplantation in patients with coronary artery disease. Results of this study were submitted to the US Food and Drug Administration with the clinical protocol, which was approved in November 2003.

**Cell Collection and Isolation**

All procedures were approved by our institutional ethics committees. Peripheral blood tMNCs were obtained from 3 healthy volunteers who underwent leukopheresis after subcutaneous administration of granulocyte colony-stimulating factor (5 \(\mu\)g · kg\(^{-1}\) · d\(^{-1}\)) for 5 days. We performed fluorescent-activated cell sorter analysis to examine the frequency of CD34\(^+\) cells in the tMNCs. CD34\(^+\) cells were isolated from tMNCs with the Isol-300 kit (Baxter, Deerfield, Ill) as an EPC-enriched fraction. Fluorescent-activated cell sorter analysis revealed that the frequency of CD34\(^+\) cells in the tMNCs was 1.7±0.9% and the purity of isolated CD34\(^+\) cells was 90.1±8.1%.

**Induction of Myocardial Ischemia and Cell Transplantation**

All procedures were performed in accordance with the policies of our Institutional Animal Care and Use committees. Female athymic nude rats (Hsd:RH-nu rats, Harlan Sprague Dawley, Indianapolis, Ind) 6 to 8 weeks of age were anesthetized with ketamine hydrochloride (75 mg/kg IP) and xylazine (10 mg/kg IP). Myocardial ischemia was induced by permanently ligating the left anterior descending (LAD) coronary artery under controlled ventilation. Ten minutes after the LAD was ligated, 100 \(\mu\)L phosphate-buffered saline (PBS), 5×10\(^4\) CD34\(^+\) cells/kg, 5×10\(^4\) cells/kg of tMNCs (low-dose MNCs [loMNCs]), or a higher dose of tMNCs (hiMNCs) calculated to contain 5×10\(^5\) CD34\(^+\) cells/kg were injected intramyocardially into 5 sites in the ischemic LAD territory with a 27G needle (20 \(\mu\)L to each site) (n=9 to 11 in each group). All cells were suspended with 100 \(\mu\)L PBS. Cell number of the hiMNC group was determined from the results of the fluorescent-activated cell sorter analysis for CD34 described above. The ischemic zone was macroscopically identified by the pale color of the anterior and lateral walls immediately after LAD ligation. Induction of myocardial ischemia and cell transplantation was performed by experienced researchers who were blinded to treatment assignment.

**Histological Assessment of Transplanted Animals**

Rats were anesthetized with ketamine hydrochloride and xylazine 3 days (n=6 to 8) and 28 days (n=8 to 11) after cell transplantation. Peripheral blood was obtained from the abdominal aorta of each rat for hematological examinations such as blood cell count, hemoglobin, and hematocrit and blood chemical examinations, including blood urea nitrogen, creatinine, alanine transaminase, aspartate transaminase, creatine kinase, lactate dehydrogenase, troponin I, and blood sugar. Immediately after blood collection, rats were killed with an overdose of ketamine hydrochloride. At necropsy, organs, comprising brain, lung, heart, liver, spleen, kidney, and ovary, from each animal were collected, weighed, and fixed with 4% paraformaldehyde. Hearts were also sliced in a bread-loaf fashion into 8 transverse sections from apex to base. In 3 additional rats in each group, heart samples collected on day 3 were similarly sliced, embedded in optimal cutting temperature compound, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen heart samples were similarly obtained on day 28 in 5 additional rats in each group.

Paraffin-embedded tissues of all organs were stained with hematoxylin and eosin to histologically examine adverse events after cell transplantation. Severity of hemorrhagic infarction in ischemic myocardium on day 3 also was evaluated semiquantitatively using the hematoxylin and eosin–stained samples as follows: 0=none, 1=mild, 2=moderate, and 3=severe. Masson-trichrome staining was performed using the paraffin-embedded heart sections obtained 28 days after transplantation to measure the average ratio of fibrosis area to the entire LV area. Histochmical staining for the murine-specific endothelial cell marker isolectin B4 (Vector Laboratories, Burlingame, Calif) was performed using the heart samples obtained 28 days after treatment.

Capillary density was evaluated morphometrically by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. Capillaries were recognized as tubular structures positive for isolectin B4.

Frozen heart samples obtained on day 3 were used for immunohistochemistry with human-specific antibody against CD45 (BD Biosciences, San Jose, Calif) to identify hematopoietic/inflammatory cells derived from transplanted human cells in rat ischemic myocardium. Frozen samples on day 28 were used for immunohistochemistry with antibodies against human nuclear antigen (HNA), cardiac troponin I, and von Willebrand factor (vWF) (all from Chemicon International, Temecula, Calif) to detect cardiomyocytes and endothelial cells derived from transplanted human cells.

All morphometric studies were performed by 2 examiners who were blinded to treatment assignment.

**Physiological Assessment of LV Function**

Transmural echocardiography (SONOS 5500, Phillips Technologies, Bothell, Wash) was performed 28 days after transplantation. Fractional shortening was measured at the middle papillary muscle level. Regional wall motion score was examined by published criteria. All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

**Statistical Analysis**

Results were statistically analyzed with the use of the Statview 5.0 software package (Abacus Concepts Inc, Berkeley, Calif). Severity scores were examined across groups through the use of the Kruskal-Wallis test, followed by the Wilcoxon rank-sum test with the simple Bonferroni method (a value of \(P<0.05\) was considered significant). Exochodigraphic and histological values on day 28 were expressed as mean±SE. Scheffé’s test was performed for the multiple comparisons after analysis of variance between groups. In Scheffé’s test, a value of \(P<0.05\) was considered statistically significant.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Exacerbation of Hemorrhagic MI Is Evident 3 Days After Transplantation of hiMNCs but Not loMNCs and Purified CD34\(^+\) Cells**

The weight of all organs was similar in all groups on days 3 and 28. Hematoxylin and eosin staining for all organs...
except the heart disclosed no abnormal findings on days 3 and 28. Results of hematologic and blood chemical tests were similar in all groups on days 3 and 28 (data not shown).

Hematoxylin and eosin staining of myocardial tissue samples on day 3 revealed that the frequency of moderate to severe hemorrhagic MI was significantly greater in rats receiving hiMNCs compared with the PBS and CD34+ cell groups (hiMNC, 87.5%; n=8; PBS, 33.3%; n=6; CD34+, 12.5%, n=8; P=0.04 versus PBS and P=0.003 versus CD34+ cell group). Frequency of severe hemorrhagic MI also was greater in the hiMNC group than in the CD34+ cell group (hiMNC, 50.0%; CD34+ cell, 0.0%; P=0.02). The severity score of hemorrhagic MI had a tendency to be greater in the hiMNC group (50.0% severe, 37.5% moderate, 12.5% mild, and 0.0% none) than in the PBS group (16.7% severe, 16.7% moderate, 50.0% mild, and 16.7% none) and CD34+ cell group (0.0% severe, 12.5% moderate, 75.0% mild, and 12.5% none); however, these differences were not statistically significant (P=0.04 versus PBS, P=0.01 versus CD34+) because a value of P<0.05/6 was considered significant by Bonferroni’s method. The severity of hemorrhagic infarction was similar in the PBS, loMNC (28.6% severe, 28.6% moderate, 28.6% mild, and 14.3% none; n=7), and CD34+ cell groups (Figure 1a through 1e).

Immunohistochemistry for human-specific CD45 revealed more abundant distribution of human CD45+ cells within the ischemic myocardium of the hiMNC group compared with the CD34+ cell and loMNC groups. The human CD45+ cells were mainly round without a tubular structure, a finding that strongly suggests differentiation of transplanted human cells into hematopoietic/inflammatory cells in the rat ischemic myocardium. Human-specific CD45+ cells were not observed in the PBS group (Figure 1f through 1i).

These results suggest that transplantation of unselected human MNCs may worsen hemorrhagic MI, perhaps via distribution of hematopoietic/inflammatory cells into the acutely ischemic myocardium. This unfavorable phenomenon was not observed after transplantation of loMNCs and CD34+ cells.

Transplanted CD34+ Cells Differentiate More Abundantly Into Cardiomyocytes and Endothelial Cells in the Infarcted Myocardium on Day 28 Compared With Unpurified tMNCs

Double immunostainings for HNA and cardiac troponin I to detect transplanted human cell–derived cardiomyocytes and for HNA and vWF to identify human cell–derived endothelial cells were performed using samples of the infarcted myocardium at day 28. These stainings revealed that double-positive cells for HNA and cardiac troponin I were identified only in rats receiving CD34+ cells but not in the hiMNC, loMNC, and PBS groups (Figure 2a through 2h). Similarly, double-positive cells for HNA and vWF were abundant in the CD34+ cell group and rare in the hiMNC group. The double-positive cells were not observed in the loMNC and PBS groups (Figure 2i through 2p).

These results suggest that purified CD34+ cell transplantation may have more potential for cardiac myoangiogenesis compared with total MNC transfer.

Transplantation of CD34+ Cells Further Augments Ischemic Neovascularization and Inhibits LV Remodeling on Day 28 Compared With That of Unpurified tMNCs

Capillary density 28 days after treatment was significantly greater in the CD34+ cell group (721.1±19.9 per 1 mm2) than in the PBS, loMNC, and hiMNC groups (384.7±11.0, 372.5±14.1, and 497.5±24.0 per 1 mm2, respectively).
Capillary density on day 28 also was significantly greater in the hiMNC group than in the PBS and loMNC groups ($P<0.003$ versus PBS group, $P<0.001$ versus loMNC group). Capillary density on day 28 in the loMNC group was not significantly different from that in the PBS group (Figure 3a through 3e).

The ratio of percent fibrosis area to entire LV area was significantly lower in the CD34$^+$ cell group (15.6±0.9%) than in the PBS, loMNC, and hiMNC groups (26.3±1.2%, 27.5±1.8%, and 22.2±1.8%, respectively) ($P=0.003$ versus PBS group, $P<0.0001$ versus loMNC group, $P=0.02$ versus hiMNC group). This ratio was similar between the PBS, loMNC, and hiMNC groups (Figure 3f through 3j).

Thus, transplantation of hiMNCs significantly augmented ischemic neovascularization; however, transplantation of CD34$^+$ cells enhanced new blood vessel formation to a greater degree than when the same dose of CD34$^+$ cells was administered within an unselected MNC population. Furthermore, only transplantation of CD34$^+$ cells significantly inhibited LV remodeling after MI.

Figure 2. a–h, Representative double immunohistochemistry for HNA (red) and cardiac troponin I (green) in PBS (a, e), loMNC (b, f), hiMNC (c, g), and CD34$^+$ cell (d, h) groups. Blue fluorescence demonstrates 4’6-diamidino-2-phenylindole (DAPI) for nuclear staining. a–d, ×10; e–h, ×40; arrows indicate nuclei of cardiomyocytes expressing HNA (purple, double positive for HNA and DAPI), which indicates differentiation of transplanted human cells into cardiomyocytes. The cardiomyogenic differentiation was identified only in the CD34$^+$ cell group. i–p, Representative double immunostaining for HNA (green) and vWF (red) in the PBS (i, m), loMNC (j, n), hiMNC (k, o), and CD34$^+$ cell (l, p) groups. Blue fluorescence demonstrates DAPI for nuclear staining. i–l, ×10; m–p, ×40; arrows show nuclei of endothelial cells expressing HNA (pale green, double positive for HNA and DAPI), which indicates differentiation of transplanted human cells into endothelial cells. The vasculogenic differentiation was abundantly detected in the CD34$^+$ cell group and rarely in the hiMNC group but not in the loMNC and PBS groups.

Transplantation of CD34$^+$ Cells Preserves LV Function After Myocardial Ischemia

By day 28 after treatment, fractional shortening was significantly higher in the CD34$^+$ cell group (30.3±0.9%) than in the PBS, loMNC, and hiMNC groups (22.7±1.5%, 23.4±1.1%, and 24.9±1.7%, respectively) ($P=0.007$ versus PBS, $P=0.02$ versus loMNC, $P=0.049$ versus hiMNC group). Fractional shortening on day 28 was similar in the PBS, loMNC, and hiMNC groups (Figure 4a and 4b). Regional wall motion score was better preserved in the CD34$^+$ cell group (21.8±0.5) than in the PBS, loMNC, and hiMNC groups (25.4±0.4, 24.9±0.4, and 24.1±0.6, respectively) ($P=0.0004$ versus PBS, $P=0.002$ versus loMNC, $P=0.02$ versus hiMNC group). Regional wall motion score was similar in the PBS, loMNC, and hiMNC groups (Figure 4a and 4c).

Thus, echocardiographic examination performed in the chronic phase after MI suggests that transplantation of...
CD34+ cells may have a favorable impact on the preservation of global and regional LV function. Transplantation of higher doses of unselected tMNCs also had a tendency to preserve LV contractility after MI, but this change was not significant.

Discussion

In the present study, dosages of CD34+ cells and tMNCs were determined on the basis of our previous animal study in anticipation of a future clinical trial. In the previous study6 evaluating intramyocardial transplantation of CD34+ cells into rats with MI, the effective cell dose for ischemic neovascularization and preservation of LV function was $10^5$ cells per rat, which is equivalent to 5 to $7 \times 10^5$ cells/kg. Previous clinical reports in the hematology field indicated that the estimated number of autologous CD34+ cells obtained by single leukapheresis after a 5-day administration of granulocyte colony-stimulating factor is 5 to $10 \times 10^5$ cells/kg. Therefore, we anticipated that transfer of $5 \times 10^5$ CD34+ cells/kg would be both an effective and a clinically realistic dose. To precisely assess the difference of safety and therapeutic potential between purified CD34+ cells and tMNCs, we also included 2 treatment groups of tMNCs: the same total dose of tMNCs (loMNC) as the CD34+ cells ($5 \times 10^5$ cells/kg) and high-dose tMNCs (hiMNC) containing an equivalent dose of CD34+ cells ($5 \times 10^5$ CD34+ cells/kg).

Histological findings in the acute phase of MI (on day 3) revealed that the incidence of moderate to severe hemorrhagic infarction, which is one of the prognostic signs of irreversible myocardial and microvascular damage after MI,16,17 was significantly greater after hiMNC transplantation than PBS or CD34+ cell injection. This unfavorable phenomenon was not observed in the loMNC group. These findings suggest that intramyocardial transplantation of tMNCs into acutely ischemic myocardium may be safe up to $5 \times 10^5$ cells/kg but may worsen hemorrhagic infarction at higher doses ($10^5$ cells/kg). The present findings also indicate that the hemorrhagic issue is not present in the case of CD34+ cell transplantation at a dose of up to $5 \times 10^5$ cells/kg. The exact mechanism of hemorrhagic infarction in the hiMNC group is unknown; however, immunohisto-
Chemical evidence of abundant human-specific CD45+ cells in rat ischemic myocardium suggests that higher numbers of hematopoietic/inflammatory cells derived from transplanted human cells may play a key role in accelerating myocardial damage after MI in the hiMNC group. Recently, we have reported differentiation of transplanted human CD34+ cells into cardiomyocytes and endothelial cells in infarcted myocardium of nude rats at day 28.18 The favorable effects of CD34+ cells were proved not only by immunohistochemistry but also by a molecular approach including fluorescent in situ hybridization. In the present study, a similar regenerative property at the chronic phase was immunohistochemically confirmed in the CD34+ cell group but not in the tMNC and PBS groups. The detailed mechanism of the different outcome in each group is unclear; however, loMNCs contain fewer CD34+ cells, which are beneficial for cardiomyogenesis. In the case of hiMNC transplantation, severe hemorrhage/inflammation in the acute phase may be harmful for survival and differentiation of the transplanted cells in the chronic phase. These results indicate that purified CD34+ cell transplantation may have more potential for cardiac myoangiogenesis in the chronic phase of MI compared with total MNC transfer.

Regarding therapeutic potential of the cell therapy after MI, morphometric analyses revealed superiority of CD34+ cell transplantation to tMNC administration. Capillary density in the ischemic myocardium was significantly greater in the hiMNC group than in the PBS and loMNC groups but was superior in the CD34+ cell group compared with the hiMNC group. LV remodeling evaluated by percent fibrosis area also was significantly reduced in the CD34+ cell group compared with all other groups. Percent fibrosis area in the hiMNC group was similar to that in the PBS and loMNC groups despite significant augmentation of ischemic neovascularization. Echocardiographic examinations also demonstrated significantly better outcomes in terms of preservation of both global and regional LV function only in the CD34+ cell group, not in the loMNC and hiMNC groups. These findings suggest that purified CD34+ cells may have more potency for preservation/recovery of LV structural integrity and function in the chronic phase after MI. Taken together with the results in the acute phase after MI, CD34+ cell transplantation may exhibit increased potency and safety in both the acute and chronic phases after MI for therapeutic neovascularization compared with tMNCs. Transplantation of loMNCs may not have significant efficacy for the histological and physiological recovery from MI in the chronic phase despite safety during the acute phase. Moreover, administration of hiMNCs may not achieve a therapeutic effect in the chronic phase equivalent to that of purified CD34+ cells despite equal dosing of CD34+ cells. The diminished effect of hiMNCs in the chronic phase may relate to increased myocardial damage during the acute phase.

A recent clinical report19 demonstrated that purified CD34+ cells incorporate more efficiently into the ischemic border-zone myocardium after intracoronary infusion compared with unselcted tMNCs. In addition to the recent report in the case of intracoronary cell infusion, the present study may provide important information regarding the superiority of CD34+ cells over tMNCs in terms of safety and efficacy after intramyocardial cell transfer.

The present findings provide additional data supporting the selection of specific cell types for applications in myocardial repair after ischemic injury and serve, along with abundant safety data, as the scientific underpinnings for a human pilot clinical trial. These data underscore one of the advantages of cell-based therapies: the ability to actually test the potency of the proposed therapeutic, ie, the human cells themselves. Existing data have documented the varying potency of cells collected from patients with vascular disease and cardiac risk factors.20 Our findings suggest that within the population of circulating cells, subsets exist that may be safer and more potent for myocardial repair. Further mechanistic data identifying the phenotypic features that define potency will move the field of cell therapy forward.

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Disclosure

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References


CLINICAL PERSPECTIVE

These preclinical studies provide evidence for increased safety and potency of CD34+ cell therapy for treatment of myocardial ischemia and form the basis for a recently completed phase 1/2 clinical trial. The selection of CD34+ cells was originally performed in the setting of stem cell transplantation for reconstitution of hematopoiesis; however, it became apparent that in many settings the unselected mononuclear cell population also was capable of achieving this goal, and the selection procedure was largely abandoned in that context. The present studies were designed to determine whether this was also the case when the CD34+ stem cell was used for neovascularization of ischemic tissue. The data reveal that all parameters of safety and efficacy are significantly improved after intramyocardial transplantation of CD34+ cells compared with treatment with an equal dose (cell number) of unselected mononuclear cells. The same was true when the mononuclear cell dose was adjusted to achieve an equivalent dose of CD34+ cells, suggesting that the unselected cells contain elements that impair the salutary effects of CD34+ cells on myocardial repair. These data provide further evidence that the CD34+ cell is a suitable platform for cell-based ischemic tissue repair and that selected cells offer a safety and potency advantage.
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