Effect of Luobuma leaves against oxidation of low-density lipoprotein : a cell culture assay

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Abstract

In a previous study, we observed an improvement in the atherosclerosis index, together with a decrease in blood cholesterol, in rats given Luobuma extract orally and fed a high-cholesterol diet. The present study was designed to examine the function of oxidized low-density lipoprotein (LDL) in atherosclerotic lesions, using cultured cells. When endothelial cells were cultured with LDL in the presence of Cu^{2+}, the release of thiobarbituric acid (TBA)-reactive substance and lactic dehydrogenase into the culture medium was increased, with a decrease in cell viability. However, when Luobuma extract was also present in the culture medium, changes in these parameters were more favorable. In another *in vitro* system using macrophages, the levels of TBA-reactive substance, total cholesterol and esterified cholesterol were all significantly lower in the presence of Luobuma extract than in its absence. There was also morphological evidence that foam cell formation through incorporation of oxidized LDL was suppressed. These findings indicate that Luobuma suppresses the progression of atherosclerosis, in which oxidized LDL is involved.

Key words Luobuma, leaf, atherosclerosis, oxidized LDL, endothelial cell, macrophage.

Introduction

Many epidemiological studies have shown that increased cholesterol in the blood is a strong risk factor for atherosclerotic diseases including coronary artery disease, and the mechanism by which atherosclerosis develops from hyperlipidemia has been studied from various aspects.[1-4] A characteristic initial feature of atherosclerosis, is the localization of conglomerates of foam cells containing large amounts of cholesterol ester beneath the vascular endothelium. Yla-Herttuala *et al.*,[5] Steinberg *et al.*,[6] and Witztun and Steinberg[7] have demonstrated that oxidized low-density lipoprotein (LDL) is specifically incorporated by macrophages, resulting in the formation of foam cells. In addition, antioxidant agents have been found to prevent atherosclerosis in an animal model of LDL hypercholesterolemia, and oxidized LDL has been detected in atherosclerotic lesions by immunohistochemistry using monoclonal antibody.[8-10] These findings also indicate the important role of oxidized LDL in the development of atherosclerosis. Thus, much attention has been focused on the possible use of antioxidant agents to inhibit the oxidation of LDL.

In a previous study, we demonstrated that Luobuma extract administered orally to rats fed a high-cholesterol diet decreased the level of cholesterol in the blood and improved the atherosclerosis index,[11] as well as inhibiting the formation of oxidized LDL.[12] Based on these findings, we speculated that Luobuma would inhibit the progression of atherosclerosis, in which oxidized LDL is involved.

In the present study, the effects of Luobuma extract on both the toxicity of oxidized LDL to endothelial cells and the formation of foam cells from

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macrophages were investigated.

Materials and Methods

Medium and reagents : RPMI 1640, Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Cell Culture Laboratories (Cleveland, OH, USA), respectively. LDL, OsO_4 and oil red O were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Penicillin and streptomycin were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Poly-L-lysine-coated coverslips were purchased from Cosmo Bio Co., Ltd., Tokyo, Japan.

Plant material : Luobuma, the leaves of *Apocynum venetum,* were collected in Shandong province, China, dried, and then roasted twice.

Preparation of Luobuma extract : Luobuma leaves were extracted with hot water at 70°C for 3 h. After filtration, the solution was evaporated under reduced pressure to give an extract at a yield of 17.8 %.

Cell culture and treatment : Endothelial cells were isolated from bovine aorta by scraping the surface of the intima. The cells were cultured in RPMI 1640 medium supplemented with 10 % FCS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 60-mm dish at 37°C in a humidified atmosphere of 5 % CO_2 in air (routine conditions). After confluence had been reached, the cells were transferred to 24-well culture plates at 6×10^4 cells/well in RPMI 1640 medium supplemented with 20 % FCS, then LDL (150 μg protein/ml) or 20 μM $CuSO_4$ and/or Luobuma extract was added to the culture medium 3 h later, and the plates were incubated under routine conditions for 24 h. Aliquots of the medium were used for determination of the level of thiobarbituric acid (TBA)-reactive substance and lactate dehydrogenase (LDH) activity, and the cells were tested for viability.

Macrophages were harvested from the peritoneal fluid of male BALB/c strain mice, 6 weeks of age, 4 days after intraperitoneal injection of 2 ml of 3 % thioglycollate medium. The cells were maintained in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin with 10 % FCS in 24-well tissue culture plates. After confluence had been reached, macrophages were plated at 1×10^6 cells/well in 6-well tissue culture plates containing poly-L-lysine-coated coverslips. The macrophages were allowed to adhere by incubation for 4 h under routine conditions. Adherent macrophages were washed three times with DMEM and cultured for a further 72 h in DMEM containing 200 U/ml penicillin and 200 μg/ml streptomycin supplemented with 20 % FCS, LDL (150 μg protein/ml) or 20 μM $CuSO_4$ and/or Luobuma extract. Aliquots of the medium and the cells were used for determination of TBA-reactive substance and oil red O staining, respectively. For determination of the cellular content of cholesterol, cells were washed three times with Ca, Mg-free phosphate-buffered saline (CMF-PBS), scraped into cold CMF-PBS and extracted with hexane/isopropanol (3:2, v:v), as described by Brown *et al.*[13] and Klinkner *et al.*[14]

TBA-reactive substance assay : The extent of lipid peroxidation was assessed by the TBA-reactive substance assay to detect malondialdehyde (MDA)-like compounds.[15] 1,1,3,3-Tetraethoxypropane was used as a standard. The amount of TBA-reactive substance was expressed as nmol MDA equivalent/mg protein.

LDH leakage : LDH activity was determined using a commercial kit (lactate dehydrogenase CII-Test Wako obtained from Wako Pure Chemical Industries, Osaka, Japan). At the end of the incubation, one aliquot of medium was removed for analysis of extracellular LDH activity.

MTT cytotoxicity assay : Cell viability was assessed using a MTT-based colorimetric assay.[16] Fifty microliters of MTT (1 mg/ml) solution was added to each well. After incubation for 4 h at 37°C, MTT solution in the medium was removed. The incorporated formazan crystals in the viral cells were solubilized with 100 μl of dimethylsulfoxide. The absorbance of each well was then read at 540 nm using a Microplate Reader (Model 3550-UV, BIO-RAD, Tokyo, Japan).

Fluorescence microscopy : Macrophages cultured on poly-L-lysine-coated glass coverslips were fixed with 6 % paraformaldehyde in 0.1 M sodium phosphate (pH 7.3) for 30 min, treated with 2.5 % potassium chromate and 1 % OsO_4 for 1 h, and then stained with oil red O, followed by examination with an Olympus

BX 50 equipped with a digital microscope camera, as described by Brown *et al.*[13]

Determination of cholesterol content : The total and free cholesterol contents were determined using commercial assay kits (Cholesterol E-Test Wako and Free Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cholesterol ester content was calculated as the difference between total and free cholesterol.

Statistics : Data are presented as mean±S.E. Differences among groups were analyzed by Dunnett's test. Significance was accepted at $p<0.05$.

Results

Endothelial cells

LDL was oxidized by $CuSO_4$, leading to peroxidation of endothelial cells. The peroxidation was suppressed when Luobuma extract was present in the incubation mixture, as shown in Fig. 1. As the concentration of Luobuma extract increased in the culture medium, the level of TBA-reactive substance decreased markedly. At a concentration of 100 μg/ml, the TBA-reactive substance level was decreased to 0.99 nM MDA/mg protein (13 % of the control value). Luobuma extract also suppressed LDH leakage significantly, as shown in Table I. When endothelial cells were incubated in the presence of LDL and $CuSO_4$, the release of LDH was about 2.1 times higher than that in the absence of $CuSO_4$, providing evidence of cell injury by copper ions. However, the LDH release in the groups treated with Luobuma extract was significantly decreased at both the 10- and 100-μg dosage levels, the latter value being 41 % lower than the control (Table I). In addition, Table I shows the inhibitory effect of Luobuma extract against the cytotoxicity induced by Cu^{2+} in endothelial cells. In the absence of Luobuma extract, Cu^{2+} killed 52.8 % of the cells. However, this cytotoxicity was dose-dependently prevented by treatment with the extract. Cell viability was 59.6 % in the presence of 100 μg/ml Luobuma extract, indicating that the extract significantly protected against the cell death caused by Cu^{2+}.

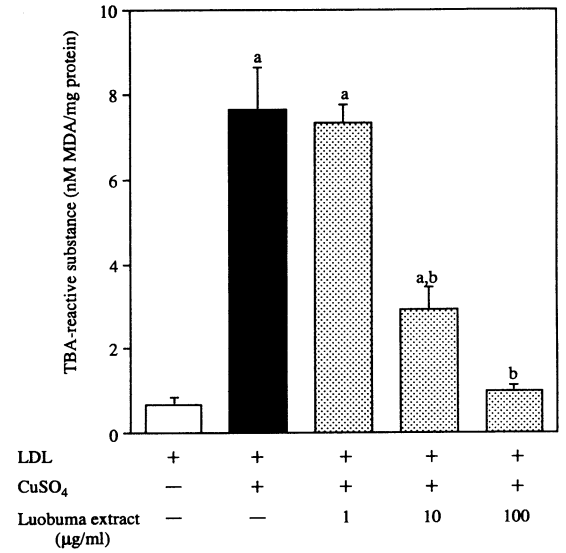

Fig. 1 TBA-reactive substance of endothelial cells. Statistical significance : $^{a}p<0.001$ *vs.* LDL-treated nonaddition values ; $^{b}p<0.001$ *vs.* LDL plus $CuSO_4$-treated nonaddition values.

Macrophages

Table II compares the levels of TBA-reactive substance in the Luobuma extract-treated groups with those in the non-treated control groups. The TBA-reactive substance level was maintained at 4.01 nM MDA/mg protein, whereas in the presence of LDL

Table I Cytotoxicity to endothelial cells.

Group	LDL	$CuSO_4$	LDH release (%)	Cell viability (%)
None	+	−	21.3±1.9	100.0±5.4
None	+	+	44.2±4.3[a]	47.2±2.0[a]
Luobuma extract (1 μg/ml)	+	+	41.8±2.3[a]	50.6±2.8[a]
Luobuma extract (10 μg/ml)	+	+	38.1±2.7[a,b]	54.8±6.5[a]
Luobuma extract (100 μg/ml)	+	+	26.0±0.8[d]	59.6±3.4[a,c]

Statistical significance : $^{a}p<0.001$ *vs.* LDL-treated nonaddition values, $^{b}p<0.05$, $^{c}p<0.01$, $^{d}p<0.001$ *vs.* LDL plus $CuSO_4$-treated nonaddition values.

Table II TBA-reactive substance in macrophages.

Group	LDL	$CuSO_4$	TBA-reactive substance (nM MDA/mg protein)
None	+	−	4.01±0.19
None	+	+	19.32±0.21[a]
Luobuma extract (10 μg/ml)	+	+	13.29±0.18[a,b]

Statistical significance : [a]$p<0.001$ *vs.* LDL-treated nonaddition values, [b]$p<0.001$ *vs.* LDL plus $CuSO_4$-treated nonaddition values.

(A) (B) (C)

Fig. 2 Oil red O-stained macrophages exposed to LDL (A), LDL plus $CuSO_4$ (B) and LDL, $CuSO_4$ plus Luobuma extract (10 μg/ml) (C). ×300.

Table III Cholesterol content of macrophages.

Group	LDL	$CuSO_4$	Total cholesterol (μg/mg protein)	Free cholesterol (μg/mg protein)	Esterified cholesterol (μg/mg protein)
None	+	−	100.9±14.9	77.2±11.1	23.8±6.8
None	+	+	140.7±10.1[b]	79.1±6.2	61.6±4.2[b]
Luobuma extract (10 μg/ml)	+	+	120.3± 6.4[a,c]	86.3±2.5	33.9±5.0[a,d]

Statistical significance : [a]$p<0.05$, [b]$p<0.001$ *vs.* LDL-treated nonaddition values, [c]$p<0.05$, [d]$p<0.001$ *vs.* LDL plus $CuSO_4$-treated nonaddition values.

and $CuSO_4$, it was increased significantly to about 4.8 times the normal value. When the effect of treatment of Luobuma extract was examined, the level of TBA-reactive substance was decreased from 19.32 to 13.29 nM MDA/mg protein (a 31 % change, $p<0.001$). On the other hand, the macrophages exposed to $CuSO_4$ had many lipid droplets in their cytoplasm, which was stained with oil red O, while cells not exposed to $CuSO_4$ were virtually devoid of the lipid droplets (Fig. 2A and B). This accumulation of lipid droplets was blocked by treatment with 10 μg/ml Luobuma extract, as shown in Fig. 2C. Under these conditions, the content of esterified cholesterol in the macrophages was 61.6 μg/mg protein when incubated with $CuSO_4$, and this was reduced to 33.9 μg/mg protein by treatment with Luobuma extract (Table III). Similarly, the total cholesterol in the group treated with Luobuma extract was significantly lower than that in the control group, as shown in Table III.

Discussion

It is considered that numerous factors contribute in a complex manner to the development of atherosclerosis through interactions of infiltrating macrophages and T lymphocytes as well as vascular endothelial cells and smooth muscle cells.[17-19] Evidence for the particular importance of LDL oxidation in the development of atherosclerosis has been provided by Steinbrecher *et al.*[20] and Steinberg.[21] In our study, in bovine aortic endothelial cells cultured with LDL in the presence of Cu^{2+}, the amount of TBA-reactive substance (an index of LDL oxidation) and LDH activity (an index of cell membrane toxicity) were both increased markedly, with a decrease in cell viability to about 50 %, as compared with cells cultured in the absence of Cu^{2+}. Thus, the high cytotoxicity of oxidized LDL was apparent. Similar results were reported by Kuzuya *et al.*,[22] who observed that such cytotoxicity was eliminated when Cu^{2+} was removed by dialysis of oxidized LDL with EDTA, but was restored when Cu^{2+} was reintroduced. They concluded that the presence of copper ions is essential for the cytotoxic activity of oxidized LDL. On the other hand, Kita *et al.*[8] reported that oral administration of probucol in WHHL rabbits, an animal model of familial hypercholesterolemia, inhibited peroxidation of LDL and halted the progression of atherosclerosis. Their results, in addition to the data reported by Parthasarathy *et al.*,[23] who observed *in vitro* inhibition of LDL peroxidation by the antioxidant probucol, demonstrate the actual mechanism of LDL peroxidation in the body and its role in atherosclerosis. These findings have provided a clearer understanding of the significance of oxidized LDL in pathogenesis of atherosclerosis. In the present study, the dose-dependent inhibitory effect of Luobuma extract on LDL oxidation, in terms of the amount of TBA-reactive substance and LDH activity, was noteworthy.

The initial stage of atherosclerosis is characterized by localized conglomerates of foam cells containing large amounts of esterified cholesterol beneath the vascular endothelium. These foam cells are thought to originate from monocyte-derived macrophages in the blood and vascular smooth muscle cells. In particular, the majority of the foam cells are presumed to be derived from macrophages in the very early stage of the disease.[24] In the present study, after macrophages had been cultured with LDL in the presence of Cu^{2+}, a great number of foamy macrophages containing esterified cholesterol were observed by fluorescence microscopy, in addition to oxidation of LDL. However, when Luobuma extract was added to the culture medium, these changes were less conspicuous. In particular, accumulation of esterified cholesterol was decreased to a degree comparable to that observed in the presence of native LDL, showing the anti-atherosclerotic effect of Luobuma extract at the cellular level.

Steinberg *et al.*[6] reported the following features of oxidized LDL in the formation of atherosclerosis. (1) It is chemotactic to monocytes in the blood, and attracts monocytes into the vascular wall. When the monocytes having infiltrated into the tunica intima of the artery become macrophages, oxidized LDL inhibits their return to the blood by suppressing their migration. (2) LDL is incorporated by macrophages *via* scavenger receptors, and causes foamy changes in the macrophages. (3) It has high cytotoxicity, and causes progression of the lesions through endothelial injury. These features suggest that oxidized LDL is involved in various stages of the disease, from the development of an atherosclerotic lesion to the establishment of an advanced focus. As the present study demonstrated that Luobuma extract exerts an inhibitory action on the oxidized LDL-induced conversion of macrophages to foam cells and on the cytotoxicity of oxidized LDL to the endothelial cells, further investigation of its effect on cell chemotaxis and migration is warranted. However, in view of the inhibitory effect on the production of oxidized LDL as observed in the oral administration experiment using rats fed a high-cholesterol diet and in a cell-free system,[25] Luobuma extract itself seems to be effective for preventing the progression of atherosclerosis.

On the other hand, it is reported by Brown and Goldstein[26] that high-density lipoprotein (HDL) draws out free cholesterol from macrophages. It has been speculated that this cholesterol is then converted to cholesterol ester by lecithin-cholesterol acyltransferase in the plasma, transported to very-low-density

lipoprotein and LDL by cholesterol ester transfer protein, and incorporated into the liver *via* LDL-receptors. Therefore, the possible role of HDL in halting the progression of atherosclerosis is now attracting attention. In addition, Nagano *et al.*[27] have reported that HDL is also oxidized, and that its ability to draw out cholesterol from macrophages is impaired by oxidation. This indicates that oxidation of lipoprotein can promote atherosclerotic changes through HDL as well as LDL. In view of the previously reported finding that Luobuma extract increases the blood HDL level,[11] we intend to further investigate the effects of Luobuma extract on HDL and macrophages.

和文抄録

先に，高コレステロール食投与ラットに羅布麻エキスを経口投与した場合，高コレステロール血症の低下とともに動脈硬化指数の改善作用が認められたので，今回，粥状動脈硬化病変への酸化 LDL の機能を細胞を用い検討した。まず内皮細胞に LDL と Cu^{2+} を添加して培養した場合，培地中へのチオバルビツール酸反応物質，総 LDH の放出が増加して，細胞生存率の低下が観察された。しかし羅布麻エキス添加群ではこれらパラメータがいずれも改善し，またマクロファージを用いた系でもチオバルビツール酸反応物質，コレステロールエステル，コレステロールエステル／遊離コレステロール比がいずれも無添加群より有意に低下し，形態学的な変化も酸化 LDL のとり込みに伴う泡沫化の形成を抑制する知見が得られた。このことから，羅布麻は酸化 LDL が関与する動脈硬化の進展過程を抑制することが明らかとなった。

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