Sustained Inhibition of Oxidized Low-Density Lipoprotein Is Involved in the Long-Term Therapeutic Effects of Apheresis in Dialysis Patients

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Objective—Low-density lipoprotein (LDL) apheresis is a potential therapy for conventional therapy–resistant peripheral artery disease. In the present study, we examined the chronic effects of LDL apheresis on clinical parameters in vivo and endothelial cell functions in vitro in hemodialysis patients who had the complication of peripheral artery disease.

Methods and Results—Twenty-five patients were enrolled, and the responses of 19 patients to LDL apheresis were analyzed. Patients were classified into 2 groups according to change in ankle-brachial pressure index (ABI) after treatment: patients with improved ABI (responders, n = 10) and patients with worsened ABI (nonresponders, n = 9). In the responders, apheresis resulted in a long-term reduction of circulating levels of oxidized LDL, C-reactive protein, and fibrinogen. In human umbilical vein endothelial cells (HUVECs), the serum from the responders increased expression of activated endothelial nitric oxide synthase protein and proliferative activity. Furthermore, there was a significant correlation between ABI and activated endothelial nitric oxide synthase protein level in HUVECs treated with responder serum (R = 0.427, P < 0.05).

Conclusion—These results demonstrate that LDL apheresis decreases oxidized LDL and inflammation and improves endothelial cell function in the responders. This may be one of the mechanisms involved in the long-term therapeutic effect of LDL apheresis on peripheral circulation in hemodialysis patients. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: atherosclerosis ■ endothelium ■ nitric oxide synthase ■ oxidized lipids ■ peripheral arterial disease ■ lipoproteins ■ oxidative stress

Cardiovascular disease is the primary cause of death in patients with end-stage renal disease. Patients on dialysis are reported to have a 10–20-fold greater risk of cardiovascular disease–associated mortality than the general population after stratification for age, gender, race, and the presence or absence of diabetes. Patients undergoing dialysis have many of the risk factors for atherosclerosis, such as hypertension, dyslipidemia, and disturbed calcium-phosphate metabolism, and, indeed, they commonly experience severe atherosclerosis, including peripheral artery disease (PAD). Low-density lipoprotein (LDL) apheresis is a potentially useful treatment for conventional therapy–resistant hypercholesterolemic patients with coronary artery disease and PAD.1,2 Previously, it was shown that a single LDL apheresis session enhanced the peripheral microcirculation, probably by increasing the production of nitric oxide (NO) and bradykinin,3 reducing blood viscosity and adhesion molecules,4 and inducing endothelium-dependent vasodilatation.5 However, the precise molecular mechanism of the long-term effects of LDL apheresis on the improvement of the peripheral circulation remains unclear and warrants further investigation.

We undertook the present study to investigate the short- and long-term effects of LDL apheresis on clinical and laboratory parameters in vivo and vascular endothelial cell function in vitro, in hemodialysis patients with PAD, and to identify factors related to the improvement of the peripheral circulation by LDL apheresis.

Methods

Patients and Study Design
The study protocol was approved by the Human Ethics Committee of Yokohama City University Hospital. A total of 25 consecutive hemodialysis patients with leg impairments and ankle-
brachial pressure index (ABI) values less than 0.9 were recruited in Yokohama City University Hospital, and written informed consent was obtained from all participating patients. All patients were being treated with cilostazol, aspirin, icosapentate ethyl, ticlopidine, or sarpogrelate. The drug therapy was not changed during the study period. Of the 25 patients enrolled in this study, 5 patients could not complete the study because of death (n=3), amputation (n=1), or percutaneous transluminal angioplasty (n=1) during the study period, and they were excluded from the analysis.

LDL apheresis was carried out once or twice a week on nonhemodialysis days, and 10 sessions of apheresis were performed for each patient. The absolute walking distance and ABI were principally estimated on nonhemodialysis days before the 1st and 10th sessions and at 3 months after the end of treatment. ABI was measured by an oscillometric method using the Form PWV/ABI (AT Co.). Because most participating patients were unable to perform treadmill exercise because of conditions such as previous heart attack or paralysis, the absolute walking distance was evaluated by medical staff on a flat floor in the hospital. Blood samples were collected before and after the first session, at the start of the 10th session, and at 3 months after the end of treatment (before regular hemodialysis).

**LDL Apheresis Technique**

LDL apheresis was performed using hollow polysulphone fibers (Sulflux, Kaneka) as the plasma separator and a dextran sulfate cellulose column (Liposorber LA-15, Kaneka) as the LDL absorber. Blood flow from the arteriovenous fistula access was in the range of 80 to 100 mL/min, the plasma flow was 25 to 30 mL/min, and 3000 to 4000 mL of the plasma volume was treated per session.

**Human Endothelial Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo. HUVECs were maintained in HuMedia-EG2 medium (Kurabo) and were cultured as described previously. Cells from the fourth to seventh passages were used for all experiments.

**Determinantion of the Total and Activated Endothelial Nitric OxideSynthase Protein Levels**

HUVECs were cultured for 24 hours in HuMedia-EG2 medium containing 50% serum from the patients. Western blot analysis was performed essentially as described previously. Briefly, whole cellular extracts from the HUVECs were loaded on 5% SDS-polyacrylamide gel electrophoresis gels. After protein transfer, membranes were immunoblotted with either anti-human endothelial nitric oxide synthase (eNOS) polyclonal antibody (Transduction Laboratories) or anti-human phospho-eNOS (Ser-1177) polyclonal antibody (Cell Signaling Technology). The protein levels were measured with densitometry using NIH ImageJ and were expressed relative to those achieved with HUVECs treated with the serum at baseline. Activated eNOS protein levels were normalized to the signal generated by the probe for the total eNOS protein.

**Cell Proliferation Assay**

HUVECs were seeded at 5×10⁴ cells per well in a 96-well collagen I–coated plate (Becton Dickinson) and cultured for 48 hours in growth factor–depleted medium for preparation of the growth-arrested condition before the experiment. The growth factor–depleted medium contained HuMedia-EB2 and 2% FBS, but neither human epidermal growth factor nor human fibroblast growth factor. The HUVECs were incubated with a fresh growth factor–depleted medium containing 50% serum from patients for 24 hours. The proliferation activity assay was conducted by 5-bromo-2′-deoxyuridine (BrdUrd) incorporation assay, essentially as described previously. The absorbance of samples was measured using a plate reader (ImmunoReader NJ-2100, InterMed) at 405 nm, with a reference wavelength at 490 nm. The mean absorbance was calculated for each of 10 samples.

**Tube Formation Assay**

Experiments on tube formation were conducted in triplicate in 24-well dishes using an angiogenesis assay kit (Kurabo) according to the manufacturer’s instructions. Briefly, HUVECs cocultured with fibroblasts were cultivated in the medium containing 5% serum from patients. After 11 days, cells were fixed in 70% ethanol and then visualized with CD31 antibody (Kurabo). Vascular endothelial growth factor (VEGF) (10 ng/mL) was also tested as a positive control. Tube length was quantified using angiogenesis imaging software (Kurabo).

**Statistical Analysis**

Data are expressed as the mean±SE. The significance of the changes before versus after treatment was analyzed by the Wilcoxon single-rank test. Differences between responders and nonresponders were determined by the Student t test, Welch t test, or Mann-Whitney test. P<0.05 was deemed to be statistically significant.

**Results**

**Effects of LDL Apheresis on Clinical Parameters**

Of the 20 patients, 1 patient experienced a worsening of leg pain despite ABI improvement and was excluded from the analysis. Nineteen patient responses were investigated. The absolute walking distance improved significantly at the 10th session of LDL apheresis compared with baseline (from 171±33 m to 294±34 m, P<0.05) and even at 3 months after the end of the treatment (from 171±33 m to 270±42 m, P<0.05). Similarly, the ABI improved at the 10th session compared with baseline (from 0.59±0.04 to 0.67±0.04, P<0.05). Subsequently, the patients were classified into 2 groups according to the changes in the ABI at 3 months after the end of treatment. The 2 groups were patients with improved ABI (responders, n=10) and patients with worsened ABI (nonresponders, n=9). In the responders, the absolute walking distance and the ABI were significantly increased at the 10th session compared with baseline (from 118±26 m to 333±45 m, P<0.05; from 0.53±0.06 to 0.69±0.06, P<0.005; absolute walking distance and ABI, respectively) and even at 3 months after the end of treatment (from 118±26 m to 297±63 m, P<0.05; from 0.53±0.06 to 0.69±0.05, P<0.005). On the other hand, neither absolute walking distance nor ABI showed any statistically significant change during the study period in the nonresponders. The baseline characteristics in each group are shown in Table 1. There was no significant difference between the 2 groups in gender, cause of renal disease, diabetes or nondiabetes, age, body mass index, hemodialysis period, duration of PAD, grade of Fontaine classification, ABI, absolute walking distance, laboratory data, or the frequency of LDL apheresis.

**Effects of LDL Apheresis on Laboratory Parameters**

The short- and long-term lipid reductions by LDL apheresis in each group are shown in Table 2. Blood collection
was performed before and after first apheresis, at the start of the 10th apheresis, and 3 months after the 10th apheresis. Therefore, lipid concentrations at the start of the 10th apheresis and 3 months after the 10th apheresis increased compared with those after the first apheresis. The serum levels of total cholesterol, LDL-cholesterol, malondialdehyde-modified (MDA)-LDL, and oxidized LDL were significantly reduced during a single session in both groups. Specifically in the responders, oxidized LDL continued to be significantly lower even at the 10th session compared with baseline. On the other hand, in the nonresponders, MDA-LDL and oxidized LDL concentrations had increased compared with the baseline levels by 3 months after the end of the treatment. These results indicate that LDL apheresis exerted a sustained lowering effect on lipid-related oxidative stress in the responders. We also measured lipid-independent oxidative stress markers, such as plasma 8-hydroxydeoxyguanosine, as well as advanced glycation end products. However, these general oxidative stress markers did not improve during the course of the study period, irrespective of the efficacy of the LDL apheresis (data not shown).

The short- and long-term changes in inflammatory markers and vasculogenerative factors induced by LDL apheresis in each group are summarized in Table 3. Although in both groups the C-reactive protein (CRP) and fibrinogen concentrations were significantly reduced during a single session, it was only in the responders that they tended to remain reduced at the 10th session compared with baseline. The plasma C-type natriuretic peptide concentration was significantly increased during a single session only in the responders. We did not find any significant short-term or long-term effects of LDL apheresis on the VEGF, hepatocyte growth factor (HGF), or interleukin-1β concentration in either group.

Effects of LDL Apheresis on Activated eNOS Expression in Vascular Endothelial Cells Exposed to Patient Serum

The total eNOS protein expression levels in HUVECs did not undergo significant change by incubation with the serum collected from either group during the course of the study period (Figure 1A and 1B). However, the serum...
collected from the responders at the 10th session significantly increased the expression level of the activated eNOS protein in HUVECs compared with the serum after the first apheresis (Figure 1A), although the increases in activated eNOS protein level at the 10th apheresis and at 3 months after 10th apheresis did not reach statistical significance as compared with baseline (10th apheresis: 138% ± 17% versus 100% ± 0%, P = 0.08; 3 months after 10th apheresis: 124% ± 14% versus 100% ± 0%, P = 0.12). In contrast, the serum from the nonresponders did not alter the expression of the activated eNOS protein in HUVECs (Figure 1B).

Effects of LDL Apheresis on the Proliferative Activity of Vascular Endothelial Cells Exposed to Patient Serum

To examine the possible effect of LDL apheresis on the proliferative activity of HUVECs, we performed a BrdUrd incorporation assay using patient serum. The synthesis of DNA in HUVECs was significantly increased by incubation with the responder serum collected 3 months after the end of the treatment compared with the serum at baseline (Figure 2A). On the other hand, the serum from the nonresponders did not alter the synthesis of DNA in HUVECs (Figure 2B).

Table 2. Short- and Long-Term Effects of LDL Apheresis on Lipid Concentrations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>After 1st Apheresis</th>
<th>At 10th Apheresis</th>
<th>Three Months After 10th Apheresis</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
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<tbody>
<tr>
<td><strong>Responders</strong></td>
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<td></td>
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</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>158 ± 9</td>
<td>94 ± 5</td>
<td>151 ± 10</td>
<td>180 ± 13</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>0.06</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>121 ± 25</td>
<td>101 ± 16</td>
<td>110 ± 20</td>
<td>107 ± 16</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>46 ± 5</td>
<td>41 ± 5</td>
<td>51 ± 6</td>
<td>60 ± 7</td>
<td>NS</td>
<td>NS</td>
<td>0.06</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>88 ± 7</td>
<td>32 ± 3</td>
<td>78 ± 9</td>
<td>98 ± 11</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MDA-LDL, U/L</td>
<td>90 ± 10</td>
<td>44 ± 5</td>
<td>87 ± 13</td>
<td>95 ± 10</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Oxidized LDL, U/L</td>
<td>38 ± 3</td>
<td>20 ± 2</td>
<td>32 ± 3</td>
<td>38 ± 4</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Nonresponders</strong></td>
<td></td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>164 ± 14</td>
<td>95 ± 10</td>
<td>146 ± 16</td>
<td>186 ± 20</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>145 ± 63</td>
<td>118 ± 40</td>
<td>88 ± 14</td>
<td>185 ± 56</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>52 ± 9</td>
<td>46 ± 8</td>
<td>53 ± 8</td>
<td>46 ± 11</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>83 ± 8</td>
<td>26 ± 2</td>
<td>75 ± 10</td>
<td>104 ± 16</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MDA-LDL, U/L</td>
<td>99 ± 20</td>
<td>40 ± 6</td>
<td>90 ± 12</td>
<td>118 ± 23</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Oxidized LDL, U/L</td>
<td>38 ± 3</td>
<td>18 ± 2</td>
<td>32 ± 4</td>
<td>46 ± 6</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Parameters are shown as mean ± SE values. P1 indicates baseline vs after the 1st apheresis; P2, baseline vs at the 10th apheresis; P3, baseline vs 3 months after the 10th apheresis. NS indicates not significant.

Table 3. Short- and Long-Term Effects of LDL Apheresis on Inflammatory Markers and Vasculogenic Factors

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>After 1st Apheresis</th>
<th>At 10th Apheresis</th>
<th>Three Months After 10th Apheresis</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
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<tbody>
<tr>
<td><strong>Responders</strong></td>
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<tr>
<td>Fibrinogen, mg/dL</td>
<td>400 ± 14</td>
<td>308 ± 18</td>
<td>337 ± 32</td>
<td>394 ± 35</td>
<td>&lt;0.01</td>
<td>0.07</td>
<td>NS</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>0.87 ± 0.40</td>
<td>0.49 ± 0.20</td>
<td>0.39 ± 0.23</td>
<td>0.75 ± 0.47</td>
<td>&lt;0.05</td>
<td>0.07</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>114 ± 14</td>
<td>87 ± 13</td>
<td>94 ± 15</td>
<td>97 ± 20</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HGF, ng/mL</td>
<td>0.36 ± 0.03</td>
<td>0.31 ± 0.03</td>
<td>0.31 ± 0.03</td>
<td>0.36 ± 0.04</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CNP, fmol/mL</td>
<td>31 ± 6</td>
<td>36 ± 7</td>
<td>33 ± 6</td>
<td>38 ± 7</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>0.25 ± 0.03</td>
<td>0.22 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Nonresponders</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>388 ± 38</td>
<td>264 ± 28</td>
<td>340 ± 45</td>
<td>427 ± 35</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>0.81 ± 0.47</td>
<td>0.34 ± 0.21</td>
<td>1.12 ± 1.02</td>
<td>0.61 ± 0.35</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>98 ± 24</td>
<td>66 ± 13</td>
<td>108 ± 34</td>
<td>101 ± 26</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HGF, ng/mL</td>
<td>0.32 ± 0.03</td>
<td>0.31 ± 0.04</td>
<td>0.38 ± 0.05</td>
<td>0.40 ± 0.06</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CNP, fmol/mL</td>
<td>27 ± 4</td>
<td>29 ± 3</td>
<td>27 ± 4</td>
<td>31 ± 4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>0.20 ± 0.01</td>
<td>0.72 ± 0.39</td>
<td>0.29 ± 0.12</td>
<td>0.46 ± 0.16</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Parameters are shown as the mean ± SE values. P1 indicates baseline vs after the 1st apheresis; P2, baseline vs at the 10th apheresis; P3, baseline vs 3 months after the 10th apheresis. CNP indicates C-type natriuretic peptide; IL-1β, interleukin-1β.
Effects of LDL Apheresis on Tube Formation in Vascular Endothelial Cells Exposed to Patient Serum

To examine the effects of LDL apheresis on HUVEC tubular morphogenesis, we cocultured HUVECs with human fibroblasts and incubated them with patient serum. The tube length and area were evidently unchanged by incubation with the serum collected during the course of the study period in either group (Supplemental Figure IA and IB, available online at http://atvb.ahajournals.org).

Relationships Among Walking Distance, ABI, Oxidized LDL, Fibrinogen, and Activated eNOS Expression

Finally, there were statistically significant correlations between walking distance and plasma oxidized LDL ($R = -0.448$, $P < 0.05$, Figure 3A) and fibrinogen ($R = -0.779$, $P < 0.05$, Figure 3B) levels and activated eNOS protein level in HUVECs ($R = 0.568$, $P < 0.01$, Figure 3C) in responders. There was also a significant correlation between the ABI and activated eNOS protein level in HUVECs ($R = 0.427$, $P < 0.05$, Figure 3D) in responders. In nonresponders, there was a significant correlation between the ABI and activated eNOS protein level in HUVECs ($R = -0.470$, $P < 0.05$, Supplemental Figure IIA) and a marginal correlation between walking distance and plasma oxidized LDL ($R = -0.329$, $P = 0.07$, Supplemental Figure IIB). On the other hand, no significant correlation between CRP or BrdUrd incorporation and ABI or walking distance was observed in either group (data not shown).

Discussion

It is now widely accepted that the endothelium exerts a critical role in the regulation of vascular tone, modification of lipoproteins, inflammation, thrombogenesis, and transformation of circulating monocytes into pathological foam cells. Accumulating evidence also indicates that proinflammatory and prothrombotic markers play an important role in the pathogenesis of atherosclerosis, and various factors are involved in the inflammatory and coagulatory processes that are active during atherosclerosis. In particular, oxidative stress is reported to be increased in patients with end-stage renal disease and has been implicated in the endothelial injury caused by oxidized LDL, a key lipid-related oxidative stress marker.
Oxidized LDL affects endothelial cell function in various ways, eg, by inducing the expression of proinflammatory cell adhesion molecules, inhibiting endothelial cell proliferation with stimulated apoptosis, and impairing endothelial vasodilator function. Atherosclerosis is also associated with increased concentrations of inflammatory hepatic markers, including CRP and fibrinogen. CRP and fibrinogen are reported to be elevated in renal insufficiency, inducing adhesion molecule expression in endothelial cells so as to provoke an inflammatory response and the atherosclerotic process, and to support the interaction of endothelial cells with matrix and inflammatory cells. Both the oxidative stress and proinflammatory conditions result in a significant decrease in eNOS activity and thus impair endothelial function and promote atherosclerotic lesions.

Previous studies demonstrated that a single LDL apheresis decreased not only the total LDL cholesterol but also the oxidized LDL, CRP, and fibrinogen concentrations as short-term effects. Although previous studies reported that LDL apheresis may induce long-term reduction of the CRP and fibrinogen concentration, there has been no report of the long-term effects of LDL apheresis on lipid-related oxidative stress. In this study, we demonstrated short-term reductions of the LDL, MDA-LDL, oxidized LDL, CRP, and fibrinogen concentrations during a single apheresis, consistent with the results of previous studies. More importantly, our results disclosed a long-term reduction of oxidized LDL, CRP, and fibrinogen specifically until the 10th apheresis in the responders. Although LDL apheresis did not cause a sustained decrease in LDL cholesterol in the responders or the nonresponders, the long-term therapeutic effects of LDL apheresis were related to the relatively sustained decrease in oxidized LDL, which is a marker of lipid peroxidation.

On the other hand, LDL apheresis did not significantly affect plasma 8-hydroxydeoxyguanosine, which is a marker of DNA oxidation injury, or advanced glycation end products, which reflect protein oxidation, in the responders. A recent study also showed apheresis-mediated reduction of thiobarbituric acid reactive substances, which is a marker of lipid peroxidation, and also production of reactive oxygen species via suppression of NADPH oxidase expression in leukocytes in hemodialysis patients. Therefore, these results suggest that LDL apheresis–mediated suppression of lipid peroxidation is a major contributing factor to its therapeutic effect on peripheral circulation in end-stage renal disease patients with PAD. With respect to the effects of LDL apheresis on high-density lipoprotein (HDL), because there was a short-term reduction of the HDL concentration during a single apheresis in nonresponders and a trend toward an increase 3 months after the 10th apheresis in comparison with baseline in responders (607 versus 465, P = 0.06), it is possible that a differential effect on the HDL profile may have a role in its therapeutic impact.

We further examined the effects of LDL apheresis on vascular endothelial cell functions in vitro by analyzing the expression of the activated form of eNOS, which is phosphorylated at Ser-1177; cellular proliferative activity, and tube formation capacity. The expression of the activated eNOS protein in HUVECs was significantly increased by incubation with the serum from the responders at the 10th session compared with the serum collected after the first apheresis. Furthermore, the proliferative activity of HUVECs was increased by the serum collected from the responders at 3 months after the end of treatment. These results suggest that a repeat of LDL apheresis is important for the responders to obtain the beneficial effect on endothelial cell function. Although the precise molecular mechanism of the LDL apheresis–mediated effects on endothelial cells needs to be further investigated, these results suggest that the long-term effects of LDL apheresis on endothelial cell functions through the activation of eNOS contribute to the improvement in peripheral circulation.

The role of angiogenesis in atherosclerosis remains controversial. Angiogenic cytokine therapy has been widely regarded as an attractive approach to treating ischemic heart disease and PAD. Furthermore, a variety of studies suggest that neovascularization contributes to the growth of atherosclerotic lesions. We did not find any change in tube formation activity in HUVECs cultured with the serum collected during the study period in either group. Although the angiogenic cytokines VEGF and HGF stimulate angiogenesis events such as tube formation, as well as the activation of eNOS and proliferation in endothelial cells, the concentrations of VEGF and HGF were not affected by LDL apheresis in this study. Previous studies have reported differential effects of antiangiogenic factors that preserved endothelial proliferation but inhibi-
The results of this study suggested that LDL apheresis increased activated eNOS protein expression and proliferative activity without a promotion of angiogenic properties and that the activating effects of LDL apheresis and the angiogenic cytokines VEGF and HGF occur through different mechanisms. Additional studies are necessary to address the molecular mechanisms of this differential activating effect of LDL apheresis on endothelial cells, including identification of the responsible factor(s) involved.

Although the absolute walking distance and ABI still remained significantly improved in the responders at 3 months after the 10th apheresis compared with baseline, the LDL apheresis–mediated decrease in the oxidized LDL, CRP, and fibrinogen concentrations lasted until the 10th apheresis, but it did not persist at 3 months after the 10th apheresis. With respect to endothelial cellular function in the responders, although the proliferative activity, as estimated by BrdUrd incorporation, was significantly activated at 3 months after the 10th apheresis in comparison with baseline (Figure 2), the increase in the activated eNOS protein level at 3 months after the 10th session did not reach statistical significance compared with baseline (Figure 1). Thus, there is a discrepancy between the long-term therapeutic effects of LDL apheresis on clinical parameters, including walking distance and ABI, and the improvements in laboratory and endothelial functional parameters, including oxidized LDL, CRP, fibrinogen, and activated eNOS expression.

However, significant correlations were found between walking distance and laboratory parameters, including oxidized LDL and fibrinogen, and significant relationships were also disclosed between the activated eNOS protein level, one of the endothelial functional parameters, and walking distance and ABI in the responders (Figure 3). These results suggest that the long-term therapeutic effects of LDL apheresis may involve the chronic reduction of oxidized LDL and fibrinogen and a relatively sustained activation of eNOS protein in endothelial cells.

On the other hand, there was no significant correlation between BrdUrd incorporation, which is an index of endothelial proliferative activity, and ABI or walking distance in the responders, and this may be one of the reasons why BrdUrd incorporation was significantly increased by incubation with the responder serum collected 3 months after the 10th apheresis, but not that collected at the 1st and 10th aphereses, compared with the serum at the baseline (Figure 2). Thus, there is a possibility that other factors that have not identified in this study play a critical role in mediating the long-term therapeutic effects of LDL apheresis on endothelial cellular function. Also, the components of the nonresponders’ serum responsible for the insufficient clinical improvement by LDL apheresis remain to be determined. Therefore, additional studies, such as microarray analysis, are necessary to address these important issues.

A limitation of the present study is that it did not examine the effects of LDL apheresis on other in vitro endothelial functions, including the expression of proinflammatory adhesion molecules, reactive species production, and NADPH oxidase expression in HUVECs, or on in vivo endothelial function, such as forearm endothelium-dependent vasodilatation. Another limitation is the lack of a control therapy group, and the general applicability of the results thus awaits further confirmation by a study with a larger number of patients. In conclusion, the results of the present study suggest that the long-term therapeutic effects of LDL apheresis on patients with end-stage renal disease who have PAD are at least partly dependent on the sustained reduction of oxidized LDL and inflammatory stress, along with the activated eNOS-mediated improvement of endothelial cell function. Because the activation of endothelial cells is an important strategy for suppression of the atherosclerotic vascular process, further efforts to improve the therapeutic efficacy of LDL apheresis are warranted.

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**Disclosures**
None.

**References**


Sustained Inhibition of Oxidized Low-Density Lipoprotein Is Involved in the Long-Term Therapeutic Effects of Apheresis in Dialysis Patients
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Supplement Material

Sustained Inhibition of Oxidized Low-density Lipoprotein Is Involved in the Long-term Therapeutic Effects of Apheresis in Dialysis Patients

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Supplemental Figure Legends

Figure I. The effects of serum from patients undergoing LDL apheresis on the tube formation of human umbilical vein endothelial cells (HUVECs).
Tube formation assay was performed for HUVECs incubated with the serum from the responders (A) or non-responders (B). Values are expressed as the mean±SE.

Figure II. Relationships between the ankle-brachial pressure index (relative ABI) and activated eNOS protein level in HUVECs (relative p-eNOS/eNOS) (A) in the non-responders, and between walking distance (relative walking distance) and plasma oxidized LDL (relative oxidized LDL) level (B) in the non-responders.
The respective values were calculated relative to those achieved at baseline in the responder or non-responder group.
Supplemental Figure I

A

![Bar graph showing tube length (pixel) over time.](image)

- **VEGF (positive control)**
- **Baseline**
- **After 1st apheresis**
- **At 10th apheresis**
- **3 months after 10th apheresis**

B

![Bar graph showing tube length (pixel) over time.](image)

- **VEGF (positive control)**
- **Baseline**
- **After 1st apheresis**
- **At 10th apheresis**
- **3 months after 10th apheresis**

**HUVECs incubated with the serum from responders**

**HUVECs incubated with the serum from non-responders**
Supplemental Figure II

A

Non-responders

Relative ABI (% of baseline)

Relative p-eNOS/eNOS (% of baseline)

$R = -0.470$

$P = 0.013$

B

Non-responders

Relative walking distance (% of baseline)

Relative oxidized LDL (% of baseline)

$R = -0.329$

$P = 0.070$