Identification of Risk Factors Related to Poor Angiogenic Potency of Bone Marrow Cells From Different Patients

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Background—Therapeutic angiogenesis induced by the implantation of autologous bone marrow–derived cells has been used for the treatment of ischemic diseases. However, as the outcomes of cell implantation obviously vary among patients, it is essential to identify patients that would benefit the most from this treatment.

Methods and Results—We collected clinical and laboratory data from 25 patients scheduled to undergo sternotomy for various surgical procedures. Then, we aspirated bone marrow cells from the sternum during the operation and investigated the cell quality in vitro by cultivation, and their angiogenic potency in vivo using an ischemic limb model of mice. The angiogenic potency of bone marrow cells differed among patients. Aging, renal failure, anemia, and high serum levels of triglyceride, C-reactive protein, interleukin-6, and type I collagen cross-linked N-telopeptide (NTX) significantly correlated with poor angiogenic potency of bone marrow cells. We assigned scores to these risk factors, and found a strong correlation between the risk scores of patients and the angiogenic potency of their bone marrow cells ($r = -0.883$, $P < 0.001$). These risk scores can predict the angiogenic potency of bone marrow cells for inducing therapeutic angiogenesis with an accuracy of 80%.

Conclusions—We have identified the risk factors related to poor angiogenic potency of bone marrow cells and developed a new scoring system to predict their angiogenic potency for the treatment of ischemic diseases. Our results may help select patients for this treatment in future clinical trials. (Circulation. 2009;120[suppl 1]:S255–S261.)

Key Words: bone marrow cells ■ angiogenesis ■ risk factor

Experimental studies have found that diabetes and other systemic diseases impair the angiogenic potency of bone marrow cells.$^6$–$^9$ However, the risk factors related to the poor outcome of bone marrow cells for inducing angiogenesis have not yet been completely defined. Thus, it is necessary to identify the risk factors related to poor outcomes and develop a risk score system to predict the angiogenic potency of bone marrow cells. This would eventually help select the best candidates for bone marrow cell therapy.

In this study, we collected clinical and laboratory data from patients with different clinical backgrounds. We then collected samples of their bone marrow and measured the angiogenic potency of the bone marrow cells using an ischemic limb model in mice. Based on the clinical and laboratory data, we identified the risk factors predictive of poor angiogenic potency of bone marrow cells. By establishing a risk score system, we found that the risk scores of patients strongly correlated with the angiogenic potency of their bone marrow cells. This enabled us to predict the angiogenic potency of bone marrow cells in different patients.
cells. Briefly, cells were incubated for 30 minutes at 4°C with c-kit, and CD133 in freshly collected bone marrow mononuclear cells. About 1.35 (0.55/H11015) mononuclear cells were isolated by density gradient centrifugation. Bone Marrow Cells

We aspirated about 15 mL of bone marrow fluid from the sternum, in the operation room, just before sternotomy, which was the procedure. Twenty-five patients with different diseases in our department were enrolled in this study (Table 1). All patients underwent a scheduled operation performed via sternotomy. Written informed consent was obtained preoperatively from every patient. The ethics review board at our university approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki.

Clinical and Laboratory Data of the Patients

We collected all clinical and laboratory data that could probably be related to poor angiogenic potency of bone marrow cells; this included demographics and clinical characteristics, routine laboratory examinations, bone metabolism markers, serum levels of cytokine and growth factors, and other specific examinations (Table 2). The demographics and clinical factors were recorded by doctors. The routine laboratory examinations were performed in our hospital. The bone metabolism markers were measured by SRL (SRL Inc) or by routine laboratory examinations, bone metabolism markers, serum levels of cytokine and growth factors, and other specific examinations (Table 2).

Markers of bone metabolism

Serum NTX (>15 ng/ml) 0.453 0.023 3
Urinary NTX (>20 nmol/BCE/nmolCRE) 0.615 0.004 *
Serum BAP (>25 μl/l) 0.393 0.052 ...
Urinary deoxypyridinoline (>5 nmol/nmolCRE) 0.317 0.173 ...
Bone mineral density (<80%) 0.250 0.306 ...
Serum osteocalcin (>10 ng/ml) 0.115 0.582 ...
Serum 1,25-Dihydroxy vitamin D (<5 pmol/L) 0.091 0.687 ...
Urinary CTX (>300 μg/nmol CRE) 0.085 0.713 ...
Serum PINP (>50 ng/ml) 0.078 0.735 ...
Serum TRACP (>500 μl/ml) 0.052 0.816 ...

Cytokine & growth factors in serum

IL-6 (>10 pg/ml) 0.412 0.041 2
TNF-α (>10 pg/ml) 0.311 0.188 ...
Erythropoietin (>10 mIU/ml) 0.320 0.135 ...
bFGF (>10 pg/ml) 0.255 0.304 ...
IL-1β (>10 pg/ml) 0.234 0.357 ...
Leptin (>4000 pg/ml) 0.168 0.422 ...
SDF-1α (>2000 pg/ml) 0.113 0.590 ...
GM-CSF (>1.0 pg/ml) 0.098 0.630 ...

(Continued)
Table 2. Continued

<table>
<thead>
<tr>
<th>Regression Index</th>
<th>P Value</th>
<th>Risk Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (&gt;200 pg/ml)</td>
<td>0.017</td>
<td>0.935</td>
</tr>
<tr>
<td>PDGF (&gt;4500 pg/ml)</td>
<td>0.005</td>
<td>0.980</td>
</tr>
</tbody>
</table>

Other specific examinations

- CD34+ cells (>0.1% of PB-MNC)
  - 0.432 0.023 †
- CD34+/flk-1 cells (>0.05% of PB-MNC)
  - 0.420 0.031 †
- CD117+ cells (>0.1% of PB-MNC)
  - 0.401 0.050 †
- CD117+/flk-1 cells (>0.05% of PB-MNC)
  - 0.356 0.077 †
- CD133+ cells (>0.02% of PB-MNC)
  - 0.223 0.386 ... |
- VE-cadherin+ cells (>50%)
  - 0.386 0.057 †
- CFU-EC (>5 unit/10⁴ PB-MNCs)
  - 0.350 0.093 †
- Total CFU (>20 units/10⁴ PB-MNCs)
  - 0.305 0.266 ... |
- Urinary 8-OHdG (>10 ng/nmol CRE)
  - 0.304 0.193 ... |
- Flow-mediated vasodilation (>10%)
  - 0.251 0.315 ... |

Risk scores were assigned as 1, 2, 3, or 4 to factors with P values less than 0.10, 0.05, 0.025, and 0.01, respectively. CRE indicates creatinine; BAP, bone specific alkaline phosphatase; CTX, collagen type I cross-linked C-telopeptide; PINP, procollagen type I N-terminal propeptide; NTx, type I collagen cross-linked N-telopeptide; TRACP, tetrat-resistant acid phosphatase; PB-MNCs, peripheral blood mononuclear cells. *The urinary NTX was not scored because the serum NTX was scored, and 6 patients with renal failure had no data on urinary NTX. †These specific examinations were not scored because they are not commonly available and the addition of these factors did not improve the predictive accuracy of the scoring system.

Furthermore, the cell quality was also evaluated by cell survival, the production of angiogenic growth factors, the formation of colonies, and the endothelial differentiation in vitro by culture as described previously. Briefly, bone marrow mononuclear cells from each patient were suspended at a density of 3 × 10⁶ cells per mL in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), and cultured at 37°C. After 3 days of culture, the supernatant was collected and all the cells were harvested. The cell survival rate was calculated by the percentage of total seeded cells on day 0. The concentration of IL-6, IL-1β, TNF-α, VEGF, bFGF, and PDGF in the supernatant was measured with ELISA kits (R&D Systems). The endothelial differentiation was identified by the expression of vascular endothelial (VE)-cadherin (Pharmingen), and the number of colony-forming units (CFU) were counted after 7 days of culture.

Assessment of the Angiogenic Potency of Bone Marrow Cells

Using an acute ischemic hindlimb model in severe combined immunodeficiency (SCID) mice, we quantitatively analyzed the potency of bone marrow cells for inducing therapeutic angiogenesis in vivo. Briefly, an ischemic hindlimb model was created in 12- to 15-week-old mice, as described previously. After the initiation of limb ischemia, bone marrow mononuclear cells of patients were injected intramuscularly at 4 points, with 1 × 10⁶ cells in 10 µL saline at each point. To reduce the individual differences, cells from 1 patient were injected into 4 to 6 mice. Control treatment was also done in another 6 mice with a saline injection only into the ischemic hindlimbs. Blood flow in the ischemic hindlimb was measured using a laser Doppler perfusion imaging system (PeriScan PIM II, Liscia AB) before and 14 and 28 days after treatment, as described previously. Blood flow was expressed as the ratio of ischemic hindlimb to nonischemic hindlimb perfusion. We also recorded the clinical scores of the ischemic hindlimbs in all mice, 14 and 28 days after treatment, as described previously. The mean blood flow and mean clinical score of 4 to 6 mice represented the data of 1 patient, and were used for statistical analysis.

Statistical Analysis

Continuous variables are expressed as means±SD. Spearman correlation coefficient was used to correlate continuous variables. Logistic regression analysis was used to identify the categorical variables, probable risk factors related with impaired angiogenic potency. In the logistic regression analysis, variables showing a difference in the univariate analysis with a probability value of ≤0.1 were considered as risk factors because of the small sample size in this study. To develop a practical risk score, we assigned 1, 2, 3, and 4 points to the risk factors when the probability values were less than 0.10, 0.05, 0.025, and 0.01, respectively. The data were analyzed using the SPSS statistical software (SPSS Inc).

Results

Angiogenic Potency of the Human Bone Marrow Cells in Ischemic Limb Model of Mice

We calculated the mean blood flow and clinical scores of 4 to 6 mice implanted with bone marrow cells from 1 patient. Each circle in all figure represents the mean blood flow of the ischemic limbs in the 4 to 6 SCID mice that received bone marrow cells from 1 patient. The bone marrow cells from different patients achieved different degrees of improvement in regional blood flow and the clinical score of the ischemic hindlimbs of mice, 28 days after cell implantation. We found a significant relationship between the mean blood flow and the mean clinical score of the ischemic limbs 28 days after treatment (Figure 1; P<0.001). Although both blood flow and clinical score in the ischemic limbs of mice are considered to be rational measures of the angiogenic potency of bone marrow cells, we selected blood flow as the primary measure of the angiogenic potency for the following statistical analysis because blood flow can be measured more objectively than clinical scores.
Relationship Between the Quality of Patient’s Bone Marrow Cells and Their Angiogenic Potency in Mice Ischemic Limb Model

The quality of patient’s bone marrow cells was evaluated by cell characteristics and their potency for survival, producing angiogenic factors, forming colonies, and differentiating into endothelial cells in vitro. We found that the blood flow of the ischemic limbs correlated closely with the number of CD34- and CD117-positive stem cells in bone marrow mononuclear cells from the patients (Figure 2A and 2B). Unexpectedly, the data from our in vitro experiments showed that the production of VEGF and PDGF from the bone marrow cells of the patients did not relate to the blood flow in the ischemic limbs of mice (Figure 2C and 2D). However, the production of IL-1β and IL-6 from the bone marrow cells of the patients correlated significantly with the blood flow of the ischemic limbs (Figure 2E and 2F).

The blood flows in the ischemic limbs of mice was also significantly associated with cell survival rate, the number of colony-forming units, and the percentages of VE-cadherin–positive cells after culture (data not shown). These results indicate that the quality of bone marrow cells differed among patients, and high quality of bone marrow cells from patients is important to induce effective therapeutic angiogenesis.

Risk Factors for Poor Angiogenic Potency of Bone Marrow Cells

To identify the risk factors, we screened about 50 factors (Table 2) that could probably be related to the poor angiogenic potency of bone marrow cells. We divided these factors into 5 categories: demographic and clinical factors, routine laboratory examinations, markers of bone metabolism, cytokine and growth factors in serum, and other specific examinations (Table 2). Univariate logistic regression analysis of these categorical variables identified 11 factors that significantly (P<0.10) impaired the improvement of blood flow in the ischemic limbs of mice after the implantation of bone marrow cells from the patients. These included an age >65 years, renal failure, anemia (hemoglobin <12 g/dL), and increased serum levels of C-reactive protein (CRP), triglyceride, type I collagen cross-linked N-telopeptide (NTX), and IL-6 (Table 2). However, diabetes, hypertension, and the serum levels of VEGF and PDGF did not significantly affect the blood flow in the ischemic limbs of mice implanted with the bone marrow cells from the patients (Table 2).

Moreover, statistical analysis using continuous variables also revealed that angiogenic potency of bone marrow cells in ischemic limb of mice was related very well with the age and hemoglobin level of patients (Figure 3).

Development of a Risk Score System

As many factors contributed to the poor angiogenic potency of bone marrow cells, it is difficult to judge the angiogenic potency by a single factor. Therefore, we assigned scores to these risk factors (Table 2). By calculating the total risk score of each patient, we found that the total risk score was closely related to the blood flow in the ischemic limbs of mice implanted with the corresponding individual’s bone marrow cells (r = -0.883, P < 0.001; Figure 4).
To predict the angiogenic potency of bone marrow cells for inducing therapeutic angiogenesis, we measured blood flow in the ischemic hindlimbs of 6 mice 28 days after control treatment with a saline injection, and the blood flow was 57.6±3.1% (the dashed line in Figure 4 shown the mean). We found that the implantation of bone marrow cells from 9 of the 25 patients improved the blood flow of the ischemic limbs by more than 10%, as compared to the control treatment (upper gray area). However, the implantation of bone marrow cells from 6 patients showed less than a 5% increase in blood flow over the control treatment (lower gray area). The blood flow after bone marrow cell implantation from the remaining 10 patients increased by 5% to 10% over that of the control treatment (gray area). Considering these variations, we classified the implantation of bone marrow cells as having a poor (<5% over that of control treatment), mild (5% to 10% over that of control treatment), and high (>10% over that of control treatment) angiogenic potency (Table 3).

According to our risk scoring criteria and the angiogenic potency definitions (Table 3), the bone marrow cells from all 9 patients with a risk score of 5 points (filled circles in Figure 4) had a high angiogenic potency in 8 patients and a mild angiogenic potency in 1 patient. Conversely, the bone marrow cells from 10 patients with a risk score >10 points (opened circles in Figure 4) had a mild angiogenic potency in 7 patients, a poor angiogenic potency in 2 patients, and a high angiogenic potency in 1 patient. For the remaining 6 patients with a risk score >10 points (dashed line circles in Figure 4), their bone marrow cells had a poor angiogenic potency in 5 patients and a mild angiogenic potency in 1 patient. The total predictive accuracy of our scoring system was up to 80% (Table 3). This suggests that bone marrow cells from patients with a risk score ≤5 points can be expected to have a good outcome for inducing therapeutic angiogenesis, whereas bone marrow cells from patients with a risk score >10 points might not offer sufficient therapeutic angiogenesis.

**Table 3. Cumulative Risk Score of Patients for Predicting the Angiogenic Potency of Their Bone Marrow Cells**

<table>
<thead>
<tr>
<th>Cumulative Risk Scores</th>
<th>Predicted Angiogenic Potency</th>
<th>Observed Angiogenic Potency</th>
<th>Predictive Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10</td>
<td>Poor (increase blood flow &lt;5%)</td>
<td>Poor in 5 patients</td>
<td>83%</td>
</tr>
<tr>
<td>&gt;5 and ≤10</td>
<td>Mild (increase blood flow 5–10%)</td>
<td>Mild in 7 patients</td>
<td>70%</td>
</tr>
<tr>
<td>≤5</td>
<td>High (increase blood flow &gt;10%)</td>
<td>High in 8 patients</td>
<td>89%</td>
</tr>
</tbody>
</table>

Mean: 80%

**Discussion**

Bone marrow–derived (stem) cells are one of the most studied stem cell sources for repairing various injured organs. Furthermore, the clinical application of autologous bone marrow–derived cells has no ethical or immunologic problems; however, many factors, such as aging and systemic diseases, contribute to the functional impairment of bone marrow cells. Indeed, the clinical application of autologous bone marrow cells for the treatment of ischemic heart diseases has a poor outcome in some patients, but the risk factors contributing to the functional impairment of bone marrow cells have not yet been defined.

Using an experimental approach, we screened the factors that probably contribute to the functional impairment of bone marrow cells for inducing therapeutic angiogenesis. We collected bone marrow cells from 25 patients with different backgrounds and estimated their angiogenic potency. The data from our study showed that the angiogenic potency of bone marrow cells had obvious individual differences among the patients. We found that bone marrow cells from patients whose health was compromised by aging or renal failure improved poorly the blood flow of the ischemic limbs in mice. Interestingly, a low hemoglobin level in the patients was found to relate significantly to the poor angiogenic...
potency of their bone marrow cells. As these patients had no signs of acute or chronic blood loss, a low hemoglobin level implicates poor hematopoietic function, which could easily be linked with the functional impairment of bone marrow stem cells.

We also found that increased serum levels of CRP and IL-6 in the patients were related to decreased angiogenic potency of their bone marrow cells. This suggests that systemic inflammation could also induce the functional impairment of bone marrow cells; however, the patient’s serum levels of angiogenic factors, including VEGF, bFGF, PDGF, and SDF-1α, were not related to the angiogenic potency of their bone marrow cells.

Considering the critical role that bone metabolism plays in regulating the niche of hematopoietic stem cells, it was not surprising that many of the markers of bone metabolism in the serum and urine of patients were closely related to the angiogenic potency of their bone marrow cells. Moreover, the angiogenic potency of bone marrow cells from patients was closely related to the number of stem (progenitor) cells in their bone marrow and circulating blood, as reported in previous studies.

In contrast to previous investigations, we could not confirm that the angiogenic potency of bone marrow cells was related to flow-mediated vasodilation (a measure of endothelial function) or the level of urinary 8-OHdG (a marker of systemic oxidative stress) in these patients. Our data also showed that diabetes, ischemic heart diseases, and smoking were not risk factors of poor angiogenic potency of the bone marrow cells, although they were reported to contribute to the decreased number and impaired function of circulating endothelial progenitor cells or bone marrow cells. These discrepancies might be attributable to the different experimental methods among studies because many previous studies used much younger healthy cohort for control, but we found that aging was the most important risk factor contributing to functional impairment of bone marrow stem cells.

Clinical trials on the implantation of autologous bone marrow cells for the treatment of various ischemic diseases have revealed favorable outcomes in some patients. However, we cannot select patients for treatment, because we are still unsure about which patients are most likely to benefit from this treatment. Therefore, the ultimate goal of this study was to predict the angiogenic potency of bone marrow cells, which would enable us to select the best candidates for treatment. Based on the data of our clinical and routine laboratory examinations (Table 2), we developed a simple risk score system to predict the angiogenic potency of autologous bone marrow cells for inducing therapeutic angiogenesis in patients. We found a strong relationship between the risk scores of patients and the angiogenic potency of their bone marrow cells (Figure 4). Furthermore, we divided the potency of bone marrow cells for inducing angiogenesis into “high,” “mild,” and “poor,” according to the improvement in blood flow in an ischemic limb model. Using our risk scoring criteria and the angiogenic potency definitions (Table 3), bone marrow cells from patients with a risk score of ≤5 points can be expected to have good angiogenic potency, that from patients with a risk score >5 and ≤10 points with mild angiogenic potency, and that from patients with a risk score >10 points with poor angiogenic potency.

Although we have identified the risk factors and developed a simple score system for predicting the angiogenic potency of bone marrow cells in patients, this study has several limitations: First, we estimated the angiogenic potency of the patients’ bone marrow cells using an ischemic limb model of SCID mice and by in vitro investigations. However, the mechanism of the induction of therapeutic angiogenesis by bone marrow cells is complex. Beyond the quality of the bone marrow cells used for implantation, the conditions of the targeted ischemic tissue, including endothelial function and the levels of growth factors, can also be important. It would be impossible to reproduce these differences among patients in our ischemic limb model in mice. Second, the small sample size of this study did not allow us to define completely the risk factors related to the functional impairment of bone marrow cells. Furthermore, these risk factors were identified from more than 50 parameters by univariate analysis. However, by multivariable analysis, only age, hemoglobin, and circulating CD34+ cells are independent factors (P<0.05) associated with blood flow of ischemic limb of mice. If we do multivariable analysis or only include parameters with P<0.05 for scoring, the relationship between risk score and blood flow of ischemic limb in mice will be decreased, which also results in a worse predictability of angiogenic potency. Third, the predictability of our risk score system warrants verification in clinical trials of autologous bone marrow cells for the treatment of ischemic diseases, although a simulation test showed a very high (80%) predictive accuracy of our scoring system (Table 3).

In summary, using an experimental approach, we screened and identified the risk factors contributing to poor angiogenic potency of human bone marrow cells. By developing a simple scoring system to evaluate these risk factors, we could predict their angiogenic potency for the treatment of ischemic diseases. These results would help select patients who may be more likely to benefit from this treatment in future clinical trials.

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Disclosures
None.

References


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