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<sup>+</sup> 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<sup>+</sup> 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<sup>5</sup> CD34<sup>+</sup> cells/kg, 5×10<sup>5</sup> tMNCs/kg (low-dose MNCs [loMNCs]), or a higher dose of tMNCs (hiMNCs) containing 5×10<sup>6</sup> CD34<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cell group (721.1±19.9 per 1 mm<sup>2</sup>) than in the PBS, loMNC, and hiMNC groups (384.7±11.0, 372.5±14.1, and 497.5±24.0 per 1 mm<sup>2</sup>) (P<0.01). Percent fibrosis area on day 28 was less in the CD34<sup>+</sup> 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<sup>+</sup> cell group (72.1±19.9%) 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<sup>+</sup> 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<sup>+</sup> cells exhibit superior efficacy for preserving myocardial integrity and function after MI than unselected circulating MNCs. (Circulation. 2006;114:2163-2169.)

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

Since endothelial progenitor cells (EPCs) were identified as circulating CD34 antigen–positive mononuclear cells, thereapeutic 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, and swine with chronic myocardial ischemia. Recent pilot clinical trials also have suggested the therapeutic potential of EPC transplantation in patients with coronary artery disease. On the other hand, tMNC transplantation has been further reported. Trials using tMNCs have demonstrated their therapeutic efficiency to enhance ischemic neovascularization in animal studies and human clinical trials. 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. 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...
induction of myocardial ischemia and cell transplantation above. The ischemic zone was macroscopically identified by the pale
in the fluorescent-activated cell sorter analysis for CD34 described
were determined from the results of
We performed fluorescent-activated cell sorter analysis to examine
induced by permanently ligating the left anterior descending (LAD)
to 8 weeks of age were anesthetized with ketamine hydrochloride (75
were performed in accordance with the policies of our
autologous CD34
with those of 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 (investi-
gational new drug) study, which supported a clinical trial of autologous CD34+ cell transplantation in patients with coronary
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
Periarterial blood tMNCs were obtained from 3 healthy volunteers
were blinded to treatment assignment.

Physiological Assessment of LV Function
Transesophageal echocardiography (SONOS 5500, Phillips Technolo-
the therapeutic potential and safety of EPC transplantation with
tMNCs compared with tMNCs in a model of rat myocardial infarction (MI) and

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. Periarterial blood was obtained from the abdominal aorta of each rat for hemato-
logical examinations such as blood cell count, hemoglo-
bin, and hematocrit and blood chemical examinations, including blood urea nitrogen, creatinine, alanine transaminase, aspartate
transaminase, creatine kinase, lactic 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% paraformalde-
hyde. 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 nitro-
gen, 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 semiquantita-
tively using the hematoxylin and eosin–stained samples as fol-
lows: 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. Histo-
chemical staining for the murine-specific endothelial cell marker
isolectin B4 (Vector Laboratories, Burlingame, Calif)4 was per-
formed using the heart samples obtained 28 days after treatment.

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-mu rats, Harlan Sprague Dawley, Indianapolis, Ind) 6
were 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<0.05/6 was considered statistically
significant). Intergroup comparison of incidence of moderate to
severe hemorrhagic infarction on day 5 was assessed by y2 test (a
value of P<0.05 was considered significant). Echocardiographic and
histological values on day 28 were expressed as mean±SE. Schef-
fe’s test was performed for the multiple comparisons after analysis of
variance between groups. In Scheffe’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 22.5±1.8%, respectively) (P=0.0003 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.

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 (23.3±1.5%, 24.3±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 study 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 7×10^5 cells/kg. Previous clinical reports in the hematology field indicated that the estimated number of autologous CD34+ cells obtained by single leukopheresis after a 5-day administration of granulocyte colony-stimulating factor is 5 to 10×10^5 cells/kg. Therefore, we anticipated that transfer of 5×10^5 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 (5×10^5 cells/kg) and high-dose tMNCs (hiMNC) containing an equivalent dose of CD34+ cells (5×10^5 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, 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 5×10^5 cells/kg but may worsen hemorrhagic infarction at higher doses (10^6 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 5×10^5 cells/kg. The exact mechanism of hemorrhagic infarction in the hiMNC group is unknown; however, immunohisto-
phase. These results indicate that purified CD34
differentiation of the transplanted cells in the chronic
in the acute phase may be harmful for survival and
hiMNC transplantation, severe hemorrhage/inflammation
which are beneficial for cardiomyogenesis. In the case of
favorable effects of CD34
human CD34
Recently, we have reported differentiation of transplanted
ating myocardial damage after MI in the hiMNC group.
transplanted human cells may play a key role in acceler-
numbers of hematopoietic/inflammatory cells derived from
chemical evidence of abundant human-specific CD45+
cells in rat ischemic myocardium suggests that higher
mechanism of the different outcome in each group is
smith, a similar regenerative property at the chronic phase
including fluorescent in situ hybridization. In the present
immunohistochemistry but also by a molecular approach
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
potency of the proposed therapeutic, ie,
myocardial repair. Further mechanistic data identifying the
phenotypic features that define potency will move the field
of cell therapy forward.

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Figure 4. a, Representative M-mode echocardiography 4 weeks
after each treatment. b, c, Echocardiographic fractional shortening
(b) and regional wall motion score (c) in all treatment groups
4 weeks after MI. *P<0.05, **P<0.01, ***P<0.001.

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 exa-
iminations 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, adminis-
tration 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 com-
pared with unselected 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
advantage of cell-based therapies: the ability to actually
test the potency of the proposed therapeutic, ie,
the human cells themselves. Existing data have docu-
mented 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 myoccardial 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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