High-Density Lipoproteins Enhance Progenitor-Mediated Endothelium Repair in Mice

Colin Tso, Gary Martinic, Wen-Hua Fan, Campbell Rogers, Kerry-Anne Rye, Philip J. Barter

Objective—We quantified endothelial progenitor cell (EPC) engraftment into the endothelial layer as an index of progenitor-mediated endothelial repair. Studies were conducted in C57BL/6J and in apolipoprotein E–deficient (apoE−/−) mice. We also investigated the possibility that high-density lipoproteins (HDL) may promote progenitor-mediated endothelial repair.

Methods and Results—Thoracic aortic sections from C57BL/6J and apoE−/− mice were analyzed for evidence of progenitor-derived endothelium as determined by the number of stem cell antigen-1–positive (Sca-1+) cells in the endothelial layer. EPCs (Sca-1+ cells) were significantly increased after endothelial damage induced by lipopolysaccharide (LPS) administration in C57BL/6J mice. The number of EPCs was greater in the aortic endothelium of untreated apoE−/− than in untreated C57BL/6J mice and was similar to the number observed in LPS-treated C57BL/6J mice. The number of EPCs in the aortic endothelium of apoE−/− mice more than doubled after intravenous infusion of reconstituted HDL.

Conclusions—EPCs are recruited into the aortic endothelial layer of mice in response to an inflammatory insult. EPCs are also increased in the aortic endothelium of untreated apoE−/− mice. The observation that number is further increased in apoE−/− mice after injection of HDL suggests a role for HDL in promoting progenitor-mediated endothelial repair. (Arterioscler Thromb Vasc Biol. 2006;26:1144-1149.)

Key Words: endothelial progenitor cells | high-density lipoproteins | mouse | endothelial damage

High-density lipoproteins (HDL) protect against the development of atherosclerosis.1 The mechanism is still uncertain, although it most probably reflects 1 of the known functions of HDL. The best known of these relates to the ability of HDL to promote the efflux of cholesterol from cells in the artery wall.2 However, other mechanisms relating to antioxidant, anti-inflammatory, and antithrombotic properties of HDL may also be important. In the study reported in this article, we consider an additional potential function by investigating a role for HDL in promoting the repair of injured endothelium by stimulating the recruitment of endothelial progenitor cells (EPCs) into the endothelial layer.

See page 965

Endothelial health reflects a balance between injury and repair and is a key component of atherosclerosis. Although there is much information about factors that cause endothelial injury, much less is known about processes that promote endothelial repair. Mitosis of neighboring endothelial cells is traditionally considered to be the exclusive source for replacing damaged cells. However, progenitor cells from bone marrow and peripheral tissues have recently been shown to function as EPCs.3-6 In the study reported in this article, we consider the possibility that EPCs contribute to the process of endothelial repair and then investigate the effects of HDL on the process.

In vivo mouse studies have shown engraftment of pluripotent progenitor cells into disease organ as a repair mechanism, resulting in restoration of organ function.7 Further, EPCs have been found to participate in re-endothelialization after wire injury to mouse carotid arteries8 and balloon denudation of rat carotid arteries.9 In the present study, we investigated the possibility that incorporation of EPCs (identified by virtue of their expression of stem cell antigen-1 [Sca-1]) into damaged endothelium contributes to endothelial repair after nondenuding endothelial damage in C57BL/6J mice and in an atherosclerosis-prone mouse strain.

Sca-1, also known as Ly-6A/E, is an 18-kDa murine cell surface glycoprotein. It is a well-recognized marker of hematopoietic stem cells and has been used for the enrichment of progenitor cells.10-12 Further, mouse EPCs also express Sca-1, and these cells differentiate into endothelial cells both in vitro and in vivo,13,14 although under normal conditions Sca-1–positive (Sca-1+) cells have not been reported in the endothelium of large arteries such as the thoracic aorta. In contrast, we find a substantial presence of Sca-1+ cells in the aortic endothelium of mice subjected to nondenuding endo-
thelial damage and in a mouse model of atherosclerosis. We also found that injection of reconstituted HDL (rHDL) into these animals further increases the proportion of Sca-1+ cells in the damaged endothelium, suggesting an additional mechanism by which HDL protect against endothelial damage.

Materials and Methods

Animals
The mice used in this study were obtained from Jackson Laboratory (Bar Harbor, Me) and Animal Resources Center (Western Australia). The animals were housed at the Heart Research Institute animal facility in Sydney, Australia, in accordance with the South West Sydney Area Health Service Animal Welfare Committee guidelines and at Brigham and Women’s Hospital in Boston, Mass, in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. Two groups of mice were studied: male C57BL/6J mice, and male apolipoprotein E–deficient (apoE−) C57BL/6JApoe<tm1Unc129> mice as an atherosclerosis-prone mouse model. All mice were fed normal chow for the entire period of the experiment. The animals were euthanized by administering an overdose of methoxyfluorane followed by right atria exsanguinations, after which their thoracic aortae were removed for further analysis.

Treatment With Lipopolysaccharide
Nondenuding endothelial injury was induced in 15-week-old C57BL/6J and apoE−/− mice by the intraperitoneal administration of lipopolysaccharide (LPS; Sigma-Aldrich) at a dose of 50 μg per animal. To establish the optimal time to investigate the effects of LPS, C57BL/6J mice were euthanized at 0 hours (untreated controls; n=4), 3 hours (n=2), 6 hours (n=2), 15 hours (n=3), and 24 hours (n=3) after receiving the treatment. On the basis of the results of these time course studies, the experiments designed to quantitate incorporation of Sca-1+ cells into the endothelium of C57BL/6J mice (n=10) and apoE−/− mice (n=7) were performed 18 hours after administration of LPS. Untreated animals from the same batches of C57BL/6J (n=5) and apoE−/− (n=5) mice served as controls.

Effects of LPS on circulating progenitor cells were assessed with flow cytometry in C57BL/6J mice euthanized 5 hours after administration of LPS (n=3). Untreated animals from the same batch served as controls (n=3). A total of 500 μL of blood was obtained from the left ventricles at the time of euthanization. Red blood cells were lysed with Red Cell Lysing Buffer (Sigma), and the remaining white blood cells were stained with R-Phycocerythrin–conjugated anti-fetal liver kinase 1 (flk-1) antibody (BD Biosciences).

Preparation of rHDL
Discoidal rHDL were prepared as described previously.15 Briefly, HDL were obtained from samples of expired human plasma (Gribbles Pathology) by sequential ultracentrifugation (1.07<density<1.21 g/mL). The HDL were delipidated and apoA-I was isolated by anion exchange chromatography on a column of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) attached to a fast protein liquid system. Discoidal rHDL containing apoA-I as their sole protein constituent and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) as their sole lipid were prepared by the cholate dialysis method and dialyzed against 5×1 L endotoxin-free PBS, pH 7.4, before use. The molar ratio of POPC:apoA-I was 100:1.

Processing of Aortic Samples
Thoracic aortae were perfusion-fixed with 5% neutral buffered formalin and embedded in paraffin. Cross-sections (4 μm) were obtained from 4 to 6 planes throughout the length of the thoracic aorta, which were then subjected to antigen retrieval by heating in Target Retrieval Solution (DakoCytomation). Serial sections 5 μm apart were peroxidase-blocked and overlaid with rat anti-mouse Sca-1 (BD Biosciences), rabbit anti-e-c-kit (DakoCytomation), rabbit anti-fox-1 (Abcam), rabbit anti-Ki67 (Abcam), and rat anti-mouse CD45 (BD Biosciences) antibodies. Rat IgG (BD Biosciences) and rabbit IgG (DakoCytomation) were used as control antibodies. The sections were incubated with biotinylated secondary antibodies and visualized with a streptavidin-peroxidase/diaminobenzidine system (DakoCytomation) and counterstained with hematoxylin. Endotheli-um was identified with biotinylated Bandeirea Simplicifolia lectin 1 (BS-1 lectin; Vector). Apoptotic studies were performed on the aortic sections from the LPS-treated and control C57BL/6J mice using a terminal deoxynucleotidyltransferase–mediated X-dUTP nick end labeling kit (TUNEL; Roche) and visualized with an Alkaline Phosphatase/Permanent red system (Dako).

Endothelial Sca-1+, TUNEL+, and total cell counts were manually counted at ×40 magnification under light microscopy on 4 to 5 evenly spaced sections throughout the length of the aorta in each animal and then averaged. Two-tailed unpaired Student t test was used to compare the results from the treatment groups, and P<0.05 was considered statistically significant.

Results

Endothelial Apoptosis and Sca-1+ Cells in the Endothelium of C57BL/6J Mice: Effects of LPS
Apoptotic (TUNEL+) cells were seen in the endothelium and media of the C57BL/6J mice 5 and 18 hours after LPS.
administration. The mean percentage of endothelial TUNEL+ cells was 33.7±29.8% in the 5-hour post-LPS mice and 15.3±23% in the control mice (P=NS). The proportion of endothelial apoptotic cells was increased to 50.7±17.5%×18 hours after LPS and 11.3±16% in the control mice (P<0.01) (supplemental data, available online at http://atvb.ahajournals.org). This is consistent with rapid and substantial LPS-induced endothelial apoptotic cell loss. Expression of the cellular proliferation marker Ki67 was seen in the media, but there was no significant endothelial Ki67 expression 5 or 18 hours after LPS (data not shown), indicating a lack of endothelial proliferative response to the insult.

In the absence of treatment with LPS, there was at most a minimal presence of Sca-1+ cells in the thoracic aortic endothelium of C57BL/6J mice (Figure 1A), nor were Sca-1+ cells seen in 3-hour post-LPS animals (Figure 1B). However, by 6 hours after receiving LPS, there was an obvious appearance of clusters of Sca-1+ cells with typical endothelial morphology in the thoracic aortic endothelial layer of these animals (Figure 1C). Sca-1+ cells were also seen in the adventitia of the vessel wall. The endothelial Sca-1+ cells were more prominent 15 hours (result not shown) and 24 hours (Figure 1D) after the LPS administration and had a patchy distribution.

There were no c-kit+ or flk-1+ cells in the thoracic aortic endothelium of control C57BL/6J mice (result not shown), but clusters of such cells were apparent 18 hours after administration of LPS (Figure 2A and 2B). Evidence that the Sca-1+ cells that appeared in the aortic endothelium after LPS stimulation (Figure 1) had an endothelial phenotype was provided by the uniform BS-1 lectin staining of the aortic endothelium of these animals (Figure 2C). There were no cells expressing the hematopoietic marker CD45 (expressed by all hematopoietic cells except red blood cells) in the endothelial layer of aortae from either control or 18 hours after administration of LPS; however, there was an appearance of CD45+ cells in the aortic adventitia 18 hours after LPS stimulation (result not shown). The presence in the endothelium of BS-1–positive cells that express the progenitor markers Sca-1, c-kit, and flk-1 but not CD45 is indicative of the presence of progenitor-derived endothelial cells.

The proportion of Sca-1+ cells in the endothelium was quantified in untreated and LPS-treated C57BL/6J mice. In this batch of animals, Sca-1+ endothelial cells were apparent in the untreated animals, but the percentage was significantly increased after LPS administration (19±13% in untreated C57BL/6J mice versus 50±19% in LPS-stimulated animals; P=0.02).

Figure 1. Time course of appearance of Sca-1+ cells in the aortic endothelial layer after administration of LPS. The photomicrographs (×60 magnification) show Sca-1+ cells (brown) in the C57BL/6J mouse aortic endothelium. A, No LPS. B, 3 hours after LPS. C, 6 hours after LPS. D, 24 hours after LPS. Each photomicrograph is representative of a study that was conducted in 2 or 3 mice.

Figure 2. Characterization of cells in the aortic endothelium after administration of LPS. The photomicrographs (×100 magnification) show immunohistochemical analyses of sections from C57BL/6J mouse thoracic aortae 18 hours after LPS stimulation. A, C-kit: the brown staining in the endothelial layer identifies c-kit+ cells. B, Flk-1: the brown staining in the endothelial layer identifies flk-1+ cells. C, BS-1 lectin: the uniform brown staining in the endothelial layer is BS-1 lectin. Each photomicrograph is representative of a study that was conducted in each of 10 mice.
Flow cytometry analysis revealed that the proportion of circulating flk-1+ cells was significantly reduced 5 hours after LPS treatment (0.2±0.06% in LPS-treated C57BL/6J mice versus 1.1±0.3% in untreated controls; P<0.01).

**Sca-1 Expression in Cultured Mouse Endothelium**

To exclude the possibility that Sca-1 expression may be attributable to upregulation by inflammatory stimuli acting on pre-existing endothelial cells, C57BL/6J mouse endothelial cells were incubated with either LPS or TNF-α for 6 hours before assessing Sca-1 expression by Western blot analysis. Neither TNF-α nor LPS had any effect on Sca-1 expression in these mature endothelial cells (Figure 3), further supporting a progenitor origin of the Sca-1+ cells that appeared in the endothelium in vivo after LPS administration.

**Sca-1+ Cells in the Endothelium of ApoE−/− Mice**

Immunohistochemical staining of the thoracic aortic endothelium of untreated apoE−/− mice showed a similar pattern of antigen expression as was observed in the LPS-stimulated C57BL/6J mice. There were clusters of Sca-1+, c-kit+, and flk-1+ cells in the aortic endothelium of these animals (result not shown), and, as in the LPS-stimulated C57BL/6J mice, the endothelial layers of these apoE−/− animals were uniform stained with BS-1 lectin (result not shown). There were no CD45+ cells in the aortic endothelial layer of the apoE−/− mice. As was concluded above from the results in the LPS-stimulated C57BL/6J mice, these findings are consistent with the presence of progenitor-derived endothelium in these animals. As shown in Figure 4, Sca-1+ cells accounted for 50±4% of the endothelial cells in the thoracic aortic endothelium of the apoE−/− mice, a level comparable to that seen in C57BL/6J mice after LPS treatment. However, in contrast to the results in C57BL/6J mice, the percentage of Sca-1+ cells in the aortic endothelium of apoE−/− mice was not significantly increased by administration of LPS (50±4% versus 57±6%; P=NS; Figure 4).

**The Effects of rHDL on Endothelial Sca-1+ Cells and Circulating Flk-1+ Cells**

rHDL infusion did not increase the percentage of Sca-1+ cells in the uninjured thoracic aortic endothelium of the C57BL/6J mice (result not shown). In contrast, rHDL infusion into apoE−/− mice more than doubled the percentage of Sca-1+ cells in the endothelium (35±8% in the untreated apoE−/− mice; 79±7% in the animals receiving rHDL; Figure 5). The endothelial phenotype of the Sca-1+ cells incorporated into the endothelium of the apoE−/− mice after administration of rHDL was confirmed by the uniform BS-1 lectin and the lack of CD45 staining of the endothelial layer in the serial sections (result not shown).

The proportion of circulating flk-1+ cells in the apoE−/− mice was reduced 5 hours after rHDL administration (0.22±0.04% in rHDL-treated apoE−/− mice versus 0.42±0.2% in the PBS-treated apoE−/− control mice; P<0.05).

The effects of rHDL on the percentage of circulating flk-1+ and endothelial Sca-1+ cells of apoE−/− mice were

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**Figure 3.** Effect of LPS and TNF-α on Sca-1 protein expression in cultured C57BL/6J mouse endothelial cells (EC). Cultured C57BL/6J mouse endothelial cells were either untreated or treated with LPS or TNF-α before determining Sca-1 protein expression by Western blot analysis. Mouse thymus extract was used as a positive control for Sca-1. This result is representative of a study that was conducted in duplicate.

**Figure 4.** Effects of LPS on the percentage of aortic endothelial cells staining positive for Sca-1. Nondenuing endothelial injury was induced in 15-week-old C57BL/6J (n=10) and apoE−/− (n=7) mice by the intraperitoneal administration of LPS. Animals were euthanized 18 hours after receiving the injections, and the aortae were removed for quantification of Sca-1+ cells. Untreated animals from the same batches of C57BL/6J (n=5) and apoE−/− (n=3) mice served as controls.

**Figure 5.** Effects of rHDL administration on the percentage of aortic endothelial cells staining positive for Sca-1. rHDL were administered intravenously via tail vein to 10- to 14-week-old male apoE−/− mice (n=6). Animals were euthanized 18 hours after receiving the injections, and the aortae were removed for quantification of Sca-1+ cells. Untreated animals (n=4) from the same batch of mice served as controls.
achieved with amounts of rHDL equivalent to <50% of the total plasma apoA-I pool, which would have increased the plasma concentration of apoA-I immediately after the injection by <50%. The precise increase in apoA-I concentration could not be measured because the apoA-I circulating after the injections would have represented a mixture of murine and human apoA-I. Eighteen hours after the rHDL injection, there was no measurable effect on either the total or the HDL cholesterol concentrations (result not shown).

**Discussion**

The results of these studies showing an increase in Sca-1+ cells in the thoracic aortic endothelium of C57BL/6J mice treated with LPS and in apoE−/− mice are consistent with a role of EPCs in repairing the endothelial damage associated with these conditions. A number of mouse studies have reported the participation of EPCs in vascular repair after nonmechanical endothelial injury.16,17 However, this process has not been quantified previously, and factors that may promote such repair have not been assessed. The additional observation that Sca-1+ cells are further increased in the endothelium of apoE−/− mice after infusion of rHDL is consistent with a novel function of HDL in promoting the repair of damaged endothelium.

Intraperitoneal LPS administration is an established method for inducing nondenuding endothelial injury and inflammation.18–21 In the current study, we find that the relatively high LPS dosage leads to rapid and substantial apoptotic endothelial cell loss in the thoracic aorta that is not matched by an endothelial cellular proliferative response. Hence, the high percentage of Sca-1+ matched by an endothelial cellular proliferative response. The precise origin of these Sca-1+ cells is not known, but they are likely to be derived from multiple sources. The bone marrow is a well-documented source of progenitor cells. However, a recent report demonstrated that the media of the vascular wall is also a rich source of progenitor cells, with up to 6% of the cells being “side population” (SP) progenitors that express Sca-1. These cells lie in close proximity to the endothelial layer and have a capacity to differentiate into endothelium in vitro. Thus, it is highly probable that these medial SP cells will participate in endothelium repair after injury. Another potential source is the adventitia Sca-1+ cells known to exist in the vascular adventitia.22 These adventitia Sca-1+ cells were also seen after LPS treatment in the present study. In this report, we did not attempt to determine the origin and relative contribution from various sources of these Sca-1+ cells that appear in the thoracic aortic endothelium. Rather, we sought to determine whether such cells (regardless of their origin) play a role in the repair of damaged endothelium and to determine the effect of HDL on this process.

Sca-1, c-kit, and flk-1 are commonly used markers to identify progenitor cells. Sca-1 is widely expressed by numerous types of murine progenitor cells, including hematopoietic stem cells,10–12 EPCs,13 mesenchymal stem cells,23 and SP cells.24–26 T27 and peripheral B lymphocytes28 also express Sca-1. Hence, it is not an ideal marker to differentiate circulating progenitor cells from other blood cells. Some nonhematopoietic cells, including the endothelium of pulmonary and renal vasculature, also express Sca-1,11,24 but Sca-1 expression has not been reported in the thoracic aortic endothelium. Thus, it is an appropriate marker to differentiate progenitor cells from mature endothelial cells.

Flk-1 (vascular endothelial growth factor receptor 2) is expressed by embryonic endothelium but is not commonly seen in the endothelium of mature animals in vivo.29 It is an established marker of circulating progenitor cells with hemangioblastic activities30 and is a useful marker to differentiate circulating EPCs from other hematopoietic cells.

In this study, the endothelial Sca-1+ cells that appeared in the LPS-stimulated C57BL/6J and apoE−/− mouse aortae are unlikely to be pre-existing endothelial cells in which Sca-1 was expressed in response to the inflammatory insult. First, the extent of LPS-induced cell loss was not matched by cellular proliferation. Second, the appearance of Sca-1 was paralleled by the endothelial appearance of other progenitor markers, in particular, c-kit, which is not upregulated by inflammatory stimulation in cultured human umbilical vein endothelial cells.31 Third, we found that stimulation with either LPS or TNF-α did not upregulate Sca-1 expression in cultured mouse endothelium. Fourth, rHDL (that have anti-inflammatory properties) increased the percentage of Sca-1+ cells in the aortic endothelium of apoE−/− mice, whereas LPS had no such effect in these animals. The absence of CD45-expressing cells in the endothelial layer excluded the presence of hematopoietic cells such as T or B lymphocytes. Thus, the Sca-1+ cells in the aortic endothelium in LPS-stimulated C57BL/6J and apoE−/− mouse aortae are unlikely to have been derived from pre-existing endothelium, and they are nonhematopoietic cells. Most likely, they represent progenitor-generated endothelial cells that have been recruited into the endothelium, consistent with a repair process.

Serum lipoproteins are known to modulate EPC functionality, but available data are scarce. EPCs isolated from patients with high serum levels of low-density lipoproteins (LDL) have lower migratory activity,32 whereas oxidized LDL have been reported to inhibit vascular endothelial growth factor–induced EPC differentiation.33 The effect of HDL on EPC functionality is also poorly understood. The endothelium of apoE−/− mice is known to be under oxidative stress,34 with increased expression of inflammatory adhesion molecules.35 The high level of Sca-1+ endothelial cells in the apoE−/− mice suggests an active progenitor-mediated endothelial repair process. The differing effects of rHDL on apoE−/− and uninjured C57BL/6J mice are consistent with a proposition that rHDL enhance progenitor-mediated endothelial repair. This is achieved by promoting progenitor recruitment rather than enhancing progenitor release from the bone marrow because the circulating level of progenitor cells was not increased after rHDL infusion. This is a potentially important atheroprotective mechanism to be added to the other known protective properties of these lipoproteins. It is worth noting that these effects were achieved by injection of relatively small amounts of the rHDL. Mice typically have an apoA-I concentration of 0.6 to 1.0 mg/mL. With a plasma volume of ~1 mL, an injection of rHDL containing 0.25 mg
per mouse would have increased the plasma apoA-I concentration by <50% immediately after the injection.

In conclusion, this study has demonstrated substantial endothelial engraftment of EPCs into the thoracic aortae of C57BL/6j mice after LPS insult and in apoE−/− mice, consistent with a process of progenitor-mediated endothelial repair that is enhanced by rHDL administration.

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