Low-density lipoprotein apheresis for haemodialysis patients with peripheral arterial disease reduces reactive oxygen species production via suppression of NADPH oxidase gene expression in leucocytes

Taiga Hara1, Hideyasu Kiyomoto1, Hirofumi Hitomi1, Kumiko Moriwaki1, Genei Ihara1, Kumiko Kaifu1, Yoshiko Fujita1, Chikako Higashiyama1, Akira Nishiyama2 and Masakazu Kohno1

1Department of CardioRenal and Cerebrovascular Medicine and 2Department of Pharmacology, Faculty of medicine, Kagawa University, Kagawa, Japan

Abstract

Background. Peripheral arterial disease (PAD) is a major complication of haemodialysis (HD), especially in patients with diabetes mellitus. Although previous reports have indicated that low-density lipoprotein apheresis (LDL-A) improves arteriosclerosis in PAD patients, the mechanism by which LDL-A affects PAD is still unclear. In this study, we tested the hypothesis that LDL-A attenuates reactive oxygen species (ROS) production in HD patients with PAD.

Methods. Twenty HD patients with PAD were investigated in this study. Clinical effects were evaluated by thermography and angiography. Oxidative stress in serum was evaluated by thiobarbituric acid reactive substances (TBARS) and expression of p22phox mRNA.

Results. Ischaemic symptoms due to PAD were gradually improved in 13 patients (65%) after LDL-A. One session of LDL-A removed ~75% of LDL from serum. Some patients exhibited dramatic improvement of severe symptoms of PAD such as skin ulcers after serial performance of LDL-A. The levels of LDL cholesterol, malondialdehyde-modified LDL, high-sensitivity C-reactive protein, vascular endothelial growth factor, international normalized ratio of pro-thrombin time and bradykinin were decreased after a single session of LDL-A, although there were no additional changes after 10 sessions of LDL-A. The levels of fibrinogen and p22phox mRNA were decreased by a single session of LDL-A, and these decreases continued over the entire period of treatment. TBARS was decreased after a course of LDL-A.

Conclusions. LDL-A improved ischaemic symptoms in HD patients with PAD by reducing ROS production in leucocytes. We conclude that LDL-A is an effective therapy for patients with HD complicated by PAD.

Keywords: NADPH oxidase; p22phox; thiobarbituric acid reactive substance (TBARS); oxidative stress; C-reactive protein (CRP)

Introduction

Peripheral arterial disease (PAD) is a serious complication of chronic haemodialysis (HD), and the number of patients with PAD has increased in recent years, particularly amongst patients with diabetic nephropathy [1,2]. PAD causes failure of microcirculation in peripheral tissues including the extremities, and is accompanied by symptoms such as numbness, feeling of cold and rest pain. Microcirculatory failure results in local inflammation and infections and sometimes sepsis, which in some cases necessitate amputation of the limb with progression of PAD. It is of critical importance to prevent and treat PAD in HD patients, although few effective therapies are available at present. In general, PAD has been treated by medications, including anticoagulants, anti-platelet agents and vasodilators, and also by surgical peripheral revascularization. Furthermore, low-density lipoprotein apheresis (LDL-A) has recently been reported to be effective against PAD in HD patients [3,4], and a reduction in LDL cholesterol (LDL-C) and coagulation factors have been suggested to underlie the efficiency of LDL-A; however, the detailed mechanisms by which LDL-A affects PAD remain unclear.

On the other hand, PAD is a phenotype of arteriosclerosis in the peripheral vessels. In addition, many reports and previous studies, including our own, have indicated that oxidative stress is involved in the progression of arteriosclerosis [5,6]. We have also revealed that oxidative stress is enhanced in patients with chronic renal failure, and further in HD patients [7]. These previous data have suggested that oxidative stress is enhanced in patients with chronic renal failure, including HD patients, and results in progression and aggravation of arteriosclerotic lesions, including PAD.

In LDL-A, blood LDL is removed by adsorption on a column. However, HD patients have been reported neither to have exceptionally high levels of serum LDL nor to
LDL-A for haemodialysis patients with PAD

Table 1. Clinical characteristics of the study population

<table>
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<tr>
<th>Age/Sex</th>
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<td>118</td>
<td>II</td>
<td>Improvement</td>
</tr>
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<td>III</td>
<td>Pain during HD</td>
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<tr>
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<td>112</td>
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<td>72</td>
<td>III</td>
<td>Rest pain</td>
</tr>
<tr>
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<td>53/M</td>
<td>84</td>
<td>III</td>
<td>Frequent arterial occlusion, rest pain</td>
</tr>
<tr>
<td>6</td>
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<td>Rest pain</td>
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<td>Ulcer of skin</td>
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Methods

Patients

Of patients undergoing chronic HD at our hospital and affiliated hospitals, a total of 20 (11 male and 9 female patients aged 48–87 years) patients who had ischaemic symptoms associated with PAD were included in the present study (Table 1). Of these patients, three had ischaemic symptoms on the fingertips of the upper extremities complicated by arteriovenous fistula (AVF). Ischaemic symptoms were graded according to the Fontaine classification of arteriosclerosis obliterans (ASO) in all patients. In the present study, the patients with symptoms of Fontaine class II or higher having poor indications for operation, including surgical bypass or percutaneous transluminal angioplasty of proximal arteries of the affected extremity, were included. In addition, LDL-A was performed only in patients in whom medication with anti-platelet agents or prostaglandin E1 preparations was not effective in improving ischaemic symptoms, and all medications were continued at the same doses during the LDL-A treatment. The present study included no patients with hypercholesterolaemia treated with any medications including statins. Six patients had smoking habits, and continued smoking during LDL-A treatment. All subjects agreed to be treated under the EDDEN protocol (effective drugs and/or dialysis evaluated NADPH oxidase) study, which was approved by the medical ethics committee of the Faculty of Medicine, Kagawa University (H15-12). Informed consent was obtained from all subjects before LDL-A was performed.

Data collection

Therapeutic efficacy was assessed by thermography and angiography focusing on the affected area before and after one course of treatment. Improvement of clinical symptoms (in Fontaine classification) was assessed by an increase in the walking distance until pain and numbness appeared in patients in Fontaine class II, by disappearance and remission of pain at rest in patients in Fontaine class III, and by a reduction of an ulcer or gangrene area in patients in Fontaine class IV.

Blood was collected before and after the 1st and 10th sessions of LDL-A. In addition to general biochemical assays, LDL-C, high-density lipoprotein (HDL) cholesterol, malondialdehyde-modified LDL (MDA-LDL), high-sensitive C-reactive protein (HSCRP), bradykinin, fibrinogen, international normalized ratio of pro-thrombin time (PT-INR), vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β) were measured by an enzyme-linked immunosorbent assay. In this study, blood samples were also collected from five healthy volunteers (three males and two females aged 30–46 years), who were not taking any medication and provided written informed consent.

TBARS measurement

TBARS levels in the plasma were measured according to the method of Kikugawa et al. [9]. Briefly, the plasma was mixed with 15% trichloroacetic acid and 0.375% thiobarbituric acid. Butylated hydroxytoluene (0.01%) was added to the assay mixture to prevent autoxidation. The mixture was centrifuged at 3500 rpm for 20 min, and the absorbance of the organic phase was measured at 535 nm.

p22phox mRNA measurement

RNA was extracted from leucocytes using PAXgene (PreAnalytiX Company, Switzerland) to determine p22phox mRNA, an essential component of NADPH oxidase, by RT-PCR, as previously described [10]. RT-PCR was performed using human p22phox primers (F: 1′-AACGAACAGGCGCTGGCCGTCGCGTCCGCG-3′ and R: 5′-CTTGGGCTCGATGCGGCTCCACT-3′) and human β-actin primers (F: 5′-CGTACCACTGGCATCGTGAT-3′ and R: 5′-GTTGTCGCGCTACAGGTCTTGT-3′). Data were normalized against β-actin expression.

Statistical analysis

Results were expressed as the mean ± S.E. The statistical significance of differences was assessed using ANOVA, followed by the Bonferroni test. Pearson’s correlation coefficient was used to evaluate the correlations between changes in p22phox mRNA and HSCRP. Findings of P < 0.05 were considered significant.

Results

Clinical and histological characteristics

Clinical symptoms disappeared in 7 of 8 patients of Fontaine class II or III, while ulceration and gangrene were improved in 6 of 12 patients of Fontaine class IV as shown in Table 1. Overall, symptoms were improved in 13 of 20 patients (65%). The cases in which thermography,
angiography and wound status exhibited improvement are presented below as representable cases.

**Case 1: male, aged 58 years, with 118-month HD history, Fontaine class II**

Intermittent claudication had slowly progressed in this patient, who complained of pain at 20 m walking. Lower extremity angiography revealed no occlusion of large or medium vessels requiring surgical treatment, and he underwent LDL-A therapy. Clinical symptoms were almost completely eliminated after completion of one treatment course, enabling him to walk 200 m. Subsequent lower extremity angiography detected marked development of superficial femoral arteries (Figure 1). Furthermore, thermography was improved after LDL-A therapy.

**Case 2: male, aged 56 years, with 23-month HD history, Fontaine class IV**

The second and third fingers had been amputated because of steal syndrome due to left AVF, and the stump remained unhealed 50 days after amputation. The wound was completely healed after completion of one course of LDL-A therapy. Thermographic and angiographic examinations before and after LDL-A treatment confirmed an obvious increase in
peripheral skin temperature and development of peripheral arteries (Figure 2).

**Case 3: male, aged 57 years, with 22-month HD history, Fontaine class IV**

The patient had ulcers and gangrenous lesions on the first, second and third toes and sole of the right foot. Although the gangrenous lesions naturally dropped off after LDL-A therapy, granulation was developed in the ulcers. The wounded area was healed 3 months after completion of LDL-A treatment, and the patient avoided lower extremity amputation (Figure 3).

**Blood examinations**

A single session of LDL-A significantly lowered levels of LDL-C and MDA-LDL cholesterol, although no differences were found in levels of these between the 1st and 10th sessions of LDL-A. The cholesterol level decreased after the first session of LDL-A, but returned again by the next session of LDL-A. The level of HDL-C did not change significantly during the course of treatment (Figure 4A–C).

Many studies have reported that oxidative stress is a trigger of arteriosclerosis [5,6]. One primary source of oxidative stress is NADPH oxidase in neutrophils, and p22phox is an essential component of this enzyme. In the present study, therefore, expression of p22phox mRNA was measured by RT-PCR, to investigate the effects of LDL-A on generation of oxidative stress. p22phox expression was not significantly decreased upon completion of the first session of LDL-A, but was significantly decreased from the 1st to the 10th session of LDL-A (Figure 5A). A comparison between the groups of patients whose clinical symptoms were improved or unchanged revealed that expression of p22phox mRNA was more significantly decreased in the improved group than in the unchanged group (Figure 5B). TBARS, marker of oxidative stress, was significantly decreased from the 1st to the 10th sessions of LDL-A (Figure 5C).

Fibrinogen levels were decreased from the 1st to the 10th session of LDL-A, although comparison between the groups with improved and unchanged symptoms revealed no difference (Figure 6A and B). PT-INR was significantly increased after the 1st and 10th sessions of LDL-A (Figure 6C). Bradykinin levels were significantly increased after the first session of LDL-A. VEGF levels were significantly reduced after the first session of LDL-A, and TGF-β levels remained unchanged over the course of treatment (Figure 7).

HSCRP was determined as a marker of inflammation. HSCRP levels were decreased after the first session of
LDL-A in each group, and thereafter remained unchanged (Figure 8). Changes in p22phox mRNA were positively correlated with those in HSCRP \( r = 0.58, P < 0.05 \).

**Discussion**

Many reports have indicated that LDL-A is effective in treating ASO patients complicated by hypercholesterolaemia [11, 12]. Recently, LDL-A has been reported to be effective even in HD patients complicated with PAD [3, 4, 13]. In the present study, 6 of 20 patients felt warmth in the peripheral extremities, apparently due to improvement of microcirculatory disorder, about 30 min after initiation of the first session of LDL-A. This improvement in microcirculatory disorder by LDL-A therapy is considered to be the result of the following mechanism: LDL-A adsorbs and eliminates serum LDL rapidly and potently by electrostatically binding positively charged amino acids of apolipoprotein B (Apo-B) contained in LDL to negatively charged dextran sulfate as a ligand to adsorb LDL. This results in a reduction in blood and plasma viscosity, improvement in blood rheology due to improvement in erythrocyte deformability, or induction of bradykinin-associated vasodilation, which eventually leads to improvement in peripheral circulation. Although bradykinin generated by LDL-A can occur vasodilation, this production may be suppressed using nafamostat, an inhibitor of kinin–kallikrein, as an anticoagulant for LDL-A. Kizaki et al. have reported that skin temperature rapidly increased in patients who underwent a

![Fig. 3. Effects of LDL-A on foot ulcer in Case 3. The patient had ulcers and gangrenous lesions on the first, second and third toes and sole of the right foot (A). Granulation was developed 1 month after LDL-A (B). The wounded area was healed 3 months after completion of LDL-A (C).](https://academic.oup.com/ndt/article-abstract/24/12/3818/1831544)

![Fig. 4. Effects of LDL-A on lipoproteins. Lipoproteins including (A) low-density lipoprotein cholesterol (LDL-C), (B) malondialdehyde-modified LDL (MDA-LDL) and (C) high-density lipoprotein cholesterol (HDL-C) were measured by biochemical and enzyme-linked immunosorbent assays. Values are the mean ± S.E (n = 20). *P < 0.05 between each group.](https://academic.oup.com/ndt/article-abstract/24/12/3818/1831544)
This finding suggests that production of bradykinin is not the only principal mechanism of the efficacy of LDL-A. In this study, the level of bradykinin, which was increased after the first session of LDL-A, exhibited no significant correlation with the use of nafamostat mesilate (data not shown). Furthermore, bradykinin returned to normal levels before the second session of LDL-A, and then remained unchanged until before completion of the 10th session of LDL-A, suggesting that bradykinin has only a small effect on improvement of arteriosclerosis by LDL-A in this study. In addition, the levels of LDL-C, MDA-LDL, HSCRP, PT-INR and fibrinogen were drastically changed after the first session of LDL-A (Figures 4 and 6), although, except for fibrinogen, they did not exhibit significant changes after completion of one treatment course, suggesting their involvement only in short-term effects. Fibrinogen was decreased not only after the first session of LDL-A, but also after completion of one full treatment course. A comparison of clinical symptoms revealed no significant difference in fibrinogen between the groups with improved and unchanged symptoms, suggesting that a decrease in fibrinogen is not directly related to the therapeutic efficacy of LDL-A.

LDL-A may act not only to rapidly improve clinical symptoms, but also to sustain improvement of condition, although the mechanism has remained unknown. In Case 3 in the present study, a tendency towards the healing of ulcers was sustained even after completion of one treatment course of LDL-A, with complete cure in 3 months (Figure 3). These findings cannot be explained by the short-term effects described above. In the present study, VEGF levels were significantly decreased by the first session of LDL-A (Figure 8). Therefore, an intensified peripheral arteriogram (Figure 1), particularly seen in Case 1, is likely to indicate improvement in peripheral arteriostenosis or recanalization of obstructed vessels resulting from inhibition of the progression of arteriosclerosis, and not from VEGF-induced neovascularization. On the other hand, since it has been reported that ROS play very important roles in the progression of arteriosclerosis [5,6,15], the sustained effects of LDL-A were examined, focusing on suppression of oxidative stress [3]. ROS are considered to be involved in arteriosclerosis in the following fashion [16–18]: firstly, oxidized...
LDL induced by ROS generates lysophosphatidylcholine. Lysophosphatidylcholine acts on endothelial cells to induce gene expression of a monocyte adhesion molecule. Monocytes that have infiltrated into the sub-endothelial layer of the vascular intima are transformed to macrophages, which ingest oxidized LDL, becoming foam cells. Aggregates of these cells form fatty streaks, the initial underlying lesion of arteriosclerosis. Therefore, transformation of LDL-C to oxidized LDL initiates arteriosclerosis, and the production of ROS responsible for this conversion is a critically important cause of arteriosclerosis. Although ROS is produced by various types of cells including vascular endothelial cells [19], vascular smooth muscle cells [20] and mesangial cells [21], leucocytes such as neutrophils are known to produce most of the ROS in the body [22]. The present study revealed that LDL-A therapy significantly decreased p22phox, an essential subunit of NADPH oxidase that can produce ROS, in leucocytes. p22phox gene expression was decreased after the first session of LDL-A, and was significantly suppressed after the completion of one full course of treatment. Additionally, a comparison between the groups with improved and unchanged clinical symptoms showed that p22phox expression was suppressed only in the responders. Moreover, levels of TBARS, which is widely measured as an index of lipid peroxidation [23], although it has some limitations [24], showed that LDL-A reduced oxidative stress during a course of treatment. These results indicated that ROS production was attenuated in HD patients treated with LDL-A. On the other hand, the relationship between inflammatory cytokine and arteriosclerosis has been reported [25]. In the present study, HSCRP was measured as an indicator of inflammation, and was augmented in the Fontaine Class IV group but significantly decreased after the first session of LDL-A. However, HSCRP levels remained statistically unchanged after completion of one treatment course, although HSCRP tended to decrease, suggesting that inhibition of inflammation might not be the main mechanism by which LDL-A improves PAD. Further studies are needed to address these issues.

Improvement of hyperlipidaemia was reported to result in reduction of cholesterol levels of the arterial wall [26]. However, a single session of LDL-A appears only able to remove ~7 g of cholesterol through adsorption, a level that is too small to improve arteriosclerosis in the whole body. It has been reported that LDL-A therapy was effective in improving the drug-resistant symptoms of...
malnutrition-based ASO patients without hyperlipidaemia [27,28], suggesting that LDL-A therapy is likely to exert anti-arteriosclerotic effects through a mechanism that reduces ROS alternative to elimination of LDL. The mechanism of efficacy of LDL-A therapy and the progression of arteriosclerosis may be elucidated in prospective studies in more patients with random assignment in the future.

Several papers showed that HD itself affects oxidative stress. Sera et al. reported that uraemia induces leucocyte priming, which is a key mediator of ROS production [29]. Although treatment of uraemia with HD may ameliorate oxidative stress, we previously showed that a single session of HD increases oxidative LDL [7], suggesting that reduction of ROS and improvement of clinical symptom are induced by LDL-A in HD patients. Meanwhile, to confirm the efficacy of LDL-A in different degree of clinical symptom, we evaluated the results in each Fontaine classification (data not shown). The statistical analysis indicated that these results did not change even if we excluded the patient of Fontaine class II. In addition, results of treatment were better in the Fontaine Class II and III groups than in the Fontaine Class IV group, suggesting that early implementation of LDL-A therapy can improve the progress of HD patients with PAD.

In summary, a total of 20 HD patients complicated by PAD underwent LDL-A therapy in the present study. LDL-A improved ischaemic symptoms in HD patients with PAD via reduction of ROS production with suppression of NADPH oxidase gene expression in leucocytes. We conclude that LDL-A is an effective therapy of HD patients complicated by PAD.

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Conflict of interest statement. None declared.

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