Objectives
This study was designed to test whether patients with coronary atherosclerosis have increases in circulating endothelial progenitor cells (EPCs) expressing an osteogenic phenotype.

Background
Increasing evidence indicates a link between bone and the vasculature, and bone marrow and circulating osteogenic cells have been identified by staining for the osteoblastic marker, osteocalcin (OCN). Endothelial progenitor cells contribute to vascular repair, but repair of vascular injury may result in calcification. Using cell surface markers (CD34, CD133, kinase insert domain receptor [KDR]) to identify EPCs, we examined whether patients with coronary atherosclerosis had increases in the percentage of EPCs expressing OCN.

Methods
We studied 72 patients undergoing invasive coronary assessment: control patients (normal coronary arteries and no endothelial dysfunction, n = 21) versus 2 groups with coronary atherosclerosis—early coronary atherosclerosis (normal coronary arteries but with endothelial dysfunction, n = 22) and late coronary atherosclerosis (severe, multivessel coronary artery disease, n = 29). Peripheral blood mononuclear cells were analyzed using flow cytometry.

Results
Compared with control patients, patients with early or late coronary atherosclerosis had significant increases (~2-fold) in the percentage of CD34+/KDR− and CD34+/CD133+/KDR− cells costaining for OCN. Even larger increases were noted in the early and late coronary atherosclerosis patients in the percentage of CD34+/CD133+/KDR− cells costaining for OCN (5- and 2-fold, p < 0.001 and 0.05, respectively).

Conclusions
A higher percentage of EPCs express OCN in patients with coronary atherosclerosis compared with subjects with normal endothelial function and no structural coronary artery disease. These findings have potential implications for the mechanisms of vascular calcification and for the development of novel markers for coronary atherosclerosis. (J Am Coll Cardiol 2008;52:1314–25) © 2008 by the American College of Cardiology Foundation

There is increasing evidence for a link between bone metabolism and the vasculature. Thus, elderly women with osteoporosis (1) or with high bone turnover (2) have increased cardiovascular mortality. In addition, mice with deletion of osteoprotegerin (OPG), which binds and neutralizes the key osteoclastogenic factor, receptor activator of nuclear factor kappa B ligand (RANKL), have not only severe osteoporosis and a marked increase in bone turnover but also diffuse vascular calcification (3). Moreover, it is now recognized that vascular calcification is not simply a dystrophic calcifying process, but rather it involves active bone formation that recapitulates the normal sequence of osteoblast development and activity on bone surfaces (4,5). These findings collectively suggest that changes in bone turnover affect the vasculature, although the possible mechanisms of this are unclear.

Potential candidates for providing a link between bone metabolism and the vasculature are endothelial progenitor cells (EPCs) that reside, at least in part, in bone marrow, are mobilized in response to vascular injury, and contribute to vascular repair (6). Although there is ongoing controversy regarding the true identity of EPCs (6), cell surface expression of CD34, CD133, and vascular endothelial growth factor receptor 2/kinase insert domain receptor (KDR) has been used to define populations of circulating cells that include cells participating in the response to vascular injury (6,7). In addition, recent work has suggested that expression of these markers may be used to classify CD34+ cells as less differentiated (CD34+/CD133+/KDR+) versus relatively more differentiated (CD34+/CD133−/KDR+) EPCs (6–8).
In addition to EPCs, osteoprogenitor cells also reside in the bone marrow, and studies by Friedenstein et al. (9) established almost 40 years ago that the bone marrow stroma contains plastic adherent cells that can give rise to a broad spectrum of fully differentiated connective tissues, including osteoblasts. Concurrent with this work, however, Long et al. (10,11) identified, over a decade ago, a nonadherent population of cells in bone marrow with osteogenic potential. These investigators used flow cytometry and magnetic activated cell sorting with an anti-osteocalcin (OCN) antibody to isolate osteoprogenitor cells that expressed bone-related proteins and were capable of mineralization in vitro (10–13). Reasoning that these nonadherent osteogenic cells likely accessed the peripheral circulation, our group used identical methods to characterize OCN⁺ cells in peripheral blood that expressed osteoblastic markers and were capable of forming mineralized nodules in vitro and bone in vivo (14,15).

Although endothelial and osteoblastic cells have traditionally been believed to derive from distinct progenitor populations, there is increasing evidence for overlap between these lineages. In fact, the STRO-1 antibody, which is widely used to identify mesenchymal stem cells in bone marrow and other tissues, was originally derived by immunizing mice with purified human CD34⁺ cells (16). Moreover, human bone marrow CD34⁺ cells can differentiate into osteoblastic cells capable of forming mineralized nodules in vitro (17), and Tondreau et al. (18) have found that CD133⁺ cells from human bone marrow, umbilical cord blood, or peripheral blood from granulocyte colony-stimulating factor–treated donors are enriched for mesenchymal stem cells capable of differentiating into osteoblasts (18). Recent studies using single cell reverse-transcriptase polymerase chain reaction have also demonstrated that ~20% of human peripheral blood CD34⁺ cells express the messenger ribonucleic acid (mRNA) for OCN (19). In addition, when infused into immunocompromised rats following femur fractures, these cells can localize to the fracture site and differentiate into endothelial cells as well as osteoblasts and enhance fracture healing (19).

Given this potential overlap between endothelial and osteoblastic lineages, in the current study, we tested the hypothesis that there is an increased percentage of circulating EPCs expressing an osteogenic phenotype, as assessed by staining for OCN (10–15), in patients with early or established coronary atherosclerosis. Our findings suggest a novel link between bone metabolism and the vasculature and may also help define new diagnostic approaches for coronary atherosclerosis.

**Methods**

**Study subjects.** The study was approved by the Institutional Review Board of Mayo Foundation, and all study subjects provided written, informed consent. A total of 72 patients who were undergoing coronary angiography and coronary endothelial function testing for clinical purposes, and met the criteria outlined here for inclusion into the 3 study groups, were recruited. Patients with acute coronary syndromes (unstable angina or acute myocardial infarction), heart failure (ejection fraction <50%), or severe renal or liver disease were excluded. The Framingham risk score was calculated as previously described (20). The 3 groups were defined as follows: control, patients without significant structural coronary lesions on angiography (<30% stenosis) and normal endothelial function, as assessed by intracoronary acetylcholine challenge (see the following section) (n = 21); early coronary atherosclerosis (ECA), defined by the absence of significant structural coronary lesions on angiography but with abnormal endothelial function (n = 22); and late coronary atherosclerosis (LCA), defined as patients with severe, multivessel coronary artery disease (CAD) (>50% stenosis in 2 or more major epicardial arteries) (n = 29).

**Coronary angiography and invasive endothelial function testing.** Patients underwent coronary angiography using standard clinical protocols. Arterial blood (20 ml) was drawn from the aortic catheter prior to cardiac catheterization for flow cytometry and biochemical analyses. After diagnostic angiography, subjects without significant structural coronary lesions underwent assessment of endothelium-dependent vasoreactivity, as previously described (21–24). In brief, a Doppler guidewire (Flowire, Volcano Corporation, San Diego, California) within a coronary-infusion catheter (Ultrafuse, SciMed Life System, Maple Grove, Minnesota) was positioned into the mid-portion of the left anterior descending coronary artery. Acetylcholine at increasing concentrations was infused into the left anterior descending coronary artery to assess endothelium-dependent vasoreactivity. Hemodynamic data, Doppler measurements, and a coronary angiogram were obtained after each infusion. Coronary artery diameter was measured by an independent investigator in the segment 5-mm distal to the tip of the Doppler wire using a computer-based image analysis system. Average peak velocity (APV) was derived from
the Doppler flow velocity spectra and coronary blood flow was determined as: \( \pi \) (coronary artery diameter/2) \(^2 \times \) (APV/2). As previously described, microvascular endothelial dysfunction was defined as an increase in coronary blood flow of <50\% and epicardial endothelial dysfunction was defined as a decrease in epicardial coronary artery diameter of >20\% in response to the maximal dose of acetylcholine (10\(^{-4}\) mol/l) (21–24). Endothelium-independent microvascular function was determined by the coronary flow reserve, which is the ratio of the APV at maximal hyperemia (induced by intracoronary adenosine [18 to 60 \( \mu \)g]) to the APV at baseline.

**Flow cytometry.** Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient, and immunofluorescent cell staining was performed using the following fluorescent conjugated antibodies: CD34-PerCP Cy 5.5 (Becton-Dickinson, Franklin Lakes, New Jersey), CD133-phycocerythrin (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and KDR-allophycocyanin (R & D Systems, Minneapolis, Minnesota) and the appropriate isotype controls. In addition, OCN (R & D Systems, Minneapolis, Minnesota) and the appropriate magnetic beads. The cells were then costained (MACS) (AutoMACS, Miltenyi Biotec GmbH) with the appropriate magnetic beads. The cells were then costained (MACS) (AutoMACS, Miltenyi Biotec GmbH) with the appropriate magnetic beads.

**Confocal microscopy.** For confocal microscopy, cells were stained for OCN–fluorescein isothiocyanate and CD34-PerCP Cy 5.5 and isolated by magnetic activated cell sorting (MACS) (AutoMACS, Miltenyi Biotec GmbH) with the appropriate magnetic beads. The cells were then costained with the CD133-phycocerythrin and KDR-allophycocyanin antibodies. Following fixation, the cells were mounted in ProLong Gold antifade reagent with 4,6-diamino-2-phenylindole, DAPI (Invitrogen, Carlsbad, California), to stain the nucleus. Samples were then examined on an LSM510 confocal laser scanning microscope (Carl Zeiss Inc., Maple Grove, Minnesota).

**Cell culture.** The CD34\(^+\) cells isolated by MACS or human bone marrow stromal cells (hMSCs) (Lonza, Basel, Switzerland) were cultured in uncoated plastic culture plates in regular culture media (alpha minimal essential medium, 10\% heat-inactivated fetal bovine serum, 1\% penicillin/streptomycin/amphoterin B [all from GIBCO/Invitrogen Corporation, Carlsbad, California]) containing an additional 5 mmol/l Ca\(^{2+}\) for the first 5 days. Subsequently, the medium was changed to osteoblast differentiation medium (alpha minimal essential medium, 10\% heat-inactivated fetal bovine serum, 1\% penicillin/streptomycin/amphoterin B plus 50 \( \mu \)g/ml ascorbic acid phosphate magnesium salt n-hydrate [Wako, Richmond, Virginia], 10 mmol/l beta-glycerophosphate, 10\(^{-8}\) mol/l 1,25-dihydroxyvitamin D\(_3\), 10\(^{-8}\) mol/l dexamethasone [Sigma-Aldrich, St. Louis, Missouri]) for an additional 7 days. The mineralization assay was done using MACS-sorted CD34\(^+\) cells and unsorted post-Ficoll PBMCNCs, which were cultured in uncoated plastic culture plates in regular culture media for 2 days and then were either maintained in regular culture medium or were changed to regular culture medium containing an additional 2.1 mmol/l Ca\(^{2+}\) for 5 days (days 3 to 7) of culture. Subsequently, the Ca\(^{2+}\) was removed and the cells were cultured in osteoblast differentiation medium (as described before) for an additional 21 days (days 8 to 28) and the presence of mineralization determined by alizarin red and von Kossa staining.

**Assessment of gene expression in CD34\(^+\) cells.** The CD34\(^+\) cells were isolated using MACS, as described earlier. Total ribonucleic acid (RNA) from CD34\(^+\) cells and undifferentiated hMSCs (Lonza) was isolated using spin columns (Micro columns, Qiagen, Valencia, California) followed by a deoxyribonuclease digestion. Because the overall number of the CD34\(^+\) cells was limited and therefore the yield of total RNA was low, we used the WT-OvationPico RNA amplification system (NuGEN Technologies Inc., San Carlos, California) to synthesize micrograms quantities of amplified complementary deoxyribonucleic acid starting with total RNA input amounts of 50 ng for all the samples. In this linear amplification system, the relative representation of each transcript species in the original sample is maintained during and after amplification. The pre-amplified complementary deoxyribonucleic acid was then used in quantitative polymerase chain reaction analyses using primer pairs for a panel of bone- and stem cell–related genes (Table 1). Normalization of the samples was performed using the geNorm method (25,26), which determines the most stable housekeeping genes within the experiment. Further analysis was performed by calculating the difference in cycle threshold (\( \Delta CT \)) values between the sample and the geometric mean cycle threshold of geNorm selected housekeeping genes. The expression level for each individual gene was determined by 2\(^{-\Delta CT} \).

**Biochemical assays.** Serum lipids were measured using enzymatic colorimetry and low-density lipoprotein cholesterol calculated from these parameters. High sensitivity C-reactive protein (hs-CRP) levels were measured using a latex particle–enhanced immunoturbidimetric assay on a Hitachi 912 automated analyzer (Hitachi America Ltd., Tarrytown, New York). Serum creatinine was measured using an enzymatic colorimetric assay (Roche Diagnostics, Indianapolis, Indiana) and glomerular filtration rate (GFR) was estimated using the isotope dilution–mass spectrometer traceable MDRD (Modification of Diet in Renal Disease) study equation: estimated GFR (ml/min/1.73 m\(^2\)) = 175 \times serum creatinine (−1.154) \times age (−0.203) \times (0.742 if female) \times (1.210 if African American).

The RANKL was measured using the ampli-sRANKL enzyme-linked immunosorbent assay (ELISA) (ALPCO Diagnostics, Salem, New Hampshire). Ampli-sRANKL has increased sensitivity compared with standard ELISA kits, because it uses a reduced nicotinamide adenine dinu-
cleotide phosphate-based enhancement system. Osteoprotegerin and matrix Gla protein (MGP) were also measured using ELISAs (ALPCO Diagnostics). The interassay coefficients of variation for the RANKL, OPG, and MGP ELISAs were 6%, 8%, and 9%, respectively.

**Statistical analyses.** Pre-specified comparisons between the ECA or LCA patients and control subjects were made using the Wilcoxon rank sum test for continuous variables and the Fisher exact test for categorical variables. Because some of these data were not normally distributed, they are presented as median (interquartile [25th to 75th percentile] range). Spearman correlations were used to describe relationships between the cell populations with circulating RANKL, OPG, and MGP levels. Logistic regression models following logarithmic (base 2) transformation, where appropriate, of the CD34+/CD133−/KDR+/OCN+ and the percentage of CD34+/CD133−/KDR+ cells costaining for OCN or of hs-CRP were used to assess the ability of these variables to predict ECA following adjustment for age and gender. Model assumptions and conclusions were checked. Receiver-operator characteristic (ROC) curve analysis was used to describe the ability of the CD34+/CD133−/KDR−/OCN+ and the percentage of CD34+/CD133−/KDR+ cells costaining for OCN or of hs-CRP to predict ECA, and the areas under the ROCs were compared using a nonparametric test (27). The gene expression data are presented as mean ± standard error of the mean. For comparison with hMSCs, the expression of each gene was normalized to the level of expression in the hMSCs and compared with 1.0 using a 1-sample t test. Differences between freshly isolated and cultured CD34+ cells were analyzed using a 2-sample t test. Any p values <0.05 were considered statistically significant.

**Results**

**Patient characteristics.** The relevant clinical and biochemical data as well as the detailed cardiac catheterization data in the study subjects are shown in Table 2. Subjects with LCA were significantly older than the control subjects and were predominantly male. However, subjects with ECA had an age and gender mix not significantly different from the control subjects. Body mass index was similar across groups, but a higher percentage of the LCA subjects were on statins compared with control subjects. The prevalence of diabetes, hypertension, and smoking was virtually identical in the control and ECA subjects, but it was higher for OCN+ than those of the LCA compared with the control subjects. There was no significant difference in the Framingham score between the subjects with ECA and control subjects, but the score was significantly higher in the subjects with LCA as compared with the control subjects. Blood lipids were similar in the ECA subjects compared with the control subjects, with the LCA subjects having lower total and high-density lipoprotein cholesterol levels compared with the control subjects. There was a nonsignificant trend for hs-CRP levels to be higher in the ECA compared with control subjects; by contrast, patients with LCA had hs-CRP levels that were significantly lower than those of control subjects. Because statin therapy is known to reduce CRP levels (28), the lower hs-CRP levels in the LCA patients were likely related to the fact that most of these patients were on statin therapy. Overall, therefore, although there were significant differences in the clinical characteristics of the LCA patients compared with subjects with normal coronary endothelial function, the patients with coronary endothelial dysfunction had clinical and biochemical features that were virtually identical to the control subjects.

<table>
<thead>
<tr>
<th>Table 1 Gene Identification and Primer Sequences</th>
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<tr>
<td><strong>Gene</strong></td>
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<tr>
<td>Runx2</td>
</tr>
<tr>
<td>Beta catenin</td>
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<tr>
<td>BMP2</td>
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<tr>
<td>Msi-2</td>
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<tr>
<td>Notch1</td>
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<td>OCN</td>
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<tr>
<td>Alkaline phosphatase</td>
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<tr>
<td>Osteonectin</td>
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<tr>
<td>Osteopontin</td>
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<tr>
<td>Osteoprotegerin</td>
</tr>
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<td>Col1 alpha1</td>
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<tr>
<td>Col1 alpha2</td>
</tr>
<tr>
<td>CD44</td>
</tr>
<tr>
<td>Nanog</td>
</tr>
<tr>
<td>HIF1 alpha</td>
</tr>
<tr>
<td>CXCR4</td>
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<tr>
<td>CD45</td>
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<tr>
<td>CD34</td>
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ACC = anterior cingulate cortex; HIF = hypoxia-inducible factor; MGP = matrix Gla protein; OCN = osteocalcin.
Table 2 also shows the detailed cardiac catheterization data in the study subjects. Based on the definition of the groups, the ECA subjects did not have significant structural CAD, whereas the LCA patients had a mean coronary artery stenosis of 90%, when calculated based on coronary arteries with at least a 50% stenosis (29,30). As expected, parameters of epicardial and microvascular endothelial function were significantly different in the ECA subjects compared with the control subjects.

Flow cytometry. We performed flow cytometry using antibodies to the EPC markers and OCN, as described in detail in the Methods section, and Figure 1 shows a representative example of the flow analysis for CD34, KDR, and OCN. In preliminary experiments, we determined that the vast majority of the CD34+ cells were in the small lymphocyte gate (Fig. 1A), as has also been noted by other investigators (29,31). Figures 1B and 1C show the flow cytometry analysis for CD34+/KDR+ cells and Figure 1D shows the further analysis of these cells with the OCN antibody.

Figure 2 shows the analysis of peripheral blood mononuclear cells for the absolute counts of CD34+ cells (per 100,000 counts) (Fig. 2A) as well as the percentage of CD34+ cells that were OCN+ (Fig. 2B). As is evident, the number of CD34+ cells were increased (by ~2-fold) in the ECA patients, with somewhat smaller increases in the LCA patients. Similarly, there were ~2-fold increases in the percentage of OCN+ of CD34+KDR+ cells in the ECA and LCA patients compared with the control subjects.

To gain further insight into these changes in CD34+/KDR+ cells in the vascular disease groups, we examined subpopulations of the CD34+/KDR+ cells based on CD133 expression. As shown in Figure 3A, the absolute number of CD34+/CD133+ cells was ~60% higher in the ECA and LCA subjects compared with the control subjects, but these differences did not achieve a statistical significance. By contrast, the percentage of OCN+ of CD34+/CD133+ cells was significantly higher in the ECA and LCA subjects compared with the control subjects. The LCA patients also had higher percentage levels of OCN+ of CD34+CD133+ cells as compared with the control subjects, but the p value for this difference was 0.07.

Figure 4 shows the corresponding results for CD34+/CD133+ cells (Fig. 4A) and percentage of OCN+ of CD34+/CD133+ cells (Fig. 4B). As is evident, there were highly significant, 5- to 7-fold increases in these parameters in the ECA patients compared with the control subjects, and 2- to 3-fold increases in the LCA patients compared with the control subjects.

Table 3 shows the absolute numbers of CD34+/KDR+, CD34+/CD133+, and CD34+/CD133+ cells in the 3 groups. In contrast to the significant differe-
Figure 1  Example of Flow Analysis

Panel A shows the forward scatter (FSC), side scatter (SSC), and the gates used in the analysis. Panel B shows the staining with the isotype controls for the CD34- and KDR-specific antibodies, and panel C shows the specific staining with these antibodies. Panel D shows the CD34+/KDR+ cells stained with the osteocalcin (OCN) antibody. We required that <0.5% of the cells were positive with the isotype controls, and for all data reported in the article, we subtracted the signal of the isotype from the signal due to the specific antibodies. APC = allophycocyanin; IgG = immunoglobulin G; KDR = kinase insert domain receptor.

Figure 2  OCN Costaining of CD34+/KDR+ Cells

(A) CD34+/KDR+/OCN+ cells, expressed as absolute counts per 100,000 counts, and (B) percentage of OCN+ of CD34+/KDR+ cells in control subjects (circles), patients with early coronary atherosclerosis (ECA, triangles), and in patients with late coronary atherosclerosis (LCA, squares). *p < 0.05 and **p < 0.01 compared with control subjects. Shown are the median values and interquartile ranges. Abbreviations as in Figure 1.

Figure 3  OCN Costaining of CD34+/CD133+/KDR+ Cells

(A) CD34+/CD133+/KDR+/OCN+ cells, expressed as absolute counts per 100,000 counts, and (B) percentage of OCN+ of CD34+/CD133+/KDR+ cells in control subjects (circles), patients with ECA (triangles), and in patients with LCA (squares). *p < 0.05 and **p = 0.071 compared with control subjects. Shown are the median values and interquartile ranges. Abbreviations as in Figures 1 and 2.


In the cells costaining for OCN noted earlier, the absolute numbers of these cells did not differ significantly in these study subjects. The CD34+/CD133+/KDR+ cells were ~10% lower in the LCA patients compared with the control subjects, but this difference was not statistically significant.

**Serum RANKL, OPG, and MGP levels.** Because changes in OPG levels have been associated with vascular disease (32) and MGP is a known inhibitor of vascular calcification (33), we also measured serum RANKL, OPG, and MGP levels in the study subjects. As shown in Table 4, although serum RANKL levels did not differ in the study subjects, serum OPG levels were significantly higher and serum MGP levels significantly lower in the LCA patients compared with the control subjects. In correlation analyses, serum RANKL or OPG levels did not correlate with any of the cell populations in the 3 study groups and serum MGP levels did not correlate with the cell populations in the control or ECA patients (data not shown). However, in the LCA patients, serum MGP levels were significantly inversely correlated with the absolute numbers of CD34+/CD133+/KDR+/OCN+ cells (Fig. 5A), with a similar trend seen for the percentage of OCN+ of CD34+/CD133+/KDR+ cells (Fig. 5B).

**Confocal microscopy.** Figure 6 shows evidence, using confocal microscopy, that each of the antibodies used in the study was binding to the surface of the cell. Note also in this figure the relatively small size (~10 μm) of these cells, as represented by a cell positive with all 4 antibodies (CD34, CD133, KDR, and OCN).

**Cell culture.** Although previous work from our group (14,15) and others (10–13) has shown that staining for OCN identifies cells with osteogenic potential both in bone marrow and in peripheral blood, we next assessed whether peripheral blood CD34+ cells were capable of mineralization in vitro. For these experiments, we used CD34+ cells isolated from anonymous blood donors and found that when cultured on uncoated plastic plates, these cells failed to adhere; by 21 days in osteoblast differentiation medium, the culture plates contained no visible cells (data not shown). Reasoning that local calcium deposition in the vasculature due to either cell death (34) or vascular microcalcifications might induce these cells to adhere, we next exposed CD34+ cells to an additional 2.1 mmol/l of extracellular Ca2+ only for 5 days at the beginning of culture (days 3 to 7). Under these conditions, we found that peripheral blood CD34+ cells routinely adhered to the

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**Table 3 Differences in CD34+ Subpopulations**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ECA</th>
<th>LCA</th>
</tr>
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<tbody>
<tr>
<td>CD34+/KDR+</td>
<td>181 (56, 378)</td>
<td>173 (70, 323)</td>
<td>180 (100, 240)</td>
</tr>
<tr>
<td>CD34+/CD133+/KDR+</td>
<td>80 (37, 156)</td>
<td>77 (36, 126)</td>
<td>73 (48, 107)</td>
</tr>
<tr>
<td>CD34+/CD133+/KDR-</td>
<td>75 (23, 200)</td>
<td>99 (29, 190)</td>
<td>98 (49, 119)</td>
</tr>
</tbody>
</table>

Absolute number (in counts per 100,000) of CD34+/KDR+, CD34+/CD133+/KDR+, and CD34+/CD133+/KDR- cells in the study subjects. Data are median (interquartile range).

KDR = kinase insert domain receptor; other abbreviations as in Table 2.

---

**Table 4 Serum RANKL, OPG, and MGP Levels in the Study Subjects**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ECA</th>
<th>LCA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANKL, pmol/l</td>
<td>0.02 (0.00, 0.12)</td>
<td>0.05 (0.00, 0.10)</td>
<td>0.06 (0.00, 0.21)</td>
</tr>
<tr>
<td>OPG, pmol/l</td>
<td>4.90 (3.20, 6.28)</td>
<td>4.80 (3.63, 6.69)</td>
<td>7.01 (4.90, 8.97)†</td>
</tr>
<tr>
<td>MGP, nmol/l</td>
<td>7.08 (6.07, 10.58)</td>
<td>8.29 (6.52, 10.18)</td>
<td>4.19 (3.35, 7.16)‡</td>
</tr>
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</table>

Data are median (interquartile range). *Serum was available in 20 of the 29 subjects in the LCA group for these measurements. †p < 0.05, ‡p = 0.01 compared with control subjects.

OPG = osteoprotegerin; RANKL = receptor activator of nuclear factor kappa B ligand; other abbreviations as in Tables 1 and 2.
plastic dishes and, when placed in osteogenic differentiation medium for an additional 21 days, formed mineralized nodules, as assessed either by von Kossa (Fig. 7A) or alizarin red (Fig. 7B) staining. Note that in these assays, from days 8 to 28 (21 days of differentiation media), the cells were in osteoblast differentiation medium without additional calcium. Because we could not obtain sufficient numbers of cells from the patients undergoing cardiac catheterization to specifically isolate CD34<sup>+</sup>/H11001 cells for culture, we next tested whether PBMNCs either from normal subjects (n = 3) or patients with LCA (n = 3) could be induced to mineralize under similar culture conditions. The PBMNCs from normal subjects, who had percentages of OCN<sup>+</sup> of CD34<sup>+</sup> cells of 4.2%, 9.9%, and 12.7%, respectively, did not form mineralized colonies, without or with calcium exposure (data not shown). Of the LCA patients, PBMNCs from the subject with the highest percentage of OCN<sup>+</sup> of CD34<sup>+</sup> cells (31.2%) were able to form mineralized deposits in the presence, but not in the absence of the brief 5-day exposure to calcium (Fig. 7C), whereas PBMNCs from the other 2 LCA patients who had lower percentages of OCN<sup>+</sup> of CD34<sup>+</sup> cells (18.3% and 24.9%) failed to mineralize under these conditions (data not shown).

Expression of bone-related genes by CD34<sup>+</sup> cells. For the gene expression analyses, we isolated CD34<sup>+</sup> cells using MACS from 6 anonymous donors from the Mayo Blood Bank and compared the expression of selected bone-related genes in these cells with the expression of the same genes in hMSCs, which are authentic osteoblast precursors (9). As shown in Figure 8A, CD34<sup>+</sup> cells expressed significantly higher levels of Runx2 and beta catenin than hMSCs did; additional bone-related genes (BMP-2, Msx-2, Notch, and...
OCN were expressed at levels similar to hMSCs. The AP, osteonectin, osteopontin (OPN), MGP, Col1 alpha 1 and Col1 alpha2 mRNAs were expressed by CD34/H11001 cells at low levels. The stem cell marker, Nanog, was expressed at significantly higher levels in CD34/H11001 compared with expression in hMSCs, whereas CD44, another stem cell marker, was expressed at similar levels. HIF-1 alpha and the chemokine receptor, CXCR4, both of which are involved in homing of cells to ischemic tissues, were expressed at very high levels in the CD34/H11001 cells. The CD34/H11001 cells also expressed mRNAs for CD34 and CD45. Following in vitro culture of the CD34/H11001 cells (first 5 days in the presence of Ca²⁺ and than an additional 7 days in osteoblast differentiation medium), mRNA expression of CD34 virtually disappeared, and CD45 mRNA was significantly decreased (Fig. 8B). Expression of OPN, a noncollagenous bone matrix protein that is known to have chemotactic properties (35), increased dramatically (by 36,000-fold) following culture (Fig. 8C). We also observed a 5-fold increase in the expression of Col1 alpha 1 under the same culture conditions (Fig. 8C). However, of the 6 CD34/H11001 cell samples, the 3 with the highest expression of OCN mRNA prior to culture significantly (by 600-fold) up-regulated Col1 alpha1 expression, whereas the 3 with low OCN mRNA levels prior to culture did not (Fig. 8D).

**Ability of OCN⁺ cells to predict early coronary atherosclerosis.** To evaluate the potential utility of OCN⁺ cells as a biomarker for ECA, we performed logistic regression models using control and ECA subjects and tested the ability of the cell population that differed most between the normal and ECA patients (CD34⁺/CD133⁻/KDR⁺/OCN⁺ cells or percentage of OCN⁺ of CD34⁺/CD133⁻/KDR⁺ cells) to predict ECA in multivariable logistic regression models that included age and gender. In these
Table 5 ROC Analysis

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Unadjusted AUC (95% CI)</th>
<th>Age-Adjusted AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-CRP*</td>
<td>0.59 (0.41–0.77)</td>
<td>0.58 (0.40–0.76)</td>
</tr>
<tr>
<td>OCN/CD34/CD133/KDR* per 100,000*</td>
<td>0.78 (0.64–0.92)</td>
<td>0.78 (0.63–0.92)</td>
</tr>
<tr>
<td>% OCN of CD34/CD133/KDR cells*</td>
<td>0.84 (0.72–0.96)†</td>
<td>0.84 (0.72–0.96)†</td>
</tr>
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Unadjusted and age-adjusted areas under the curve (AUCs) for receiver-operator characteristic (ROC) curves for prediction of ECA, using the OCN/CD34/CD133/KDR* per 100,000, percentage of OCN of CD34/CD133/KDR cells, or hs-CRP levels as predictors. This analysis was done using the control and ECA patients. *Log2 used; †p < 0.05 versus corresponding AUC for hs-CRP.

Abbreviations as in Tables 1, 2, and 3.

We demonstrate in the present study that, compared with control subjects with normal endothelial function and no structural CAD, patients with ECA, characterized by coronary endothelial dysfunction, or those with established LCA, characterized by severe, multivessel CAD, have a greater percentage of CD34+/KDR+ cells containing OCN. Similar increases in the percentage of OCN+ of CD34+/CD133+/KDR+ cells were present in the vascular disease patients, with these subjects having even more marked increases in the percentage of OCN+ of CD34+/CD133+/KDR+ cells. In addition, the absolute numbers of CD34+/CD133+/KDR+/OCN+ cells were also significantly higher in the vascular disease compared with the control subjects. These findings have potentially important implications for our understanding of the mechanisms of coronary vascular calcification and of the link between osteoporosis and vascular disease, as well as for the development of possible new markers for vascular disease.

Although novel, our findings are nonetheless consistent with recent reports by Gabbasov et al. (36,37) comparing circulating cells staining with an antibody for another bone-related protein, osteonectin, in patients with CAD compared with normal subjects. In those studies, osteonectin-positive cells were not characterized as EPCs; however, the investigators did find that osteonectin-positive cells were increased in the circulation of CAD compared with control patients. In the context of the increasing overlap between endothelial and osteoblastic lineages noted earlier (17–19), our findings and those of Gabbasov et al. (36,37) suggest that vascular injury or disease may be associated with activation of osteogenic genes by EPCs. Because circulating EPCs may constitute the initial response to vascular injury (6,7), the expression of an osteogenic “transcriptosome” by these cells may promote vascular calcification rather than normal repair processes. Consistent with this, our gene expression analysis demonstrated that human circulating CD34+ cells expressed a spectrum of bone-related genes, including Runx2, beta catenin, BMP-2, Msx-2, Notch, and OCN at levels similar to (or even higher than) cultured hMSCs, which are authentic osteoblast precursors (9).

Our cell culture studies using MACS-sorted CD34+ cells suggest that increases in local calcium concentrations in the vessel wall, perhaps due to cell death (34) or micocalcifications, may play a role in the adherence of circulating EPC populations and perhaps their differentiation toward an osteogenic phenotype. Along these lines, it is also of interest that, even using exposure to increased extracellular Ca2+, we could not successfully culture mineralizing cells from unsorted PBMCs of normal subjects, but were able to do so from PBMCs of a patient with LCA who had the highest percentage of OCN+ of CD34+ cells of the 3 LCA patients tested, which is consistent with the hypothesis that there are increased concentrations of cells with mineralizing ability in the circulation of patients with advanced atherosclerosis. Our culture data are also consistent with a recent preliminary report by Bick et al. (38) showing that circulating EPCs from sheep and humans can be differentiated into osteoblastic cells, thus providing further support for a possible role for these cells in vascular calcification.

Of particular interest, OPN expression was markedly up-regulated (by 36,000-fold) following in vitro culture in the presence of Ca2+. Osteopontin is a noncollagenous bone matrix protein produced by osteocytes, osteoblasts, and osteoclasts that can interact with the cell surface receptor, CD44 (39). However, it also appears that OPN is associated with the presence and the extent of cardiovascular disease (40), and it may serve as a chemoattractant for a number of cell types (35,41). Thus, production of OPN by EPCs at sites of tissue injury may modulate proliferation, migration, and accumulation of endothelial and vascular smooth muscle cells, thereby promoting vascular repair, but also perhaps initiating vascular calcification.

age- and gender-adjusted models, both parameters were significant (p = 0.003 for both) predictors of ECA, with odds ratios (95% confidence interval) of 1.9 (1.3 to 3.0) and 3.0 (1.5 to 6.1) for the CD34+/CD133+/KDR+/OCN+ and percentage of OCN+ of CD34+/CD133+/KDR+ cells, respectively. Because in these models a log2 transformation of both the CD34+/CD133+/KDR+/OCN+ and the percentage of OCN+ of CD34+/CD133+/KDR+ cells was used, the odds ratio represents the increase in risk associated with a doubling in the percentage of these cells. Results were similar when models were adjusted for any of the following: statin use, diabetes, hypertension, smoking, hs-CRP, cholesterol, high-density lipoprotein, triglyceride, low-density lipoprotein, creatinine, and glomerular filtration rate.

Finally, we also performed ROC analyses to further assess the ability of these cell populations to predict ECA and placed this in the context of a similar analysis using the currently available marker, hs-CRP. As shown in Table 5, both for unadjusted and for age-adjusted models, the percentage of OCN+ of CD34+/CD133+/KDR+ cells performed better than hs-CRP as predictors of ECA, with the CD34+/CD133+/KDR+/OCN+ having intermediate areas under the curve.

Discussion

We demonstrate in the present study that, compared with control subjects with normal endothelial function and no structural CAD, patients with ECA, characterized by coronary endothelial dysfunction, or those with established LCA, characterized by severe, multivessel CAD, have a greater percentage of CD34+/KDR+ cells containing OCN. Similar increases in the percentage of OCN+ of CD34+/CD133+/KDR+ cells were present in the vascular disease patients, with these subjects having even more marked increases in the percentage of OCN+ of CD34+/CD133+/KDR+ cells. In addition, the absolute numbers of CD34+/CD133+/KDR+/OCN+ cells were also significantly higher in the vascular disease compared with the control subjects. These findings have potentially important implications for our understanding of the mechanisms of coronary vascular calcification and of the link between osteoporosis and vascular disease, as well as for the development of possible new markers for vascular disease.

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Our cell culture studies also revealed that CD34+ cells with higher expression of OCN mRNA prior to culture were the ones that up-regulated expression of Col1 alpha1 mRNA following in vitro culture. Thus, the level of OCN expression by CD34+ cells may be a predictor of the ability of these cells to develop an osteogenic phenotype. Clearly, further studies are needed to address this possibility. In addition, studies using animal models are needed to determine whether these circulating cells lead to vascular calcification or whether they initiate this process, resulting in the recruitment of local cells, such as vascular smooth muscle cells or pericytes (42,43), to develop an osteogenic phenotype, or both. These additional studies should help to define the true biological role in vivo of EPC populations in regulating the process of vascular calcification. Additionally, further studies using coronary histopathology are needed to identify whether the EPC populations differentially impact microcalcifications of fibrous versus lipidic origin (44) and the outward remodeling that characterizes acute coronary syndromes (45).

Interestingly, we also found that in the LCA patients, CD34+/CD133−/KDR+/OCN− cells were significantly inversely correlated with serum MGP levels. Because MGP is a known inhibitor of vascular calcification (33), these findings suggest a biological connection between the cell populations we have identified and the process of vascular calcification that warrants further investigation. In addition, the use of newer assays for undercarboxylated MGP (46), which has impaired biological function, may identify further associations between this form of MGP and the cell populations described here and may also provide additional risk stratification to identify patients who progress from early to late coronary atherosclerosis.

The current study also demonstrates for the first time that the percentage of OCN+ of EPCs, particularly the CD34+/CD133−/KDR+/OCN− and percentage of OCN+ of CD34+/CD133−/KDR+ cells, can serve as peripheral markers of ECA. In fact, from a diagnostic standpoint, these cells outperformed the commonly used systemic marker, hs-CRP, in ROC analyses. We recognize, however, that additional studies using larger numbers of subjects are needed to formally compare the predictive ability of these cells with other biological markers for coronary atherosclerosis.

Although previous studies (29,47) have found associations between the numbers of EPCs defined as CD34+/KDR+ and vascular outcomes, we did not find significant differences in these cells between our study groups. The reasons for this are somewhat unclear, but may have to do with the specific patient populations studied, including the fact that the majority of our patients with LCA were on statins, which are known to increase circulating EPCs (48).

In this context, our findings suggest that the addition of OCN as a biomarker to CD34+/KDR+ or CD34+/CD133−/KDR+ cells may be of use in predicting cardiovascular outcomes, and prospective studies addressing this possibility are needed.

Conclusions

Our findings demonstrate that a higher number and percentage of circulating cell populations containing EPCs costain for OCN in patients with early or late coronary atherosclerosis compared with control subjects with normal endothelial function and no structural CAD. These data suggest that the activation of an osteogenic program by EPCs may play a role in the response to vascular injury and contribute to vascular calcification, as opposed to noncalcifying vascular repair. Additionally, the same proinflammatory factors involved in the pathogenesis of osteoporosis may lead to the expression of an osteogenic phenotype by endothelial lineage cells, providing a potential mechanism for the link between osteoporosis and vascular calcifications. Finally, our finding that the number of CD34+/CD133−/KDR+/OCN− cells and percentage of OCN+ of CD34+/CD133−/KDR+ cells can predict coronary atherosclerosis suggests that these (or similar) cell populations may hold promise as novel markers for vascular disease.

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Reprint requests and correspondence: Dr. Sundeep Khosla, Endocrine Research Unit, College of Medicine, Mayo Clinic, 200 First Street SW, Guggenheim 7, Rochester, Minnesota 55905. E-mail: khosla.sundeep@mayo.edu.

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