Aging, Progenitor Cell Exhaustion, and Atherosclerosis

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Background—Atherosclerosis is largely attributed to chronic vascular injury, as occurs with excess cholesterol; however, the effect of concomitant vascular aging remains unexplained. We hypothesized that the effect of time in atherosclerosis progression is related to obsolescence of endogenous progenitor cells that normally repair and rejuvenate the arteries.

Methods and Results—Here we show that chronic treatment with bone marrow–derived progenitor cells from young nonatherosclerotic ApoE−/− mice prevents atherosclerosis progression in ApoE−/− recipients despite persistent hypercholesterolemia. In contrast, treatment with bone marrow cells from older ApoE−/− mice with atherosclerosis is much less effective. Cells with vascular progenitor potential are decreased in the bone marrow of aging ApoE−/− mice, but cells injected from donor mice engraft on recipient arteries in areas at risk for atherosclerotic injury.

Conclusions—Our data indicate that progressive progenitor cell deficits may contribute to the development of atherosclerosis. (Circulation. 2003;108:r9-r15.)

Key Words: aging ■ atherosclerosis ■ stem cells

Recent studies have revealed an association between risk factors for atherosclerosis and low circulating levels of bone marrow (BM)–derived endothelial progenitor cells involved in vascular repair.1 We hypothesized that in aging, the most powerful risk factor for atherosclerosis, an organism’s supply of BM cells capable of vascular repair is exhausted. Exhaustion of this component of BM in number, function, or both could produce a disequilibrium between vascular injury and vascular repair that leads to atherosclerosis.2–7 Furthermore, exhaustion may represent a cellular basis for how chronic but not acute exposure to risk factors contributes to atherosclerosis. In this report, we provide the first direct evidence that chronic injury and age-related exhaustion of BM-derived vascular repair cells likely constitutes a novel and pivotal step in the pathogenesis of atherosclerosis.

Methods

Animals

All mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and handled according to Duke University animal care and use regulations. Animals fed a high-fat diet were given diet #88137 (Harlan-Teklad; 42% fat, 1.25% cholesterol) beginning at 3 weeks of age. BM injections were via the internal jugular vein under ketamine anesthesia or via intraperitoneal cavity (controls).

Cells

BM isolated from tibiae and femora was cultured in minimum essential medium alpha (Invitrogen) with 12.5% fetal calf and 12.5% equine serum and 2 μmol/L hydrocortisone. After 2 days, hematopoietic-enriched (nonadherent) cells were suspended in 0.9% NaCl and immediately used for injection. Stromal-enriched (adherent) cells were expanded for 2 weeks before injection.

Pathology

Aortic arches were photographed through a Leica M-650 microscope. Whole aortas, opened lengthwise, and microscopic frozen sections of aortic root were stained with oil red O and quantified. Means and SEMs for atherosclerosis data were compared by ANOVA and Tukey tests with significance set at P<0.05.

Fluorescence-Activated Cell Sorting

Hematopoietic- and stromal-enriched BM cells were stained with FITC-conjugated rat anti-mouse CD45 (leukocyte common antigen, Ly5, clone 30-F11) and phycoerythrin-conjugated rat anti-mouse CD31 (Clone MEC 13.3) antibodies (Pharmingen). Labeled cells were sorted with a dual-laser fluorescence-activated cell sorter (FACS; Becton-Dickinson), and analysis was performed with FlowJo software (version 4.2, Tree Star). Mean results were compared by Student’s t test, with significance assumed at P<0.05.

Telomere Length Assay

We isolated DNA (4 to 6 μg) from cells bluntly scraped from whole aortic intima using DNAzol (Invitrogen). Terminal restriction frag-

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ments were prepared and probed as described previously, followed by electrophoretic separation on a 0.3% agarose gel, transfer to filter paper, and phosphorimagery.

**ELISA for IL-6**

Six-month-old ApoE\(^{-/-}\) mice were injected intravenously with 2×10\(^6\) hematopoietic-enriched BM cells or combined hematopoietic- and stromal-enriched cells from 6-month-old wild-type (WT), 6-month-old ApoE\(^{-/-}\), or 4-week-old ApoE\(^{-/-}\) donors. Donors were maintained on either regular or high-fat diets. For each donor type, 7 to 8 recipients were treated. At 0, 15, or 30 days after cell injection, plasma interleukin 6 (IL-6) levels were measured by ELISA (R&D Systems). (See the Data Supplement for more details about the methods used in this study.)

**Results**

**Age-Dependent Antiatherosclerotic Effect of BM Cells**

To test our hypothesis, we first sought to compare the efficacy of old versus young donor BM treatment in atherosclerosis prevention in aging ApoE\(^{-/-}\) mice. BM cells from severely atherosclerotic, 6-month-old ApoE\(^{-/-}\) mice and from recently weaned 4-week-old ApoE\(^{-/-}\) mice that had not yet developed detectable atherosclerosis were isolated and cultured. To encompass a host of diverse cell types likely involved in vascular repair, we enriched the isolated BM for both hematopoietic and stromal BM fractions. Hematopoietic- and stromal-enriched cells from either young or old ApoE\(^{-/-}\) donors were then injected intravenously into unirradiated ApoE\(^{-/-}\) mice on a high-fat diet (1×10\(^6\) cells/injection) every 2 weeks beginning at 3 weeks of age.

Recipient ApoE\(^{-/-}\) mice were killed at 14 weeks of age, after they had received a total of 6 injections. The atherosclerotic burden was determined by 3 complementary techniques: (1) histological analysis of aortic root cross sections, (2) morphometric analysis of transilluminated aortic arches, and (3) en face staining of the aortas with oil red O. Each analysis revealed significantly less atherosclerotic burden in mice that had received combined hematopoietic- and stromal-enriched
cells from young ApoE<sup>−/−</sup> donors (n=6) than in those that had received the same cells from old ApoE<sup>−/−</sup> donors (n=6; Figure 1, A through F, and Figure 1G, groups a and b). These findings indicated that (1) aged cells had atherogenic properties, (2) BM-derived cells had atheroprotective properties that were lost with aging and exposure to atherosclerosis, or (3) there was a combination of both processes.

To help address this question, we used 2 negative controls: (1) ApoE<sup>−/−</sup> mice that received no cells (n=6) and (2) ApoE<sup>−/−</sup> mice that received 6 injections of young WT BM cells (1×10<sup>6</sup> cells every 2 weeks, combined hematopoietic enriched and stromal enriched), but this time intraperitoneally (n=6; Figure 1G, groups c and d). We also used 2 positive controls, WT hematopoietic-enriched cells alone and WT stromal-enriched cells alone, each delivered intravenously (Figure 1G, groups e and f). Whenever male donor BM cells were injected into female recipients, we consistently detected Y-chromosome–positive DNA in the peripheral blood and BM of recipients if the cells were given intravenously but not if the cells were given intraperitoneally (polymerase chain reaction findings up to 14 days after injection; data not shown). Levels of atherosclerosis in the negative control groups (Figure 1G, groups c and d) were similar to the atherosclerotic burden in mice that received old ApoE<sup>−/−</sup> cells (Figure 1G, group a). In contrast, mice that received young ApoE<sup>−/−</sup> or WT cells (Figure 1G, groups b, e, and f) had less atherosclerotic burden at each anatomic location analyzed. These data indicate that BM cells derived from young, prediseased, animals have an atheroprotective effect, which requires vascular distribution.

**Age-Related Loss of Progenitor Cells**

The reduced atheroprotective effect of old BM cells suggested that loss of cells with repair capacity might occur with aging. To test this possibility, we studied the effect of chronic hypercholesterolemia on BM-cell content. Using FACS, we compared the percentage of BM cells that expressed established vascular progenitor markers (CD31+CD45−) in healthy 1-month-old WT mice, young ApoE<sup>−/−</sup> mice, and 6-month-old ApoE<sup>−/−</sup> mice with advanced atherosclerosis. FACS revealed that CD31+CD45− cells (Figure 2) were significantly diminished in BM from 6-month-old ApoE<sup>−/−</sup> mice (3.79±0.20% gated cells, n=5) compared with 1-month-old ApoE<sup>−/−</sup> mice (7.03±0.81% gated cells, n=5) and WT mice (6.36±1.02% gated cells, n=5). This loss of vascular progenitor cells in BM obtained from older ApoE<sup>−/−</sup> mice may explain, at least in part, the loss of antiatherosclerotic effect of the older ApoE<sup>−/−</sup> BM cells.

In contrast, FACS analysis of BM from these same groups for the hematopoietic stem cell marker c-kit and the generalized murine stem cell markers sca-1 and CD34 did not reveal any significant deficiencies in old ApoE<sup>−/−</sup> mice (see Data Supplement). Furthermore, to discern whether differences in vascular progenitor content might reflect a difference in the vascularity of the BM, we performed FACS analysis for VEGFR-2 (Flk-1), a marker of mature endothelial cells. This analysis revealed an 8.8% (nonsignificant) increase in old ApoE<sup>−/−</sup> mice relative to young ApoE<sup>−/−</sup> mice and a slight nonsignificant decrease relative to WT (supplemental data). These data confirm that a specific depletion of intermediate vascular progenitor cells (CD31+/CD45−), without parallel changes in more primitive stem cells (sca-1+, c-kit+, or CD34+) or mature vascular cells (VEGFR-2+), most likely accounted for the age-related loss of BM-derived vascular repair capacity.

To determine whether repeated BM cell injections could replenish the decreased number of CD31+/CD45− cells in aging ApoE<sup>−/−</sup> mice, we performed FACS for CD31 and CD45 on the recipients’ bone marrow. We found that chronic injection (2 million cells every 1 week for 14 weeks) of combined hematopoietic- and stromal-enriched cells did not significantly restore the deficiency of CD31+/CD45− cells in the BM of aging ApoE<sup>−/−</sup> mice (data not shown). The
presence of donor cells was, however, detected in the recipient BM by polymerase chain reaction for Y chromosome. These data suggest that rather than reconstituting stem cells in the BM, CD31+/CD45− cells may be actively involved in a vascular repair process with ongoing consumption.

Localization of Donor Cells
To enable identification of donor-derived cells in recipient mice, we intravenously injected combined hematopoietic- and stromal-enriched BM cells (1 × 10^6 cells/injection every 2 weeks for 3 injections) from donor mice that expressed β-gal into ApoE−/− recipients on high-fat diet (n=4) or WT recipients on normal chow diet (n=4). En face aortic β-gal staining revealed donor cell localization to the most atherosclerosis-prone regions of the aorta, including the arch, branching points, and distal abdominal region (Figure 3A). These data, in conjunction with the oil red O staining of paired aortas shown in Figure 1, revealed significantly less lipid deposition in BM-treated animals (versus untreated), particularly in those regions with the most donor cell engraftment (Figure 3, A through E). Consistent with previous work,10 histological sections of aortic segments with positive β-gal localization revealed vascular differentiation of donor BM cells (Figure 3F). β-Gal–positive cells were found to overlie the intima. The predominant phenotype of engrafted cells was endothelial, as demonstrated by colocalization of staining for β-gal and CD31, an endothelium-specific cell marker (Figure 3, F and G). Administration of β-gal–positive BM cells to WT recipients resulted in much fainter en face aortic β-gal staining, with slightly enhanced localization to the arch (Figure 3C). Untreated ApoE−/− and WT mice had no aortic β-gal staining (Figure 3B).

Although engrafted cells predominantly expressed CD31, nonendothelial β-gal–positive cells were also observed. A quantitative phenotyping of 220 β-gal–positive cells on aortic histological sections revealed the following: 138 cells (62%) were CD31+/CD45−; 49 (22%) were CD31−/CD45+; 5 (2%) were CD31+/CD45+; and 28 (13%) were CD31−/CD45+−. This mixed population of engrafted BM-derived cells might indicate that a variety of cells, including leukocytes, could be involved in vascular repair. As observed previously,10 the present data also highlight the possibility that BM-derived cells, when depleted of endothelial progenitors, could instead participate in inflammation and neointima formation. This possibility could theoretically become a more important concern with aging, as the BM becomes exhausted of presumably more salutary CD31+ progenitor cells.

Potential Mechanisms of BM-Derived Atheroprotection
We considered the potential mechanisms by which injection of young BM-derived cells could delay the progression of atherosclerosis. We first turned to cholesterol, the presumed source of atherogenic injury in ApoE−/− mice. We found that although plasma cholesterol levels varied strikingly with diet and genotype (Figure 4A), elevated plasma cholesterol levels in ApoE−/− mice (1420 ± 170 mg/dL, n=6, for untreated mice) were not significantly suppressed after injection of any type of BM cells used (eg, 1300 ± 130 mg/dL after 6 injections of WT BM, n=12; additional data for injection of other diet/cell combinations shown in Figure 4B). These data indicated that the atheroprotective outcome after cell injection was not due to elimination of the hypercholesterolemic source of vascular injury in these mice. The protective mechanism must therefore differ fundamentally from that previously observed in ApoE−/− mice after complete BM ablation and WT reconstitution,11,12 in which correction of hypercholesterolemia explained, at least in part, the suppression of atherosclerosis.

Having observed that BM-derived cells engraft on and “endothelialize” recipient arteries in vivo, we turned our attention to the possibility that the cells could locally mediate antiatherosclerotic effects at the level of the arterial wall. One possible mechanism of the antiatherosclerotic impact of engrafted BM cells might be the replacement of senescent endothelial cells by younger cells. Endothelial senescence refers to the acquisition of proinflammatory and proatherosclerotic properties among endothelial cells that have under-
Figure 4. Suppression of IL-6 by BM cell injection. A–D, Six-month-old ApoE−/− mice were injected intravenously with $2 \times 10^6$ combined hematopoietic- and stromal-enriched cells from 6-month-old WT or 6-month-old ApoE−/−. Donors were maintained on either regular (R) or fat-rich (F) diets. For each donor type, 7 to 8 recipients were treated, and blood was drawn for analysis 15 days after cell injection. A and B, Plasma cholesterol levels in untreated mice (A) and ApoE−/− mice treated with BM from WT and ApoE−/− mice (B). C and D, Plasma IL-6 levels in untreated mice (C) and in ApoE−/− mice treated with BM from WT and ApoE−/− mice (D). E, Six-month-old ApoE−/− mice were injected intravenously with $2 \times 10^6$ hematopoietic-enriched cells from 6-month-old WT, 6-month-old ApoE−/−, or 4-week-old ApoE−/− donors. Donors were maintained on either regular (R) or fat-rich (F) diets, and 7 to 8 recipients were treated for each donor type. We measured plasma IL-6 levels at 0, 15, or 30 days after cell injection. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with control (leftmost bar on each graph). †$P<0.05$ compared with WT donors on regular or fat-rich diet.
significantly weaker when the donor cells originated from the telomeres of intimal cells in untreated ApoE mice (lanes 1 to 4), BM-treated ApoE mice (1×10⁶ WT cells/injection, combined hematopoietic- and stromal-enriched cells, every 2 weeks for six injections; lanes 5 to 10), and congenic, untreated, nonatherosclerotic WT mice (lanes 11 and 12). BM-treated mice had significantly longer telomeres than untreated mice, indicating attenuated vascular senescence.

Therefore, we hypothesized that the BM might contain endothelial progenitors that help repair areas of vascular senescence, a function which, if lost with aging and risk factors, would lead to accelerated atherosclerosis. We measured the average telomere lengths on DNA from cells scraped from the whole aortic intima, comprising not only endothelial but potentially also inflammatory cell DNA. This assessment revealed that ApoE−/− mice had shorter telomeres than healthy age-matched mice (Figure 5, lanes 1 to 4 versus lanes 11 to 12), whereas the telomeres of intimal cells in ApoE−/− mice that received combined hematopoietic- and stromal-enriched BM cells (1×10⁶ WT cells/injection, every 2 weeks for 6 injections) were significantly longer than those of untreated ApoE−/− mice (Figure 5, lanes 5 to 10 versus lanes 1 to 4). These data indicate that one potential mechanism of BM-derived vascular repair could be a local effect on reducing endothelial senescence.

Finally, we considered potential humoral effects that could additionally account for the observed amelioration of atherosclerosis. Recent studies indicate that acute phase proteins, additionally account for the observed amelioration of atherosclerosis. Reduced vascular progenitor content in aging BM could additionally result in a disequilibrium between reparative endothelial cells and inflammatory leukocytes, tipping the balance of injury and repair.

Although a precise determination of the molecular mechanism for IL-6 suppression in this model will require substantial additional work, it is likely that the injected cells either suppressed the production of IL-6 by diminishing local and/or systemic vascular inflammation or that a humoral feedback loop was interrupted by cell injection. IL-6 levels fell by a factor of 10 within 15 days after a single cell injection (Figure 4D). Presumably, this time course is too rapid to have reduced the atherosclerotic burden by such a factor. One tempting explanation is that the persistent elevation of IL-6 and other inflammatory proteins in aging and atherosclerotic disorders could be linked to a lack of BM cells capable of arterial repair. “Injured” blood vessels may trigger the secretion of cytokines, such as IL-6, and growth factors that might help mobilize or “recruit” BM-derived cells for vascular repair. Consistent with this hypothesis are recent findings that circulating levels of endothelial progenitor cells dramatically increase during episodes of active vasculitis,17 potentially to aid in repair of ongoing vascular damage. The present data indicate that atherosclerosis, and perhaps other chronic inflammatory processes, may, with aging, eventually deplete the BM of progenitor cells. This might then lead to an exhaustion of the vascular repair process, loss of the “negative feedback loop” on IL-6 production, and a consequent heightened cytokine release. This increase in inflammatory cytokines, a signature of advanced atherosclerosis, could then itself participate in further vascular injury and atherosclerosis progression.13,15

Discussion

In this mouse model of atherosclerosis, we have established that there is an atheroprotective property of the BM that is “exhausted” with aging and prolonged exposure to risk factors. Several findings indicate that this exhaustion likely involves progenitor cell–mediated vascular repair. FACS analysis of BM indicated an age-related decline in cells simultaneously expressing endothelial progenitor and lacking leukocyte markers. Moreover, after treatment, more than half of the donor-derived BM cells that engrafted in recipients’ arterial vessels exhibited endothelial progenitor characteristics. Although FACS analysis did not reveal quantitative deficiencies of any leukocyte lineages (data not shown), age-related qualitative or functional differences in leukocytes and other BM-derived cell types may contribute as well. These qualitative traits could include alterations in cholesterol metabolism that do not change the plasma cholesterol, other local biochemical effects on the blood vessel wall, cytokine expression by immune-competent cells, or the acquisition of primed immune cells that exacerbate atherosclerosis. Reduced vascular progenitor content in aging BM could additionally result in a disequilibrium between reparative endothelial cells and inflammatory leukocytes, tipping the balance of injury and repair.

Although it is possible that a single “therapeutic” cell type is exhausted with aging, it appears more plausible that multiple types are affected, each one a component of the vascular repair process. Consistent with previous
work,10,18–21 the present study has identified the apparent importance of CD31+/CD45− cells in vascular repair. However, because of the mixture of cells injected, we cannot rule out the role of other cell types. Potential confounders could include the effects of self-renewing “true stem cells” or side lineages, such as leukocytes. Much remains to be learned about the repair process and the various cells involved. By optimizing dose and timing of delivery, identifying the cell lineages with the greatest capacity for vascular repair, and eliminating possible proatherosclerotic “contaminant” cells, it is possible that the atheroprotective effects of BM cell injection could be even greater. Identification and restoration of potential age-related qualitative deficiencies in BM cell function could, in the future, facilitate atheroprotection without the need for actual cell transfer.22

Taken together, these results support a novel model of atherosclerosis in which deficient vascular repair, secondary to obsolescence of BM cells, is a critical determinant of disease initiation and progression. Whereas previous treatments for atherosclerosis have focused on eliminating multiple sources of vascular injury,23,11,12 we now provide evidence in support of an alternative approach, vascular repair, that can attenuate atherosclerosis progression even in the continued presence of vascular injury. Once optimized, BM-derived progenitor cell restoration could have important antiatherosclerosis applications in humans.

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