Notch Signaling Regulates Endothelial Progenitor Cell Activity During Recovery From Arterial Injury in Hypercholesterolemic Mice

Masaaki Ii, MD, PhD; Kyosuke Takeshita, MD, PhD; Kayoko Ibusuki, MD; Corinne Luedemann, BS; Andrea Wecker, BS; Elizabeth Eaton, BS; Tina Thorne, BS; Takayuki Asahara, MD, PhD; James K. Liao, MD; Douglas W. Losordo, MD

Background—Little is known about the role of endothelial progenitor cells (EPCs) in atherosclerosis. Accordingly, we performed a series of assessments with hypercholesterolemic (apolipoprotein E–null [ApoE<sup>−/−</sup>]) and wild-type (WT) mice to evaluate how cholesterol influences reendothelialization, atherosclerosis, and EPC function after arterial injury.

Methods and Results—Unexpectedly, reendothelialization (assessed by resistance to Evans blue staining) and circulating EPC counts (EPC culture assay) were greater in ApoE<sup>−/−</sup> mice than in WT mice, and transplantation of ApoE<sup>−/−</sup> bone marrow in WT mice accelerated endothelial recovery and increased recruitment of bone marrow–derived EPCs to the neointima. Cholesterol concentration-dependently promoted the proliferation (MTS assay) of both ApoE<sup>−/−</sup> and WT EPCs, and the concentration dependence of EPC adhesion (to vitronectin-, collagen type I–, fibronectin–, and laminin-coated plates), migration (modified Boyden chamber assay), and antiapoptotic (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling stain) activity was biphasic. Cholesterol enhanced the messenger RNA expression (quantitative, real-time reverse-transcription polymerase chain reaction) of vascular endothelial growth factor and inhibited Notch1 messenger RNA expression in both ApoE<sup>−/−</sup> and WT EPCs, whereas endothelial nitric oxide synthase messenger RNA expression increased in ApoE<sup>−/−</sup> EPCs and declined in WT EPCs after cholesterol exposure. EPC activity was greater in Notch1<sup>−/−</sup> EPCs than in WT EPCs, and transplantation of Notch1<sup>−/−</sup> bone marrow accelerated endothelial recovery after arterial injury in WT mice.

Conclusion—the results presented here provide novel insights into the role of EPCs during atherosclerosis and suggest that cholesterol and Notch1 may be involved in the regulation of EPC activity. (Circulation. 2010;121:1104-1112.)

Key Words: atherosclerosis • endothelial progenitor cells • hypercholesterolemia • nitric oxide synthase • Notch receptors

Hypercholesterolemia has repeatedly been identified as a risk factor for atherosclerosis; however, its influence on reendothelialization (ie, the recovery of endothelial integrity) after arterial injury is uncertain. Animal studies and clinical reports provide conflicting evidence relative to the importance of cholesterol after angioplasty or stenting and on the relationship between cholesterol levels and the risk of restenosis.1–4 The loss of endothelial integrity that occurs during angioplasty/stenting appears to induce the accumulation of inflammatory cells and the proliferation and migration of vascular smooth muscle cells, which can lead to neointimal thickening.5 Reendothelialization inhibits neointimal thickening, thereby suppressing the development of the substrate for lipid deposition and macrophage accumulation that is believed to induce the formation of atherosclerotic lesions and may contribute to restenosis. Drug-eluting stents have significantly reduced the rate of restenosis; however, drug-eluting stents also appear to delay reendothelialization.6 This delay results in excessive rates of thrombosis, which could increase the occurrence of acute coronary syndromes.

Clinical Perspective on p 1112

Endogenous reendothelialization occurs through the proliferation and migration of endothelial cells adjacent to the site of arterial injury and via the activity of endothelial progenitor cells (EPCs). Studies performed in our laboratory and others...
indicate that both exogenously infused EPCs and EPCs mobilized from the bone marrow (BM) are recruited to the sites of arterial injury, where they promote reendothelialization. Mobilization occurs in response to ischemia, physical training, and the administration of statins, estrogen, and a variety of cytokines. Then, the mobilized cells are recruited to ischemic tissue, where they form a structural component of the new vasculature and promote the proliferation and migration of local endothelial cells by secreting signaling molecules and growth factors.

Kwon et al have recently reported that a knockout mutation of the Notch ligand Jagged-1 inhibits EPC-mediated angiogenesis by reducing EPC differentiation and bioactivity. Blood flow recovery and postnatal neovascularization after hind-limb ischemia are also impaired in haploinsufficient Notch1 mice; however, similar results were not observed in mice lacking expression of another Notch ligand, Delta-like 1, and Notch1 activation in endothelial cells reduced the expression of vascular endothelial growth factor (VEGF) receptor 2 and VEGF-mediated endothelial cell proliferation. Thus, Notch signaling appears to modulate adult angiogenesis, but whether it acts as a positive or negative regulator is somewhat unclear. Mutations in Notch family members have also been linked to an adult-onset vascular dementia that is accompanied by an increased risk of coronary occlusion.

The number and function of circulating EPCs are inversely correlated with many risk factors for atherosclerosis and cardiovascular disease; however, the relationship between EPC function, gene expression, hypercholesterolemia, Notch1, and reendothelialization after arterial injury has not been completely characterized. Accordingly, we performed a series of investigations with hypercholesteremic (apoE−/−) mice; however, results were not obtained in mice lacking expression of another Notch ligand, Delta-like 1, and Notch1 activation in endothelial cells reduced the expression of vascular endothelial growth factor (VEGF) receptor 2 and VEGF-mediated endothelial cell proliferation. Thus, Notch signaling appears to modulate adult angiogenesis, but whether it acts as a positive or negative regulator is somewhat unclear. Mutations in Notch family members have also been linked to an adult-onset vascular dementia that is accompanied by an increased risk of coronary occlusion.

The number and function of circulating EPCs are inversely correlated with many risk factors for atherosclerosis and cardiovascular disease; however, the relationship between EPC function, gene expression, hypercholesterolemia, Notch1, and reendothelialization after arterial injury has not been completely characterized. Accordingly, we performed a series of investigations with hypercholesteremic (apoE−/−) mice; however, results were not obtained in mice lacking expression of another Notch ligand, Delta-like 1, and Notch1 activation in endothelial cells reduced the expression of vascular endothelial growth factor (VEGF) receptor 2 and VEGF-mediated endothelial cell proliferation. Thus, Notch signaling appears to modulate adult angiogenesis, but whether it acts as a positive or negative regulator is somewhat unclear. Mutations in Notch family members have also been linked to an adult-onset vascular dementia that is accompanied by an increased risk of coronary occlusion.

**Methods**

**Animals and Surgical Procedure**

All procedures were approved by St Elizabeth’s Institutional Animal Care and Use Committee. Male ApoE−/− (B6.129P2-Apoem1Mae/J), WT (C57BL/6J), and FVB-Tg(Tie2-LacZ)182Sato mice were obtained from The Jackson Laboratories (Bar Harbor, Me). Tie2-LacZ mice were generated in our laboratory by backcrossing FVB-Tg(Tie2-LacZ)182Sato mice with C57BL/6J mice for >10 generations. Tie2-Cre Notch1+/− mice were provided by Dr Liao. Wire-induced carotid denudation was performed as described previously in animals 10 to 12 weeks of age. Mice were euthanized for histological analyses via cervical dislocation.

**EPC Counts**

The number of EPCs in the BM and circulation of ApoE−/− and WT mice was determined via the EPC culture assay as described previously.

**BM Transplantation**

Donor mice were strain and age matched to recipient mice 6 to 8 weeks of age. The BM transplantation procedure was performed as described previously and as summarized in the Methods section of the online-only Data Supplement.

**Incorporation of BM-Derived EPCs**

The incorporation of BM-derived EPCs was evaluated in ApoE−/− and WT mice transplanted with BM from Tie2-LacZ donor mice, which express LacZ from the endothelium-specific Tie2 promoter. Carotid arteries were harvested from mice euthanized 14 days after carotid denudation for en face histological assessments. BM-derived endothelial cells were identified and quantified by X-gal staining as described previously.

**Reendothelialization**

Reendothelialization was evaluated as described previously and as summarized in the Methods section of the online-only Data Supplement; assessments were performed in ApoE−/− and WT mice, ApoE−/− mice transplanted with BM from ApoE−/− or WT donor mice, WT mice transplanted with BM from ApoE−/− or WT donor mice, and WT mice transplanted with BM from Tie2-Cre Notch1+/− or Tie2-Cre donor mice.

**Cellular Contributions to Atherosclerotic Lesion Formation**

The formation of atherosclerotic lesions was evaluated in ApoE−/− and WT mice transplanted with BM from Tie2-LacZ donor mice. Assessments of lesion size, apoptosis, β-galactosidase expression (signaling the presence of BM-derived endothelial cells), and CD68 expression (signaling the presence of monocytes/macrophages) were performed as summarized in the Methods section of the online-only Data Supplement.

**EPC Function and Apoptosis**

Proliferation, adhesion, migration, and apoptosis were assessed in EPCs isolated from the BM of ApoE−/− mice, WT mice, Tie2-Cre mice, and Tie2-Cre Notch1+/− mice. The effect of cholesterol on EPC function was determined by repeating the assays in the presence of 0, 50, 150, 300, and/or 500 mg/dL cholesterol (Sigma-Aldrich Co, St Louis, Mo). Assays were performed as summarized in the Methods section of the online-only Data Supplement.

**Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction**

Messenger RNA (mRNA) expression was evaluated in EPCs isolated from the BM of ApoE−/− and WT mice as summarized in the Methods section of the online-only Data Supplement (primer and probe sequences are also provided). Relative mRNA expression was calculated with the comparative Ct method (relative expression = 2−ΔΔCt) and normalized to the expression of the endogenous 18S gene.

**Statistical Analysis**

All values were expressed as mean ± SEM. Statistical analyses were performed with commercially available software (Statview, Abacus Concepts, Berkeley, Calif). Comparisons between 2 groups were tested for significance with the Student t test; comparisons between multiple groups were tested by ANOVA followed by posthoc testing with the Tukey procedure; measurements obtained at multiple time points were evaluated via repeated-measure analysis. A value of P<0.05 was considered significant.
EPCs was significantly greater in ApoE mice before injury (ApoE mice; 73.9 ± 8.4 cells per HPF; WT, 48.9 ± 3.4 cells per HPF; P < 0.05; n = 5 in each group) than in WT mice before injury (ApoE mice, 73.9 ± 8.4 cells per HPF; WT, 48.9 ± 3.4 cells per HPF; P < 0.05; n = 5 in each group), but the number of circulating EPCs present in (A) and WT mice was similar (ApoE mice, 20.2 ± 10.6 cells per HPF; n = 5) than in WT mice transplanted with WT Tie2-LacZ BM (LacZBMWT mice; 74.0 ± 6.6 cells per HPF; n = 5; Figure 1A), but the difference did not reach statistical significance, and circulating EPC levels in LacZBM/ApoE mice declined to preinjury levels by day 14. Nevertheless, significantly more (P < 0.001; n = 5 in each group) X-gal–positive cells were observed on the luminal surface of carotid arteries from LacZBM/ApoE mice (60.4 ± 5.4 cells per HPF) than in arteries from LacZBMWT mice (20.2 ± 4.1 cells per HPF; Figure 2B and 2C). When DiI-labeled ApoE or WT EPCs were administered systemically, significantly more (P < 0.001; n = 5 in each group) ApoE EPCs than WT EPCs were observed in the neointimal layer of either mouse strain (ApoE mice, 35.0 ± 3.5 ApoE EPCs per HPF versus 20.4 ± 3.3 WT EPCs per HPF; WT mice, 41.4 ± 4.9 ApoE EPCs per HPF versus 22.0 ± 3.9 WT EPCs per HPF; 74.0 ± 6.6 cells per HPF; n = 5; Figure 2A), but the difference did not reach statistical significance, and circulating EPC levels in LacZBM/ApoE mice declined to preinjury levels by day 14. Nevertheless, significantly more (P < 0.001; n = 5 in each group) X-gal–positive cells were observed on the luminal surface of carotid arteries from LacZBM/ApoE mice (60.4 ± 5.4 cells per HPF) than in arteries from LacZBMWT mice (20.2 ± 4.1 cells per HPF; Figure 2B and 2C). When DiI-labeled ApoE or WT EPCs were administered systemically, significantly more (P < 0.001; n = 5 in each group) ApoE EPCs than WT EPCs were observed in the neointimal layer of either mouse strain (ApoE mice, 35.0 ± 3.5 ApoE EPCs per HPF versus 20.4 ± 3.3 WT EPCs per HPF; WT mice, 41.4 ± 4.9 ApoE EPCs per HPF versus 22.0 ± 3.9 WT EPCs per HPF;
transplantation surgery; reendothelialization was significantly
higher in ApoE−/− mice than in WT mice (48.6 ± 3.6%, 41.3 ± 2.4%, and 31.5 ± 5.0% vs. 37.1 ± 2.2%, 29.3 ± 2.1%, and 14.3 ± 9.0%, respectively). These results suggest that reendothelialization was greater in ApoE−/− mice than in WT mice, but the difference between the two groups was not statistically significant. Therefore, the study suggests that reendothelialization is accelerated in ApoE−/− mice and is dependent on the genotype of the BM cells, whereas the effect of progressively greater cholesterol concentrations on EPC adhesion and migration was biphasic (Figure 5B), suggesting that the plaque was surrounded by BM-derived EPCs shortly after the onset of reendothelialization. Evidence of apoptosis and EPC accumulation persisted through week 4, but the plaque core contained only amorphous β-galactosidase protein, and no viable EPCs, at week 6. Similar staining patterns were observed in sections double immunostained for evidence of apoptosis and monocytes/macrophages (ie, cells stained positively for CD68 expression); macrophages accumulated through week 4 but were not present in the plaque core by week 6 (Figure 4D). Thus, both EPCs and inflammatory cells contributed to the formation of the atherosclerotic lesion.

**Cholesterol Modulates EPC Function**

In the absence of cholesterol, proliferation (ApoE−/− EPCs, 1.15 ± 0.02 OD; WT EPCs, 1.00 ± 0.00 OD; *P=0.01*) and adhesion (ApoE−/− EPCs, 57.4 ± 2.4 cells per HPF; WT EPCs, 51.3 ± 3.2 cells per HPF; *P=0.05*) were significantly greater in ApoE−/− EPCs than in WT EPCs (Figure 5A and 5B), but the migration of ApoE−/− and WT EPCs (51.3 ± 1.5 and 47.0 ± 3.1 cells per HPF, respectively) was similar (Figure 5C). Cholesterol enhanced the proliferation of both ApoE−/− EPCs and WT EPCs in a concentration-dependent manner (Figure 5A), whereas the effect of progressively greater cholesterol concentrations on EPC adhesion and migration was biphasic (Figure 5B and 5C). Peak adhesion and migration were observed at 150 mg/dL, and measurements were significantly lower (*P=0.05*) at 500 mg/dL than in the absence of cholesterol.

**Cholesterol Influences the Expression of VEGF, Endothelial Nitric Oxide Synthase, and Notch1 mRNA in EPCs and EPC Apoptosis**

The influence of cholesterol on the mRNA expression of VEGF and endothelial nitric oxide synthase (eNOS), which promote angiogenesis and EPC activity, and Notch1, which promote reendothelialization after carotid artery injury. Carotid arteries were harvested 3, 7, and 14 days after carotid artery injury in WT and ApoE−/− mice; mice were injected with Evans blue dye 10 minutes before death. A, Representative Evans blue-stained arteries from mice euthanized 7 days after carotid artery injury; reendothelialized regions are resistant to the dye and appear white (bar = 1 mm). B, Quantification of reendothelialization (*P=NS; *P<0.05; n = 5 in each group). C and D, Carotid arteries were harvested 7 days after carotid artery injury in WT and ApoE−/− mice transplanted with BM from WT or ApoE−/− mice; mice were injected with Evans blue dye 10 minutes before death. C, Representative Evans blue-stained arteries; reendothelialized regions are resistant to the dye and appear white (bar = 1 mm). D, Quantification of reendothelialization (*P=NS; *P<0.001; n = 5 in each group).
determines cell fate (including apoptosis), was assessed via real-time reverse-transcription polymerase chain reaction. In the absence of cholesterol, there was no significant difference between ApoE−/−/− and WT EPCs in VEGF, eNOS, or Notch1 expression (Figure 5D through 5F). Cholesterol concentration-dependently increased VEGF expression (Figure 5D) and decreased Notch1 expression (Figure 5E) in both ApoE−/− EPCs and WT EPCs, whereas eNOS expression was significantly higher (P<0.05) in ApoE−/− EPCs but declined in WT EPCs after exposure to cholesterol (Figure 5F).

The effect of progressively greater cholesterol concentrations on EPC apoptosis was biphasic (Figure 5G). Apoptotic cells were significantly less frequent (P<0.05) in 50 mg/dL cholesterol (29.0±2.9% of ApoE−/− EPCs and 22.3±3.4% of WT EPCs) and 150 mg/dL cholesterol (33.1±2.8% of ApoE−/− EPCs and 25.9±1.9% of WT EPCs) than in the absence of cholesterol (44.5±8.1% of ApoE−/− EPCs and 35.8±2.7% of WT EPCs); however, apoptosis was significantly higher (P<0.0001) in 300 mg/dL (67.2±6.4% of ApoE−/− EPCs and 60.4±3.7% of WT EPCs) or 500 mg/dL (90.3±3.1% of ApoE−/− EPCs and 84.2±8.1% of WT EPCs) cholesterol than in 0 mg/dL cholesterol.

**Moderate Reduction in Notch1 Expression Enhances EPC Function, Reduces EPC Apoptosis, and Accelerates Reendothelialization**

The cholesterol-independent effects of Notch1 were investigated by comparing EPCs obtained from Tie2-Cre Notch1−/− mice, in which Notch1 expression is reduced by 50%,22 with EPCs from Tie2-Cre (ie, Notch1+/−) mice. All EPC functional measures were significantly greater (P<0.05) in Tie2-Cre Notch1−/− EPCs than in Tie2-Cre EPCs (proliferation: Tie2-Cre Notch1−/−, 1.57±0.12 OD; Tie2-Cre, 1.0±0.0 OD; adhesion: Tie2-Cre Notch1−/−, 102.6±4.0 cells per HPF; Tie2-Cre, 38.8±3.9 cells per HPF; migration: Tie2-Cre Notch1−/−, 115.6±13.2 cells per HPF; Tie2-Cre, 27.8±3.9 cells per HPF); Tie2-Cre Notch1−/− EPCs also exhibited less apoptosis (Tie2-Cre Notch1−/−, 13.1±0.7%.;
Furthermore, exposure to 150 mg/dL cholesterol, which decreased Notch1 expression by \( \approx 50\% \) (Figure 5E), increased Tie2-Cre EPC function (proliferation, 1.65±0.43 OD; adhesion, 89.6±8.8 cells per HPF; migration, 139.3±22.5 cells per HPF) and decreased Tie2-Cre EPC apoptosis (18.4±2.0%) to levels similar to those measured in Tie2-Cre Notch1\(^{+/−}\) EPCs. Treatment of Tie2-Cre Notch1\(^{+/−}\) EPCs with 150 mg/dL cholesterol enhanced proliferation, impaired adhesion and migration, and increased apoptosis, which is consistent with the results obtained when Notch1 expression declined to \(<50\%\) of baseline levels in WT and ApoE\(^{−/−}\) EPCs treated with 300 to 500 mg/dL cholesterol (Figure 5A through 5C). The potential contribution of Notch1 expression to endothelial recovery was evaluated in WT mice transplanted with BM from Tie2-Cre Notch1\(^{+/−}\) or Tie2-Cre mice. Evans blue staining of whole-mount carotid arteries revealed significantly greater (\( P<0.01; n=4 \) in each group) evidence of reendothelialization on day 7 after carotid artery injury in mice transplanted with Tie2-Cre Notch1\(^{+/−}\) BM (75.2±3.2%) than in mice transplanted with Tie2-Cre BM (48.8±5.5%) (Figure 6E and 6F), suggesting that a moderate reduction in Notch1 expression in BM-derived cells promotes endothelial recovery.

**Discussion**

Results from the series of experiments presented here indicate that the number of circulating EPCs and their recruitment to the site of arterial injury are greater in ApoE\(^{−/−}\) mice than in WT mice and that the enhanced recruitment likely evolves from the properties of the EPCs themselves as well as the physiological environment. ApoE\(^{−/−}\) mice also displayed greater evidence of reendothelialization than WT mice, which was somewhat surprising because ApoE\(^{−/−}\) mice are prone to atherosclerosis, and accelerated reendothelialization is commonly believed to inhibit the formation of atherosclerotic lesions.\(^{33–35}\)
general, ApoE\(^{-/-}\) and WT EPCs responded similarly to the presence of cholesterol in vitro. Progressively greater cholesterol concentrations enhanced VEGF expression and EPC proliferation, whereas Notch1 expression declined and apoptosis was biphasic. However, eNOS expression increased with cholesterol exposure in ApoE\(^{-/-}\) EPCs and declined in WT EPCs. In vivo, the recruitment of WT EPCs derived from transplanted BM was higher in ApoE\(^{-/-}\) mice than in WT mice, whereas the recruitment of systemically administered WT EPCs was similar in both strains, perhaps because the duration of exposure to high serum cholesterol levels (ie, the length of time between BM transplantation or EPC injection and the evaluation of recruitment) was 6 to 8 weeks for the transplanted cells compared with just 2 weeks for the injected cells.

The concentration-dependent enhancement of VEGF expression by cholesterol could explain the greater EPC proliferation observed at high cholesterol levels. The secretion of VEGF and other growth factors by EPCs is believed to enhance angiogenesis in ischemic tissues by promoting the proliferation and migration of local endothelial cells,\(^{17-20}\) and this paracrine mechanism could contribute to reendothelialization in response to arterial injury. However, VEGF has also been linked to lesion formation and the progression of atherosclerosis.\(^{36-38}\)

Although mild impairment of Notch1 expression was accompanied by declines in apoptosis both in Tie2-Cre Notch1\(^{-/-}\) EPCs and in EPCs exposed to low cholesterol levels, excessive impairment of Notch1 expression by high cholesterol levels increased apoptosis. This potential biphasic relationship between Notch1 expression and apoptosis has been implied by results from previous investigations indicating that both Notch1 deletion\(^{39}\) and overexpression\(^{40}\) increase apoptosis in endothelial cells. Notch1 may also regulate EPC activity in the presence of cholesterol because moderate cholesterol-induced reductions in Notch1 expression were associated with greater EPC adhesion and migration; however, confirmation of this potential regulatory mechanism requires additional investigation.

**Figure 6.** The influence of Notch1 expression on EPC function, EPC apoptosis, and reendothelialization. A through D, Assessments of EPC function and apoptosis in the presence and absence of 150 mg/dL cholesterol were performed with EPCs obtained from Tie2-Cre mice and from Tie2-Cre Notch1\(^{-/-}\) mice, in which Notch1 expression is reduced by \(\sim 50\%\). A, Proliferation was assessed with optical density (OD) measurements obtained via the colorimetric MTS assay and normalized to the mean OD of Tie2-Cre EPCs in the absence of cholesterol. B, Adhesion was assessed by incubating EPCs for 1 hour on plates coated with the extracellular matrix protein fibronectin and then washing away the nonadherent cells with PBS and counting the adherent cells. C, Migration was assessed with a modified Boyden chamber assay; cell suspensions were placed in the upper chamber; the lower chamber was filled with 50 ng/mL recombinant mouse VEGF protein; and the EPCs that had migrated to the lower chamber were counted 16 hours later. D, Apoptosis was assessed as the number of TUNEL-positive cells observed after 48 hours in culture. E and F, Carotid arteries were harvested 7 days after carotid artery injury in WT mice transplanted with BM from Tie2-Cre or Tie2-Cre Notch1\(^{-/-}\) mice; mice were injected with Evans blue dye 10 minutes before death. E, Representative Evans blue–stained arteries; reendothelialized regions are resistant to the dye and appear white (bar=1 mm). F, Quantification of reendothelialization (n=4 in each group). *\(P<0.05\) vs all other groups; **\(P<0.05\) vs Tie2-Cre Notch1\(^{-/-}\), 0 mg/dL cholesterol and vs Tie2-Cre, 150 mg/dL cholesterol.
Cholesterol increased the expression of eNOS in ApoE<sup>−/−</sup> EPCs and decreased eNOS expression in WT EPCs. eNOS is essential for the mobilization of stem and progenitor cells from the BM, and reendothelialization after carotid artery injury is retarded in both eNOS knockout mice<sup>12</sup> and diabetic mice,<sup>28</sup> in which eNOS expression is attenuated. Thus, the higher circulating EPC counts and accelerated reendothelialization observed in ApoE<sup>−/−</sup> mice might be attributable to enhanced eNOS expression under hypercholesterolemic conditions. However, the recruitment of transplanted WT (Tie2-LacZ) EPCs was greater in the hypercholesterolemic ApoE<sup>−/−</sup> mice than in WT mice despite the cholesterol-induced inhibition of eNOS expression, so the potential relationship between cholesterol, eNOS expression, and EPC recruitment remains uncertain.

Transplanted, BM-derived WT (Tie2-LacZ) EPCs were found at the border of atherosclerotic lesions in ApoE<sup>−/−</sup> mice and were accompanied by ample evidence of apoptosis. Although these results could imply that EPCs participate in the formation of atherosclerotic lesions, the mechanism and consequences associated with the cholesterol-mediated modulation of EPC activity have yet to be determined. Our in vitro results indicate that hypercholesterolemia likely enhances EPC adhesion, migration, and VEGF expression, but these effects might be offset (at least partially) by cholesterol-induced EPC apoptosis, perhaps caused by very high local cholesterol concentrations at the luminal surface. Furthermore, EPCs comprise a heterogeneous cell population, and individual subpopulations of EPCs may function differently during recovery from vascular injury.

**Conclusions**

This investigation provided novel insights into the role of EPCs during recovery from arterial injury and identified the potential importance of cholesterol and Notch1 for the regulation of EPC activity. EPCs appear to participate in reendothelialization, which is enhanced in hypercholesterolemic mice, and BM-derived EPCs and monocyte/macrophages may also contribute to the formation of atherosclerotic lesions. Both cholesterol and Notch1 influence EPC function and viability; however, the influence of cholesterol on many EPC functions is biphasic, so the consequences of EPC activity under hypercholesterolemic conditions warrant further investigation.

**Acknowledgments**

We thank W. Kevin Meisner, PhD, ELS, for editorial support and M. Neely and Ashley Peterson for administrative assistance.

**Sources of Funding**

This study was supported in part by National Institutes of Health grants HL-53354, HL-57516, HL-77428, HL-95874, HL-80137, PO1HL-66957 (to Dr Losordo), and HL-052233 (to Dr Liao).

**Disclosures**

None.

**References**


Abundant evidence has linked hypercholesterolemia with the advent and progression of atherosclerosis. To facilitate the study of the mechanisms by which hypercholesterolemia leads to atherosclerosis, animal models have been developed. Most notably, the apolipoprotein E (ApoE)–null and low-density lipoprotein receptor–null mice have been used extensively in attempts to gain a better understanding of the effects of high lipid levels on atheroma formation. In recent years, traditional cardiovascular risk factors have also been associated with declines in human EPC levels. Collectively, our findings emphasize the need for additional experiments in other species to further investigate the contribution to reendothelialization versus plaque formation in ApoE–/– knockout mice after arterial injury, further investigations of which must be interpreted with caution, particularly because circulating EPC levels were higher in ApoE–/– mice, which was somewhat surprising because ApoE–/– mice are deficient in bone marrow derived EPCs. These findings indicate that EPC recruitment was higher in ApoE–/– mice than in wild-type mice, whereas hypercholesterolemia, hypertension, and many other risk factors for cardiovascular disease are associated with declines in human EPC levels. Collectively, our findings emphasize the need for additional experiments in other hypercholesterolemic animal models and underscore the potential for genetic mouse models of human disease to yield data that do not necessarily reflect clinical reality. To clarify the paradoxical observation on the direct effect of cholesterol on EPC contribution to reendothelialization versus plaque formation in Apo E knockout mice after arterial injury, further investigations using different types of hypercholesterolemic animals and, importantly, additional clinical correlation are required.
Notch Signaling Regulates Endothelial Progenitor Cell Activity During Recovery From Arterial Injury in Hypercholesterolemic Mice
Masaaki Ii, Kyosuke Takeshita, Kayoko Ibusuki, Corinne Luedemann, Andrea Wecker, Elizabeth Eaton, Tina Thorne, Takayuki Asahara, James K. Liao and Douglas W. Losordo

Circulation. published online February 22, 2010;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2010/02/22/CIRCULATIONAHA.105.553917.citation

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2010/02/18/CIRCULATIONAHA.105.553917.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/
**SUPPLEMENTAL MATERIALS**

**Supplemental Methods**

*Bone-marrow transplantation (BMT)*

Donor mice were strain- and age-matched to recipient mice aged 6-8 weeks. The BMT procedure was performed as described previously\(^1,2\) with minor modifications. Briefly, the BM in recipient mice was ablated with lethal irradiation (12 Gy), then mice were transplanted with $5 \times 10^5$ donor BM cells, and the transplanted BM was allowed to regenerate for 4-6 weeks before subsequent experimental procedures were performed. ApoE\(^{-/-}\) mice were fed a 1% cholesterol-containing Western diet after transplantation to maintain elevated serum cholesterol levels.

*Incorporation of infused EPCs*

The incorporation of systemically infused ApoE\(^{-/-}\) and WT EPCs was evaluated in both ApoE\(^{-/-}\) mice and WT mice. The infused EPCs were cultured from mouse BM\(^1,2\) and EPC transfusion was performed as described previously\(^3\). Briefly, mice were splenectomized to prevent homing of the transfused EPCs to the spleen. Seven days later, the carotid denudation procedure was performed, and $1 \times 10^6$ Dil-acLDL-labeled EPCs were injected into the tail vein. Mice were sacrificed 14 days after carotid denudation, and carotid arteries were harvested for *en face* histological assessments. Transfused EPCs were visualized with fluorescent microscopy as described previously\(^3\).
Re-endothelialization

Re-endothelialization was evaluated as described previously\textsuperscript{4,5}; assessments were performed in 1) ApoE\textsuperscript{−/−} and WT mice, 2) ApoE\textsuperscript{−/−} mice transplanted with BM from ApoE\textsuperscript{−/−} or WT donor mice, 3) WT mice transplanted with BM from ApoE\textsuperscript{−/−} or WT donor mice, and 4) WT mice transplanted with BM from Tie2-Cre Notch1\textsuperscript{+/−} or Tie2-Cre donor mice. Briefly, Evans blue dye (Sigma-Aldrich Co., St. Louis, MO, USA) diluted with saline (5\%) was injected into the heart, the heart was perfused with saline to wash out the whole blood and excess dye, then the stain was fixed via perfusion of 4\% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Ten minutes after PFA perfusion, mice were sacrificed, and carotid arteries were harvested for en face histological assessments; regions of damaged endothelium incorporate the stain and appear blue whereas re-endothelialized tissue is resistant to the dye. The areas stained blue and the unstained areas were outlined and measured with Image J software.

Cellular contributions to atherosclerotic lesion formation

The formation of atherosclerotic lesions was studied in ApoE\textsuperscript{−/−} and WT mice transplanted with BM from Tie2-LacZ donor mice. Mice were sacrificed 2, 4, and 6 weeks after carotid artery denudation, then carotid arteries were harvested, paraffin embedded, and sectioned. Lesion formation was visualized with Masson’s trichrome staining, then the intima/media ratio was calculated and reported as a percentage. Evidence of apoptosis and β-galactosidase (β-gal) expression (signaling the presence of BM-derived endothelial cells) or apoptosis and CD68 expression (signaling the presence of monocytes/macrophages) was evaluated via double immunofluorescent
staining. Sections were stained for apoptosis by using a fluorescein in situ cell-death detection kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland) according to the manufacturer’s instructions. β-gal expression was visualized by overnight incubation with rabbit polyclonal anti-β-gal antibody (1:1000, Cappel Laboratories Inc., Cochranville, PA, USA) at 4°C followed by a 30-minute incubation with Cy3-goat anti-rabbit IgG antibody (1:2000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). CD68 expression was visualized by overnight incubation with anti-CD68 antibody (1:100) (AbD Serotec, Oxford, UK) at 4°C followed by a 30-minute incubation with Cy3-rabbit anti-rat IgG antibody (1:500, Jackson ImmunoResearch Laboratories Inc.). Nuclei were counterstained with DAPI (1:5000, Sigma-Aldrich, Co.), then sections were mounted in aqueous mounting medium, and images were examined under a fluorescent microscope (Nikon ECLIPSE TE200, Nikon Inc., Melville, NY, USA).

**EPC function (proliferation, adhesion, and migration) and apoptosis**

EPC functional assays were performed with EPCs isolated from the BM of ApoE−/− mice, WT mice, Tie2-Cre mice, and Tie2-Cre Notch1+/− mice. The effect of cholesterol on EPC function was determined by repeating the assays in the presence of 0, 50, 150, 300, and/or 500 mg/dL cholesterol (Sigma-Aldrich Co.).

Proliferation was evaluated by using a colorimetric MTS assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer’s instructions. Cells (1×10^4 cells/well) were seeded on 96-well plates and cultured in 0.5% fetal bovine serum (FBS) for 24 hours, then the culture medium was replaced with 5% FBS/EBM-2
medium supplemented with cholesterol. After 72 hours in culture, proliferation was visualized with MTS dye, and the optical density (OD) at 490-nm wavelength was measured in 8 wells with a plate reader. Proliferation was reported as the mean relative OD (i.e., normalized to the mean OD of WT or Tie2-Cre EPCs in the absence of cholesterol).

Adhesion was evaluated as described previously. Briefly, EPCs (2.5×10^4 cells/well) in 5% FBS/EBM-2 medium supplemented with cholesterol were seeded on 96-well plates precoated with vitronectin, collagen type I, fibronectin, and laminin (Sigma-Aldrich, Co.) and incubated for 1 hour at 37°C under 5% carbon dioxide, then washed 3 times with PBS. The attached cells were fixed and stained with DAPI, then visualized under a fluorescent microscope (10× magnification). The adhered cells were counted in 8 wells, and adhesion activity was reported as the mean number of attached cells.

Migration was evaluated with a modified Boyden’s chamber assay as described previously. Briefly, cells suspended in EBM-2 medium supplemented with cholesterol were placed in the upper chamber (5×10^4 cells/chamber), and the lower chamber was filled with medium containing 50 ng/mL recombinant mouse VEGF protein (R&D Systems, Inc., Minneapolis, MN, USA). The chamber was incubated for 16 hours at 37°C under 5% carbon dioxide, then the EPCs that had migrated into the lower chamber were fixed with 2% PFA/PBS for 10 minutes and stained with DAPI. Migrated cells were viewed under a fluorescent microscope (40× magnification) and counted in 4 chambers,
5 HPFs per chamber. Migration activity was reported as the mean number of migrated cells.

Apoptosis was evaluated by the TUNEL method with an *in situ* cell-death detection kit (F. Hoffmann-La Roche Ltd) according to the manufacturer’s instructions. Briefly, cells (1×10⁵ cells/well) were seeded on 4-well glass slides and cultured in 0.5% FBS for 24 hours, then the culture medium was replaced with 5% FBS/EBM-2 medium supplemented with cholesterol. After 48 hours in culture, cells were fixed with 2% PFA/PBS, TUNEL stained, and viewed under a fluorescent microscope (20× magnification). The number of TUNEL positive cells and the total number of cells were determined in 5 HPFs per well. Apoptosis was reported as the mean percentage of TUNEL-positive cells per HPF.

*Quantitative real-time RT-PCR*

EPCs were isolated from the BM of ApoE−/− and WT mice and lysed with RNA-Stat (Tel-Test, Inc., Friendswood, TX, USA), then RNA was extracted and reverse transcribed by using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Amplification was performed with a Taqman 7300 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Primer and probe sequences are indicated below. Relative mRNA expression was calculated with the comparative C_T method (relative expression=2^ΔCT) and normalized to the expression of the endogenous 18S gene.
VEGF primers: forward, CATCTTCAAGCCGTCCTGTGT; reverse, CAGGGCTTCATCGTTACAGCA. VEGF probe: CCGCTGATGCGCTGTGCAGG.

Endothelial nitric oxide synthase (eNOS) primers: forward, TCTGCGCGATGTCACTATG; reverse, CATGCCGCCCTCTGTGTTG. eNOS probe: AACCAGCGTTCCTGCAAACCGTG.

Notch1 primers: forward, CGTGGTCTTCAAGCGTGATG; reverse, GCTCTTCTCGTGCCCATAG. Notch1 probe: CAAGGCCAGCAGATGATCTTCCCG.
Supplemental Table. *Serum Cholesterol Levels (mg/dL)*

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Before Injury</th>
<th>Time After Arterial Injury (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>WT</td>
<td>51.4±4.4</td>
<td>52.8±6.8</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>458.0±38.6</td>
<td>445.9±30.7</td>
</tr>
</tbody>
</table>

*Within each mouse strain, cholesterol levels before injury did not differ significantly from the levels observed at subsequent time points.*
Supplemental References


