Mobilized Endothelial Progenitor Cells by Granulocyte-Macrophage Colony-Stimulating Factor Accelerate Reendothelialization and Reduce Vascular Inflammation After Intravascular Radiation

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Background—Endothelial progenitor cells (EPCs) play a pivotal role in repair and regeneration of damaged vessels. We investigated the role of mobilized EPCs in the healing process after intravascular radiation therapy.

Methods and Results—One iliac artery of hypercholesterolemic rabbits was subjected to balloon injury and intravascular radiation with a Re-188 balloon and the contralateral iliac artery to balloon injury only. Rabbits received granulocyte-macrophage colony-stimulating factor (recombinant human GM-CSF) (60 μg/d subcutaneously) daily for 1 week, either 7 days before the angioplasty or at the time of angioplasty. Control rabbits received human albumin. GM-CSF significantly increased the double-positive (CD31+ and KDR+) fraction in peripheral blood monocytes and showed a higher number of EPCs than albumin after culture and, furthermore, enhanced migration and incorporation of EPCs. In the albumin group, intravascular radiation therapy reduced neointimal hyperplasia but delayed reendothelialization and aggravated monocyte infiltration. GM-CSF treatment significantly accelerated the reendothelialization and inhibited monocyte infiltration (reendothelialization index, 81±13% in the GM-CSF radiation [n=7] versus 30±11% in the control radiation [n=9] at 2 weeks, P<0.01). GM-CSF treatment produced an additional significant reduction in neointimal formation at 14 and 28 days after injury in the intravascular radiation groups (intima to media ratio, 0.14±0.11 in the GM-CSF radiation [n=5] versus 0.36±0.07 in the control radiation [n=5] at 4 weeks, P<0.01).

Conclusions—GM-CSF treatment mobilizes EPCs, accelerates reendothelialization, and reduces monocytes infiltration after intravascular radiation therapy, suggesting that stem cell mobilization is a promising strategy for enhancing the vascular healing process after cytotoxic angioplasty. (Circulation. 2003;108:2918-2925.)

Key Words: endothelium ■ angioplasty ■ restenosis ■ radioisotopes
regeneration after intravascular radiation and inhibit the infiltration of inflammatory cells, leading to an additional reduction in neointimal formation.

Methods

Animal Experiment and Intravascular Radiation
Male New Zealand White rabbits (3 to 3.5 kg; Yonam Laboratory Animals, Cheonan, Korea) received a 1% cholesterol diet (Oriental East Co) for 2 weeks before arterial injury. Balloon injury was administered to bilateral iliac arteries using a 3.0×30-mm balloon, as previously reported.6 After baseline overstretch balloon injury, unilateral iliac arteries were treated with intravascular radiation with Re-188-DTPA/contrast-filled balloon. The target dose on the arterial segment was 18 Gy at a depth of 1 mm from the balloon-arterial interface. The GM-CSF pretreatment group (n=15) received recombinant human GM-CSF (60 μg/d injected subcutaneously, LG Chemical Co) daily for 1 week, beginning 7 days before the procedure. The control group (n=18) received subcutaneous injections of human albumin. Another experimental group received GM-CSF treatment immediately after injury for 7 days, whereas controls received albumin. (n=5, GM-CSF group; n=5, albumin group). Animals were killed at 3 and 14 days after injury in the GM-CSF pretreatment group and at 28 days after injury in the GM-CSF simultaneous injury group to assess vessel morphometry and histology. This study was approved by the Experimental Animal Committee of Clinical Research Institute, Seoul National University Hospital.

Vessel Morphometric and Immunohistochemical Analyses
Arteries were stained with H&E and elastic fiber staining and subjected to immunohistochemistry for the detection of macrophage (RAM-11, DAKO) and endothelium (CD31, DAKO). In addition to CD31 staining, scanning electron microscopy was performed to evaluate endothelial recovery and morphology. Samples for electron microscopy were fixed in glutaraldehyde and processed using standard technique.9 Morphometric analysis was performed with a computerized digital image analysis system (DP50 digital camera and Image Pro version 4.5, MediaCybernetics) by a single observer who was blinded to prior treatment as previously described.10 The neointimal to media ratio was calculated as the ratio of the neointimal area to the media area, which was determined by computerized image analysis (Tamas; Uniscan, Ltd). Endothelial and smooth muscle cell layers were distinguished by elastic fiber staining and trichrome staining, respectively. To assess in vivo EPC incorporation into injured arteries, we performed dual staining of mononuclear cells with EPC markers and H&E or elastic fiber staining. The total number of adherent cells was counted in each well. The neointimal to media ratio was calculated as the ratio of the neointimal area to the media area. The neointimal area was determined by computerized image analysis (Tamas; Uniscan, Ltd).

Statistical Analysis
All data are presented as mean±SD. Continuous variables were compared using the Student t test and the Mann-Whitney U test, and

<table>
<thead>
<tr>
<th>14 Days</th>
<th>Control Group (n=9)</th>
<th>GM-CSF Group (n=7), Pretreatment</th>
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</thead>
<tbody>
<tr>
<td>Lumen, mm²</td>
<td>1.09±0.49</td>
<td>1.24±0.65</td>
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<tr>
<td>Neointima, mm²</td>
<td>0.27±0.11*</td>
<td>0.09±0.03</td>
</tr>
<tr>
<td>Media, mm²</td>
<td>0.44±0.11</td>
<td>0.46±0.14</td>
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<tr>
<td>I/M ratio</td>
<td>0.61±0.23*</td>
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<table>
<thead>
<tr>
<th>28 Days</th>
<th>Control Group (n=5)</th>
<th>GM-CSF Group (n=5), Simultaneous Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumen, mm²</td>
<td>0.67±0.27</td>
<td>0.91±0.24</td>
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<tr>
<td>Neointima, mm²</td>
<td>0.52±0.18§</td>
<td>0.25±0.10</td>
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<tr>
<td>Media, mm²</td>
<td>0.49±0.24</td>
<td>0.70±0.29</td>
</tr>
<tr>
<td>I/M ratio</td>
<td>1.12±0.35*</td>
<td>0.36±0.07</td>
</tr>
</tbody>
</table>

Values are mean±SD. I/M indicates neointima to media ratio.
*P<0.01 vs control radiation.
†P<0.01 vs GM-CSF radiation.
§P<0.05 vs control radiation.
||P<0.05 vs GM-CSF radiation.

Fluorescence-Activated Cell Sorting Analysis and Rabbit EPC Culture
Peripheral blood mononuclear cells (PB-MNCs) were isolated by Histopaque-1083 (Sigma) density gradient centrifugation. FACS was used to detect the expression of cell-surface antigens for endothelial lineage in peripheral blood, as previously described.11,12 Primary antibodies to CD31 (DAKO) and CD34 (BD-PharMingen) were used with secondary detection using an FITC-conjugated antibody (Becton-Dickinson) in each case. We performed also dual staining with PE- and FITC-labeled anti-CD31 (Becton-Dickinson) and KDR (Sigma) antibodies. PB-MNCs were cultured in EGM-2 media (Clonetics) with 5% FBS on gelatin-coated plates up to 4 weeks. Cells were assayed by staining with DiI/acLDL (Molecular Probes) and FITC-conjugated BS-1 lectin (Sigma) at 2 and 4 weeks, as previously described.12,13

EPC Migration Assay (Scratch-Wound Assay)
After 3 weeks culture, in vitro scratched wounds were created by scraping cell monolayers with a sterile disposable rubber policeman, as previously described.14,15 The remaining cells were washed with medium (EBM, Clonetics) and incubated with or without GM-CSF (0.1 and 10 ng/mL) over 12, 24, and 48 hours. EPC migration into denuded area was quantified with a computerized digital image analysis system.

EPC Incorporation
For in vitro analysis of incorporation, DiI-labeled (Molecular Probes) cultured EPCs were incubated on a monolayer of HUVECs with or without GM-CSF (0.1 and 10 ng/mL). Five hours after incubation, nonadherent cells were removed by washing with PBS.16 The total number of adhesive EPCs in each well was counted in a blinded manner under a 100 magnification field of a fluorescent microscope. To assess in vivo EPC incorporation into injured arteries, 3×10⁶ cultured EPCs were infused systemically to balloon-injured rabbit.
multiple comparisons were performed using the Kruskal-Wallis test and ANOVA with a Bonferroni correction using SPSS 10.0. P<0.05 was considered significant.

**Results**

**Serum Cholesterol Level and Hematologic Data**
After 2 weeks on a 1% cholesterol diet, the baseline serum total cholesterol levels were similar in the albumin and the GM-CSF groups (824±165 versus 851±197 mg/dL, P=0.7), and no significant difference in the number of total white blood cells (6224±2177 versus 5691±1885/μL, P=0.6), red blood cells, or platelets was observed after administering human albumin or GM-CSF for 7 days. The total white blood cell count was unchanged by injury.

**Effect of Radiation on Neointimal Proliferation, Endothelial Regeneration, and Macrophage Infiltration**
Morphometric analysis at 14 and 28 days showed that intravascular radiation after balloon injury significantly reduced neointimal thickening and the intima to media ratio compared with balloon injury alone (Table). However, radiation aggravated the infiltration of foam cells derived from macrophages (Figure 1). Such macrophage infiltration was associated with the delayed endothelial regeneration after intravascular radiation, as shown in Figures 2A and 2B.

**Effect of GM-CSF Injection on Neointimal Proliferation and Macrophage Infiltration**
As shown in Figure 1B, GM-CSF pretreatment reduced foam cell infiltration that was observed after radiation therapy. Furthermore, pretreatment or simultaneous treatment with GM-CSF potentiated the efficacy of radiation therapy, showing additional reduction of neointimal formation or intima to media ratio at both 14 and 28 days after injury (Table and Figure 1). In the balloon injury alone group, the effect of GM-CSF was neither harmful nor beneficial (Figure 1 and Table). At 28 days after injury, intimal hemorrhage and fibrin deposition were observed in irradiated arteries of control animals but not in animals that received GM-CSF (Figure 1C).
Effect of GM-CSF on Reendothelialization

Reendothelialization indices were determined by CD31 immunohistochemical staining and scanning electron microscopy (Figure 2). Reendothelialization after radiation therapy was significantly accelerated by GM-CSF compared with albumin treatment at 14 days in the pretreatment regimen and at 28 days in the simultaneous treatment regimen. This difference was apparent as early as 3 days after radiation therapy (51±12% versus 34±11%, GM-CSF [n=5] versus albumin [n=7], respectively, P<0.05). The effect of GM-CSF to enhance the endothelial regeneration was notable only after intravascular radiation therapy, when endothelial recovery was seriously impaired, and it had no detectable effect in the balloon injury alone condition, when endothelial regeneration had not been impaired (Figure 2).

GM-CSF Increases Endothelial Progenitor Cell Mobilization and Growth

To demonstrate that GM-CSF increases the number of EPCs, PB-MNCs were isolated for FACS analysis and culture assay. FACS analysis showed that the CD31-positive and CD34-positive fractions were greater in the GM-CSF than albumin group. These data were corroborated by dual FACS analysis with KDR as an additional endothelial marker (Figure 3A).
PB-MNCs from GM-CSF and control rabbits were cultured for 4 weeks in EPC growth media. EPCs, defined as double-positive cells showing the phenotypes of DiI/acLDL uptake and FITC-conjugated BS1-lectin binding, were quantified (Figures 3B and 3C). The number of EPCs was significantly greater in PB-MNCs from the GM-CSF-treated group than from those treated with albumin (33/1100615 versus 6/110064/mm2, P<0.05 at 2 weeks; 446/11006101 versus 58/1100629/mm2, P<0.01 at 4 weeks; n=5).

Effect of GM-CSF on Endothelial Progenitor Cell Migration
Migration was assessed by scratch-wound assay for EPCs that were derived from GM-CSF–treated or albumin-treated rabbit and cultured for 3 weeks (Figure 4A). There was no significant difference of migration distances between 2 groups of EPCs at 3 weeks in vitro culture when GM-CSF was not present (Figure 4B). However, addition of GM-CSF in culture media (0.1 and 10 ng/mL) enhanced EPC migration dose dependently (Figures 4A and 4C). This effect was observed even at low doses of GM-CSF and more apparent at high doses of GM-CSF (GM-CSF 10 ng, 1.26±0.14 mm; GM-CSF 0.1 ng, 1.18±0.17 mm; and medium only, 0.76±0.13 mm; P<0.01; Figures 4A and 4C).

Incorporation of Endothelial Progenitor Cell
DiI-labeled cultured EPCs were incubated on a monolayer of HUVECs with or without GM-CSF (0.1 and 10 ng/mL). Five hours after incubation, nonadherent cells were gently removed. Incorporation of EPCs with HUVECs under 10 ng/mL GM-CSF exceeded that under GM-CSF 0.1 ng/mL or medium only (Figures 5A and 5B; n=5; GM-CSF 10 ng, 28.2±3.1; GM-CSF 0.1 ng, 19.8±4.7; 15.6±5.9 cells per 100 magnification field of a fluorescent microscope, P<0.05, GM-CSF 10 ng versus medium only).

Finally, to confirm that circulating EPCs home to balloon-injured arterial segment, cultured EPCs were labeled and infused systemically to rabbit after balloon injury. Tissues were harvested at 3 days after infusion of the EPCs and analyzed by fluorescence microscopy. As shown in Figure 5C, DiI-labeled red fluorescent EPCs were homed to the denuded arterial site and incorporated into endothelial regeneration at injured arteries. Labeled EPC incorporation was also observed in the reticuloendothelial system (liver and spleen) but not in uninjured arteries, lung, kidney, and heart.

Discussion
Intravascular irradiation reduces neointimal proliferation after coronary intervention.1–3 However, this procedure is associated with incomplete healing, the infiltration of inflammatory cells, and thrombosis.4,5 Irradiated arteries display poor endothelial regeneration even at 1 month after angioplasty, whereas control arteries with balloon-injury alone have complete regeneration of a monolayer of endothelial cells. Delayed endothelial regeneration and thrombosis require the prolonged administration of antiplatelet agents as
adjuvant therapy for patients who undergo intravascular radiation therapy.17

Recently, statin therapy has been reported to mobilize EPCs, resulting in accelerated endothelial regeneration and suppression of neointimal hyperplasia after balloon-denudation injury in the rat carotid artery.12 Enhanced endothelial regeneration by gene therapy has been also shown to suppress thrombogenic property and neointimal hyperplasia in a rabbit vein graft model.18,19 Cytokines such as GM-CSF have been suggested to exert a potent stimulatory effect on EPC kinetics, and cytokine-induced EPC mobilization has been reported to enhance neovascularization of ischemic tissues.13 However, the effects of cytokine-induced EPC mobilization on the healing process after angioplasty or intravascular radiation therapy has not been examined.

In this study, pretreatment or simultaneous treatment with GM-CSF diminished the adverse effects of intravascular radiation therapy by accelerating reendothelialization. Furthermore, it potentiated the efficacy of radiation therapy to suppress neointimal proliferation in the injured arteries. We hypothesize that under these conditions, EPCs contribute to the early endothelial regeneration and the suppression of inflammation. GM-CSF was shown to mobilize EPCs under these conditions, and EPCs homed to the denuded luminal surface of irradiated arteries. When EPCs were harvested and cultured for 3 weeks in GM-CSF–withdrawn condition, 2 groups of EPCs harvested from GM-CSF–treated or albumin-treated rabbit did not show functional difference in the aspect of migration and incorporation. But the incubation with GM-CSF enhanced migration and incorporation function of EPCs. Taken together, GM-CSF not only increased the number of circulating EPCs from bone marrow but also enhanced their migration and incorporation function.

Figure 4. GM-CSF enhances endothelial progenitor cell migration in vitro. A, EPC migration was enhanced in the presence of GM-CSF in culture medium. Representative images of EPC migration after 48 hours of incubation in scratch-wound assay. B, Migration distances from scratch-wound edges were not significantly different between the 2 groups of EPCs that were harvested from GM-CSF–treated or albumin–treated rabbit and cultured for 3 weeks. (n=5, respectively). C, EPC migration significantly increased under GM-CSF (10 ng/mL) in the medium compared with under 0.1 ng/mL GM-CSF or medium only after 48 hours of incubation, (n=5; *P<0.05, 0.1 ng/mL GM-CSF vs medium; †P<0.01, 10 ng/mL GM-CSF vs medium).
GM-CSF is relatively inexpensive and safe, and it has been used extensively in patients. Therefore, a randomized clinical trial to test the efficacy of GM-CSF after intravascular radiation may be warranted.

In conclusion, our study demonstrates that the cytokine-induced EPC mobilization is a promising strategy to normalize the healing process after angioplasty that may be impaired by the coadministration of cytotoxic or cytostatic agents, including intravascular radiation therapy or drug-eluting stents.

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