Hematopoietic Progenitor Cells and Restenosis After Carotid Endarterectomy

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Methods

—HPC number (CD34+/CD133+ cells), early colony-forming units, migratory capacity, and senescence were measured. Stenosis was assessed by duplex scanning. Results—HPC numbers (P<0.001) and early colony-forming unit count (P=0.001) fell rapidly 24 hours postoperatively. Restenosis at 6 months correlated negatively with the magnitude of postoperative falls in HPC numbers (R=-0.38, P=0.013) and early colony-forming unit counts (R=-0.42, P=0.008). The migratory capacity of preoperative HPCs correlated negatively with restenosis (R=-0.48, P=0.007). Preoperative SDF1 levels correlated with falls in HPC number (R=0.42, P=0.044) and early colony-forming unit counts (R=0.56, P=0.004).

Conclusions—HPC function appears to be linked to the development of carotid artery restenosis after endarterectomy. These data support the concept that HPCs have a role in regulating remodeling of the injured arterial wall. (Stroke. 2012;43:00-00.)

Key Words: carotid endarterectomy ■ hematopoietic progenitors ■ restenosis

Acutal arterial injury caused by angioplasty or endarterectomy denudes the endothelium, which may lead to neointimal hyperplasia.1 Maintaining endothelial integrity and function may attenuate the response to acute and chronic vascular injury. Bone marrow-derived endothelial progenitor cells differentiate into mature endothelial cells and maintain endothelial function.2–4 Enhancing their mobilization after injury promotes re-endothelialization and decreases neointima formation in animal models of arterial injury.2–4 Recent studies suggest, however, that these cells are likely to represent a heterogeneous group of primitive hematopoietic progenitor cells (HPCs) that express both endothelial and myeloid lineage markers.5,6 The response of bone marrow cells to arterial injury appears to depend on the type of injury induced.7 Circulating HPC numbers rise acutely after coronary artery bypass grafting8 and angioplasty9 but fall after carotid endarterectomy (CEA).10 The aim of this study was to determine whether circulating HPC number and function is related to the likelihood of developing restenosis after CEA.

Methods

Patient Recruitment and Sample Collection
Consecutive (symptomatic and asymptomatic) patients presenting for CEA at St Thomas’ Hospital were recruited (46 patients with a mean age of 71±9 years). Blood samples were collected immediately preoperatively, 24 hours, and 6 weeks postoperatively. The degree of restenosis was assessed by duplex scanning at 6 months.

Measurement of progenitor cell numbers and function (detailed methodology in the online-only Data Supplement; http://stroke.ahajournals.org) consisted of: (1) CD133+ve/CD34+ve circulating HPCs were enumerated using flow cytometry; (2) HPC early colony-forming units (ECFUs) were measured after 5 days in culture (ECFU assay); (3) fluorescently labeled HPC migration toward vascular endothelial growth factor was examined using a modified Boyden chamber. Migrated cells were enumerated as percentage increase in fluorescence intensity over the migration in control wells; and (4) senescence β-galactosidase activity in HPCs isolated from the ECFU assay were measured.

Cytokines
Plasma concentrations of vascular endothelial growth factor, SDF1, granulocyte-macrophage colony-stimulating factor, and PLGF were analyzed by multiplex enzyme-linked immunosorbent assay.

Received January 4, 2012; accepted January 23, 2012.
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The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.111.649673/-/DC1.

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Stroke is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.111.649673
Statistical Analyses

Data were tested for normality (SPSS 14.0) and analyzed using t test. Correlations were carried out by Spearman rank or Pearson rank as appropriate.

Results

There was no difference in major clinical variables in symptomatic compared with asymptomatic patients with the exception of the increased prevalence of heart disease in the asymptomatic group (online-only Data Supplement Table I).

HPC and ECFU counts fell by 28% (P<0.001) and 18% (P<0.001) by 24 hours postoperatively. Both counts rose to preoperative levels by 6 weeks (online-only Data Supplement Table II).

The pre- or postoperative HPC or ECFU numbers were not associated with the development of restenosis (online-only Data Supplement Table III). The degree of restenosis was negatively correlated with the percentage acute fall in HPC number (R = -0.38, P=0.013) and ECFU count (R = -0.42, P=0.002) by 24 hours postoperatively. Both counts rose to preoperative levels by 6 weeks (online-only Data Supplement Table II).

The acute fall in HPC numbers (CD133+/CD34+ cells and ECFUs) after CEA, followed by a recovery to preoperative levels at 6 weeks in this report, confirms the work of others but is in contrast with the rise in HPC numbers that occurs after bypass surgery or balloon angioplasty as well as in the acute phase injury after ischemic stroke. It is possible that angioplasty and coronary bypass induce some degree of ischemia that leads to mobilization of HPC numbers, because coronary angioplasty does not promote mobilization of progenitors in the absence of myocardial necrosis. The circle of Willis protects the cerebral circulation from ischemia during CEA and so the main stimulus is that of endothelial damage alone. CEA also results in denudation of an area of endothelium much greater than that seen after angioplasty. We postulate that this may lead to recruitment of HPCs to the site of endarterectomy in an attempt to re-endothelialize this area. In the absence of an ischemic stimulus, this may overwhelm the capacity to mobilize these cells from the bone marrow within the first 24 hours after surgery, leading to a fall in circulating HPC numbers.

In contrast to the finding that patients who develop restenosis after coronary angioplasty have lower circulating HPC numbers and increased senescence, we found no association between circulating HPC numbers, ECFU count, and continued to rise by 6 weeks (P=0.04; online-only Data Supplement Table IV). Preoperative SDF1 levels correlated with postoperative fall in HPC and ECFU numbers (R=0.42, P=0.04 and R=0.56, P=0.004, respectively; Figure 2A–B).

Discussion

The acute fall in HPC numbers (CD133+/CD34+ cells and ECFUs) after CEA, followed by a recovery to preoperative levels at 6 weeks in this report, confirms the work of others but is in contrast with the rise in HPC numbers that occurs after bypass surgery or balloon angioplasty as well as in the acute phase injury after ischemic stroke. It is possible that angioplasty and coronary bypass induce some degree of ischemia that leads to mobilization of HPC numbers, because coronary angioplasty does not promote mobilization of progenitors in the absence of myocardial necrosis. The circle of Willis protects the cerebral circulation from ischemia during CEA and so the main stimulus is that of endothelial damage alone. CEA also results in denudation of an area of endothelium much greater than that seen after angioplasty. We postulate that this may lead to recruitment of HPCs to the site of endarterectomy in an attempt to re-endothelialize this area. In the absence of an ischemic stimulus, this may overwhelm the capacity to mobilize these cells from the bone marrow within the first 24 hours after surgery, leading to a fall in circulating HPC numbers.

In contrast to the finding that patients who develop restenosis after coronary angioplasty have lower circulating HPC numbers and increased senescence, we found no association between circulating HPC numbers, ECFU count,

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**Figure 1.** Relationship between restenosis and postoperative fall in: (A) circulating HPC numbers; (B) ECFU number and at 6 months (n=40, Spearman rank correlation). HPC indicates hematopoietic progenitor cell; ECFU, early colony-forming unit.

**Figure 2.** Relationship between preoperative SDF-1 levels and postoperative change in: (A) HPC numbers; (B) ECFU number (n=24, Spearman correlation). HPC indicates hematopoietic progenitor cell; ECFU, early colony-forming unit.
or HPC senescence and the development of restenosis after CEA. These conflicting data may be the result of the variable use of combinations of markers (including CD31, CD34, vascular endothelial growth factor receptor-2, CD133, cKit, and CD45) that make it difficult to compare studies associating HPCs with various cardiovascular conditions and outcomes. We did, however, find a significant negative correlation between restenosis and the magnitude of the postoperative fall in HPC and ECFU numbers as well as a significant relationship between a reduced incidence of restenosis and a higher functional (migratory) capacity of HPCs. A significant correlation was maintained in symptomatic but not asymptomatic patients. The latter may be the result of a Type II statistical error because of the low patient numbers in this group.

SDF-1α stimulates progenitor cell mobilization, growth, and differentiation. Preoperative SDF-1 levels correlated strongly with the postoperative fall in both circulating HPC and ECFU number. These data, taken together with our earlier results, lead us to speculate that higher preoperative SDF1 leads to increased HPC homing to the site of injury, resulting in a greater postoperative fall in HPC number, enhanced neointimal hyperplasia, and reduced neointima formation. This paradigm merits further investigation to better define the HPC phenotypes using multiple markers and to confirm recruitment of specific phenotypes to the site of endothelial injury in humans. This could be done using autologous HPCs isolated by flow cytometry with clinical-grade antibodies and noninvasive imaging of cells labeled with radiotracers such as indium or technetium.

Sources of Funding
Supported by a clinical fellowship grant from the British Heart Foundation.

Disclosures
None.

References
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Stroke. published online April 17, 2012;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2012/04/17/STROKEAHA.111.649673

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SUPPLEMENTAL MATERIAL

Haematopoietic progenitor cells and restenosis following carotid endarterectomy

METHODS

Measurement of progenitor cell number and function

(i) Flow Cytometry
Cluster of differentiation designation markers, CD34 and CD133, are found on haematopoietic lineage cells, while CD133 is expressed by early progenitor cells(1). Cells that co-express these markers have a macrovascular phenotype in vitro(2) and may therefore have the potential to facilitate re-endothelialisation following vessel injury. Whole blood (0.5ml) was treated with peridinin chlorophyll protein (PerCP) conjugated anti-CD34 (0.5µg/ml, BD, UK) and phycoerythrin (PE) conjugated anti-CD133 (0.33µg/ml, Miltenyi, UK). Fluorescence isotype-matched antibodies were used as controls. The fluorescence intensity from 200,000 mononuclear cells was measured using a flow cytometer (FACScalibur, Becton Dickinson, BD, UK) and analysed using Win MDI software in order to measure the proportion of these cells expressing CD34+/CD133+.

(ii) Early colony forming unit (ECFU) assay
The ECFU assay was carried out as previously described(3). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated, whole blood by Ficoll gradient density centrifugation(4). The cells were placed in fibronectin-coated (10µg/ml, Sigma, UK) 6well plates (BD, UK) at a concentration of 1x10^6 PBMCs per well. Cells were cultured at 37°C and 5% CO2 in endothelial basal medium (EBM) 2 supplemented with the Bullet Kit (Clonetics, UK) and 5% foetal calf serum (FCS, Sigma, UK). Colony numbers were counted under phase contrast microscopy after 5 days.

(iii) Migration
HPCs were isolated and cultured as described for the ECFU assay. After 5 days the cells were serum depleted and fluorescently labeled with Calcien AM
(Molecular Probes, UK). The cells were removed from the culture dish using Accutase (Invitrogen, UK) and $3 \times 10^5$ cells (suspended in Dulbecco’s modified eagle medium – DMEM, Cambrex, UK) placed in the top well of a Boyden Chamber(5). Human VEGF solution (50ng/ml recombinant human VEGF, R&D systems diluted in DMEM medium, Cambrex, UK) or control solution (DMEM medium alone) was placed in the bottom well, separated from the top well by a 1µm filter (BD Falcon HTS Fluoroblok). The plate was incubated at 37°C for 4 hours after which the fluorescence in the bottom chamber was quantified using a bottom reading fluorimeter and expressed as a percentage increase over the background migration seen in the control well.

**(iv) Senescence**

HPC senescence was assessed by staining cells isolated from the ECFUassay for senescence-associated β-galactosidase activity(6) (SA-Bgal, Ima-Gene Green Kit, Molecular probes, UK). Endogenous B-galactosidase activity was blocked by incubating cells with cloroquine (300µM in EBM-2 medium) on day 5 of culture. The cells were stained with the fluorogenic β galactosidase substrate, C12FDG (33µM in EBM-2 at pH6.0, Ima-Gene Green Kit), and co-stained with the blue fluorescent nuclear marker, DAPI. Cells were counted under fluorescence microscopy and HPC senescence quantified as the percentage of the total number of cells staining positive for β galactosidase.

**Cytokine measurement**

The mechanism by which progenitor cells are mobilised into the circulation and localise to sites of injury is not fully understood. There are, however, a number of cytokines that are thought to be important in this process including, vascular endothelial growth factor (VEGF)(7), granulocyte macrophage colony stimulating factor (GM-CSF)(8), stromal derived factor-1(SDF-1)(9), and placental growth factor (PIGF)(10). The plasma concentrations of the cytokines VEGF, GM-CSF, SDF-1, and PLGF were therefore analysed by multiplex ELISA (Searchlight, Pierce Biotechnology, UK).
RESULTS

Cells isolated from symptomatic patients developed a significantly higher number of ECFU (18±0.6, n=30) compared with cells from asymptomatic patients (13±0.6, n=16, P<0.001), although circulating CD133+/CD34+ HPC population numbers were also higher (131 (53-429) n=30 vs 84 (32-457), n=16), this did not reach statistical significance (P=0.12).

Supplementary Table 1. Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic (30)</th>
<th>Asymptomatic (16)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>71 ± 8</td>
<td>69 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Male sex</td>
<td>66 %</td>
<td>88 %</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>66 %</td>
<td>81 %</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>17 %</td>
<td>25 %</td>
<td>NS</td>
</tr>
<tr>
<td>Heart disease</td>
<td>33 %</td>
<td>69 %</td>
<td>P=0.005</td>
</tr>
<tr>
<td>Current Smoker</td>
<td>20 %</td>
<td>25 %</td>
<td>NS</td>
</tr>
<tr>
<td>High cholesterol</td>
<td>87 %</td>
<td>100 %</td>
<td>NS</td>
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</tbody>
</table>

Supplementary Table 2. HPC and ECFU number pre, post and 6 weeks post-operatively

<table>
<thead>
<tr>
<th></th>
<th>Pre-op (n=46)</th>
<th>Post-op (n=46)</th>
<th>6 weeks (n=34)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC number median(range)</td>
<td>81(32-457)</td>
<td>55(8-252)</td>
<td>69 (20-436)</td>
<td>P=0.05</td>
</tr>
<tr>
<td>ECFU number Mean(SE)</td>
<td>17(±0.6)</td>
<td>13(±0.6)</td>
<td>16(±0.7)</td>
<td>P=0.02</td>
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</tbody>
</table>
**Supplementary Table 3. Relationship between pre-operative HPC numbers and restenosis**

<table>
<thead>
<tr>
<th></th>
<th>HPC number (median, range)</th>
<th>ECFU number (mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restenosis &lt;50%</td>
<td>102 (32-429) n=28</td>
<td>16±0.6 n=28</td>
</tr>
<tr>
<td>Restenosis ≥50%</td>
<td>122 (34-457) n=12</td>
<td>18±1 n=12</td>
</tr>
<tr>
<td>P value</td>
<td>0.43</td>
<td>0.07</td>
</tr>
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</table>

**Supplementary Table 4. Serum concentrations of GM-CSF, PIGF, SDF-1 and VEGF (pg/ml), pre, post and 6 weeks post-operatively (n=24).**

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Pre-operative</th>
<th>1 day post-operative</th>
<th>6 weeks post-operative</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>458(±1177)</td>
<td>211(±232)</td>
<td>358(±585)</td>
<td>0.54</td>
</tr>
<tr>
<td>PIGF</td>
<td>42(±86)</td>
<td>25(±24)</td>
<td>39(±68)</td>
<td>0.62</td>
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<tr>
<td>SDF-1</td>
<td>951(±312)</td>
<td>763(±270)</td>
<td>924(±262)</td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td>VEGF</td>
<td>43(±51)</td>
<td>52(±70)</td>
<td>112(±160)</td>
<td><strong>0.04</strong></td>
</tr>
</tbody>
</table>
Reference List


(10) Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. FASEB J 2006;20:1495-1497.