行政院國家科學委員會專題研究計畫　期中進度報告

氧化性低密度脂蛋白之結構及其臨床應用研究(2/3)

計畫類別：個別型計畫
計畫編號：NSC93-2314-B-002-031-
執行期間：93年02月01日至94年01月31日
執行單位：國立臺灣大學醫學院內科

計畫主持人：李源德
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摘要

本計畫延續第一年成果，持續自粥狀動脈硬化斑塊中萃取 oxLDL，或取自循環血液中的 LDL 經由 FPLC 分離 LDL 子類。這些來自不同狀態不同氧化程度的 LDL，在經 trypsin 消化之後，以 MALDI-TOF 質譜儀來確定受氧化修飾的區域。然因國人之粥狀硬化斑塊取得有困難度，特別是量的限制，通常要匯集許多病人的檢體才得以進行分析。目前除了持續收集病人檢體，並尋求靈敏度較高功能較吻合需求的質譜儀。本機構已陸續購入 SELDI, MALDI-TOF, MALDI-QTOF, LC-QTOF 四種不同功能的質譜儀, MALDI-TOF 鑑定出的蛋白質, 可將它的抗體 coating 於 chip, 再用 SLEDI 進行大量篩選，以作為臨床的應用；MALDI-QTOF 則可進行 peptide 定序；而 LC-QTOF 則可以在奈米的等級下進行極微量的蛋白質後修飾位置之研究。藉由這些先進設備的陸續設置完成，即可達成確認氧化修飾區域的既定目標。

此外, 本年度亦著手研究 oxLDL 對細胞功能性的調控影響, 乃是藉由 proteomic 方法研究體外銅氧化 LDL 與血液分離出的 LDL 對人類臍帶靜脈內皮細胞 (human umbilical vein endothelial cells, HUVEC) 蛋白質之調控，並比較兩種處理方式對 HUVEC 細胞蛋白質調控方式的異同。針對被調控的未知蛋白質以質譜儀加以鑑

定，並將探討其與粥狀硬化形成之關聯性，作為未來研究的新方向。

步驟：HUVEC 與 serum-free medium, 正常 LDL，銅氧化 LDL (氧化 2 或 24 小時) 及血液分離出的 LDL (L1, L3, and L5) 培養 24 小時後, 監測 MCP-1 產量以作為細胞發炎的標準。同時將細胞蛋白質經二維電泳分析得 protein profiles，再以 MALDI-TOF 質譜儀鑑定蛋白質。

結果：不同濃度的 24 小時氧化 LDL 作用對 HUVEC 細胞數與 MCP-1 濃度並沒有明顯的影響。HUVEC 與不同氧化程度的 LDL 培養 24 小時，細胞存活數與 MCP-1 產量並無變化。但經血液分離出的 LDL 與 HUVEC 作用 24 小時則可發現 L5 的細胞數量較 L1 與 L3 少，而 MCP-1 則較高，顯示 L5 可以引起較強的免疫發炎反應及具有較強的 atherogenic 能力。初步結果顯示 protein profiles 不盡相似，可能是因為銅氧化 LDL 及血液分離出的 LDL 對 HUVEC 調控的方式不同，不過仍有待進一步的量化統計。經由質譜儀鑑定出有調控的蛋白質大多與氧化還原相關；以 peroxiredoxins 與 heat shock proteins 為大宗。

經由 proteomic 方法鑑定出的蛋白質，為探討粥狀動脈硬化的形成，提供了新的研究方向。我們將針對特定蛋白質加以調控，以觀察它在粥狀硬化斑塊中扮演的角色。

Abstract

The objective of this study was to study the protein profiles of HUVEC regulated by oxLDL and LDL circulating subfraction by proteomic approaches. In addition, the candidate proteins which were regulated by oxidized LDL and LDL subfractions were identified. Human umbilical vein endothelial cells (HUVECs) were incubated with serum-free medium, native LDL (100 ug/mL), oxLDL (2hr- and 24 hr-oxidized; 100 ug/mL), LDL circulating subfraction (L1, L3 and L5; 50 ug/mL) for 24 hrs, respectively. MCP-1 was measured as an indicator of inflammation. Cytosolic proteins were applied in 2D electrophoresis and identified by MALDI-TOF mass-spectrometer. No

differences of cell viability and MCP-1 production were observed on dose responses of 24hr- oxLDL treatment. Various grades of copper oxidation of LDL had the same effects on MCP-1production and cell viability. Circulating subfraction L5 had a highest inflammatory reaction and caused lower cell viability, suggested that L5 had a higher atherogenic capability than L1 and L3 did. The protein profiles of oxLDL and LDL subfraction were different in the preliminary test. However, more replications and statistical analysis are required to make the conclusion. For those proteins had been identified at present, most of them were redox-related proteins, peroxiredoxins and heat shock proteins were the majority.

Introduction

Low-density lipoprotein (LDL) oxidation plays a crucial role in atherogenesis. Studies shown that oxidized LDL induced the proinflammation (interleukine-8 (IL8) and monocyte chemotactic protein 1 (MCP-1); Sonoki et al., 2002); stimulated the expression of adhesion molecules (vascular cell adhesion molecule-1, VCAM-1) (Takei et al., 2001); inhibited the proliferation and nitric oxide synthesis in endothelial cells (Chen et al., 2000a; Nuszkowski et al. 2001).; induced apoptosis of endothelial cells (Santa and Walsh, 1998). Also, oxidized LDL bound with high affinity to scavenger receptors on the macrophage plasma membrane, induced macrophage proliferation (Biwa et al. 2000) and foam cell formation (Itabe 2003).

A circulating subfraction of electronegative LDL (LDL (-)) was another type of modified LDL. Studies shown that patients with diabetes mellitus and/or hypercholesterolemia had increased serum levels of LDL(-) than normal subjects (Vidie et al., 1998; Yano et al., 2004). LDL (-) contributed to atherogenesis via several mechanisms, including proinflammatory, proapoptotic and anti-angiogenesis properties. Ziouzenkova et al (2003) demonstrated that LDL (-) increased tumor necrosis factor

alpha (TNF-alpha)-induced inflammatory responses with corresponding increases in VCAM-1 expression. In addition, LDL (-) involved in early phases of leukocyte recruitment by inducing the production of chamomiles (IL8 and MCP-1) (Sanchez-Quesada et al., 2004). LDL (-) impaired angiogenesis and increased apoptosis by decreasing DNA synthesis and intracellular fibroblast growth factor 2 production.

Many researches have studied the role of oxLDL on atherogenesis by using catalyst-oxidized LDL in vitro. However, the representation of oxLDL as circulating LDL in physical environment is still doubtable. Since very few studies have compared the mechanism of oxLDL and LDL subfraction on endothelial cells. The objective of this study was to elucidate the differences between oxLDL and LDL subfraction by study the protein profiles of HUVEC. In addition, the proteins which were regulated by oxidized LDL and LDL circulating subfractions were identified by proteomic approach.

Materials and Methods

Cell culture

Umbilical cord was obtained from pregnant females with the caesarian section in National Taiwan University Hospital. HUVEC was isolated from umbilical cord using 0.1 % collagenase. Cells were grown in M199 medium supplemented with 10 % fetal calf serum, 50 ug/mL endothelial cell growth supplement, 5 IU/mL heparin (Leo, Ballerup, Denmark), cocktail of antibiotics (100 IU/mL penicillin, 0.1 mg/mL streptomycin, 0.25 ug/mL amphotericin B) in a humidified atmosphere containing 5% CO_2. The cells were used between 3-5 passages.

Isolation and oxidation of LDL

LDL was isolated from human plasma using density-gradient ultracentrifugation. In brief, blood sample was collected into tubes containing EDTA from healthy volunteers after an overnight fast. After centrifugation, the plasma were adjusted the density to

1.063 g/mL by adding NaBr. The solution was centrifuged at 58,000 rpm for 11.5 hrs, and the supernant was LDL. LDL was incubated in 10 umol $CuSO_4$/ L PBS at 37°C for various periods. After the incubation, reaction was terminated by EDTA (0.5 mg/mL) and stored in 4°C. Precautions were taken to prevent endotoxin contamination.

LDL subfraction

LDL was chromatograhed with an ion-exchange FPLC system (Pharmacia Biotech Co) through a UnoQ12 column (BioRad) that had been equilibrated with buffer A (0.02 mol/L Tris-Cl, pH8.0, containing 1 mmol/L EDTA). Subfractions were eluted by use of a multistep gradient of buffer B (1 mol/L NaCl in buffer A). Samples equilibrated with buffer A were eluted with a linear gradient program at a flow rate of 2 mL/min. Effluent was monitored at 280 nm and protected from ex vivo oxidation with 5 mmol/L EDTA. Protein concentrations were detected by the Lowry method. Thiobarbituric acid- reactive substances (TBARS), agarose electrophoresis and formation of conjugated diene at absorbance 234 nm were assayed as a measure of oxidative lipid modification was measured.

Protocol

Cell cultures grown to 90% confluence were starving for 24 hrs. After starvation, cells were incubated with serum-free medium, native LDL (100 ug/mL), oxLDL (2hr- and 24hr- oxidized; 100 ug/mL), LDL subfraction (L1, L3 and L5; 50 ug/mL) for 24 hrs, respectively. MCP-1 was measured as an indicator of inflammation.

ELISA

To measure the secretion of MCP-1, ELISA was performed in medium by use of an immunoassay kit, and MCP-1 was estimated spectrophotometrically at 450 nm.

Two-dimension electrophoresis

Cytosolic protein was extracted by lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris base, 12.5 M DTT and 0.5% carrier ampholytes). The protein concentration was

assayed using 2-D quant kit (Amersham Bioscience, Piscataway, NJ). Protein (1 mg) was added to rehydration solution (7M urea, 2 M thiourea, 4% CHAPS, 10M DTT, 0.5% Triton X-100, 0.4% carrier ampholytes and a trace of bromophenol blue). Strips were rehydrated for 6 hrs and then IEF was performed for a total 24500 Vhr. 10% SDS-PAGE was applied to the second dimension electrophoresis. After electrophoresis, gels were silver stained and analyzed using ImageMaster 2D Elite 3.10 software (Amersham Bioscience, Piscataway, NJ).

In-gel digestion and mass spectrometer

Protein spots were destained with 50% acetonitril and air dried. The dried gel was then rehydrated in trypsin solution and incubated for 20 hrs at 37℃. After the peptides were eluted with TFA of different concentrations, the peptide mixtures were analyzed by using Voyager-DE time-of-flight (MADLI-TOF) mass spectrometer (Perseptive Biosystems, Framingham, MA) with delayed extraction and a reflector. MS/MS spectra were searched automatically against the protein database by using MOSCOT software (http://www.matrixscience.com/). Multiple peptides from each protein generally were detected, adding confidence to the protein identifications.

Results and discussions

Figure 1 is immunostained HUVEC by von Willebrand Factor (VWF). The cells identified by VWF confirmed them as HUVEC in this study. Schwachula et al. (1994) characterized the immunophenotype of HUVEC and fibroblast, and found CD31 and VWF were HUVEC- specific proteins. Therefore, VWF was used in this study as an identification marker for HUVEC.

Table 1 was results of formation of conjugated dienes and TBARS. There were slightly increase in diene and TBARS between 0 and 2 hrs of oxidation; and thereafter, large amount of dienes were accumulated. Also, similar results were observed in the

SDS-PAGE gel and agarose electrophoresis (**Figure 2 and 3**). As the oxidative period increased, LDL degraded more in SDS-PAGE gel. In addition, LDL was more electronegative when severity of oxidation increased. Therefore, 2 hr was the critical point for LDL oxidation by copper. Diene for circulating subfractions showed no differences on lipid peroxidation among L1, L3 and L5 (Table 1).

Dose response of oxLDL in HUVEC culture was shown on **Table 2**. There were no differences observed among the treatments, suggested these concentrations did not cause serious damages.

Protein profiles of HUVEC treated with oxLDL and LDL subfraction were shown on **Figure 4**. Protein profiles were different between oxidized LDL in vitro and LDL subfraction. HUVEC treated with LDL subfraction induced more protein modifications. However, it required more replications and statistical analysis on protein expression to make a conclusion.

Figure 6 was the protein profiles for HUVEC identified and **Table 5** was the list of identified proteins by mass spectrometer at present. Most of them were redox relative proteins (peroxiredoxin 2 (Prdx2), peroxiredoxin 3 (Prdx3), peroxiredoxin 6 (Prdx6), glutathione transferase) and family of heat shock proteins (GP96, HSP70RY, HSP27, ORP150).

Protein expressions were quantified as the ratio of native LDL treatment (**Table 6**). Comparing with 24hr-oxLDL, L5 had higher expressions of HSP70RY, ORP150, and lower expression of glutathione transferase. Treatment of 24hr-oxLDL detected a higher expression of Prdx6, and lower expressions of Prdx3 and HSP27. However, L5 did not show any differences. Since this was the result of preliminary test, more replications are required.

Peroxiredoxins are thiol-specific antioxidant (TSA) family of proteins, which possess peroxidase activity that protect cells from oxidative damage resulting from

metal-catalyzed oxidation system. Prdxs had involved in atherogenesis by several ways: blocking inflammatation in endothelial cells (Shau et al., 1998); reducing plasma lipid hydroperoxide levels and decreasing aortic root lesions (Wang et al., 2004); behaving like glutathione peroxidase and helping recruit macrophages (Chen et al., 2000b); causing apoptosis (Chen et al., 2004).

The relation between HSPs and atherosclerosis has been studied widely. HSP is a potential biomarker for atherosclerosis (Martin-Ventura et al., 2004). Over-expression of HSPs protected the intact heart against myocardial injury by reducing infarction size (Efthymiou et al., 2004; Griffin et al., 2004), decreased apoptosis of cardiac cells against thermal or hypoxic stress (Brar et al., 1999, Suzuki et al., 2000).

Both of GP96 and Oxygen regulated protein 150 (ORP150) are glucose- related proteins. GP96 combined non-specific signals for the innate immune system and specific signals for the adaptive immune system, thus played a crucial role in proinflammation of atherogenesis (Schild and Rammensee, 2000). Suppression of ORP150 expression attenuated survival of mononuclear phagocytes (Tsukamoto et al., 1996), and caused a lower survival rate of mice (Bando et al., 2004).

CapZα1, a key component for F-actin capping, was involved in smooth muscle cell function (McGregor et al., 2004). ER60, cysteine protease in ER, is one member of major histocompatibility complex (MHC) class I. ER60 induce the defense system against intracellular pathogens by assembling in the ER with chaperones, and binding to the transporter associated with antigen processing (Morrice and Powis, 1998).

The ubiquitin-proteasome system (UPS) had different functions on early/mid/late stages of atherosclerosis. A high activity of UPS in coronary artery was detected in the early stage of atherosclerosis (Hermann et al., 2003). UPS enhanced the proliferation of smooth muscle cells and the formation of foam cell at the progression of atherosclerosis (Hermann et al., 2004). UPS was involved in the complication of atherosclerosis by

activating the formation of T cells (Hannson 2001).

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Figures and Charts

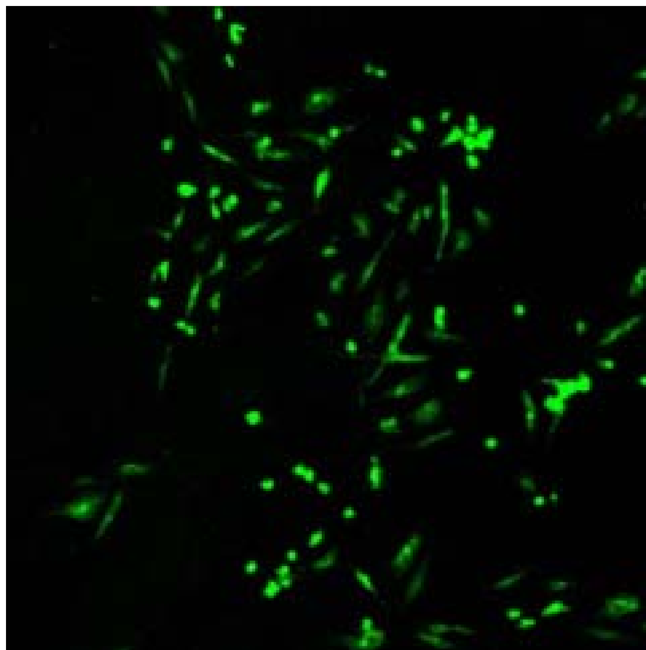

Figure 1. Identification of HUVEC by VWF (1:1000).

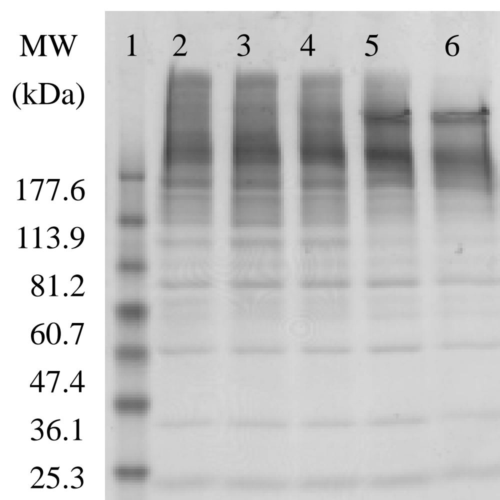

Figure 2. Protein expression of HUVEC treated with different oxidative grades of LDL for 24 hrs. Lane 1 was marker, lane 2 was native LDL, lane 3 was 1hr-oxidized LDL, lane 4 was 1.5hr-oxidized LDL, lane 5 was 2hr-oxidized LDL, and lane 6 was 24hr-oxidized LDL.

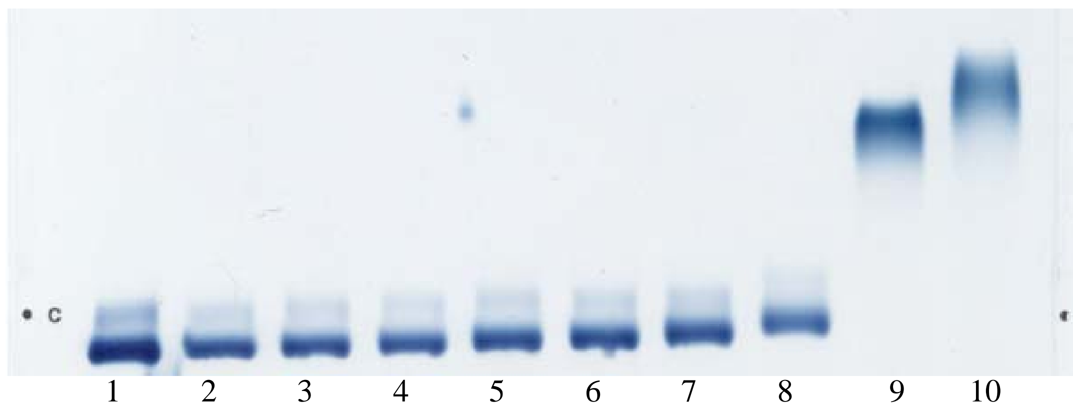

Figure 3. Electronegativity of HUVEC treated with different oxidative grades of LDL. Lane 1 to 10 were native LDL, 20min-oxidzed LDL, 40 min-oxidized LDL, 60 min-oxidized LDL, 80 min-oxidized LDL, 100 min-oxidized LDL, 2 hr-oxidized LDL, 3 hr-oxidized LDL, 8 hr-oxidized LDL and 24 hr-oxidized LDL, repectively.

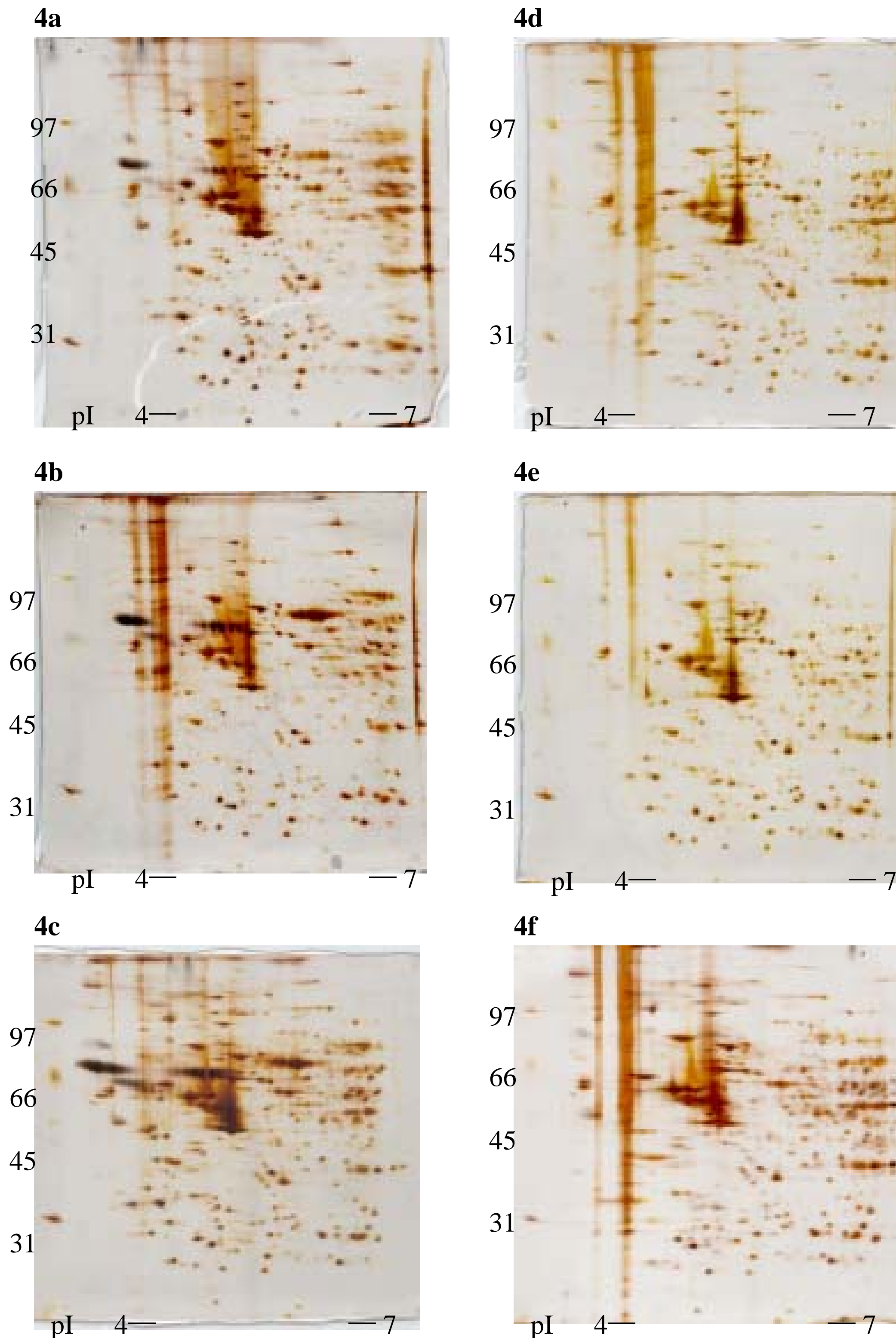

Figure 4. The protein profiles of HUVEC treated with different oxLDL. A: native LDL; b: 2 hr-oxidized LDL; c: 24 hr-oxidized LDL; d: circulating subfraction LDL- L1; e: circulating subfraction L3; f: circulating subfraction L5. Cytosolic proteins (130ng) was loaded on pI 4-7 linear IPG strips (11 cm), with 10% vertical SDS-PAGE as the second dimension. The gel was visualized by silver staining.

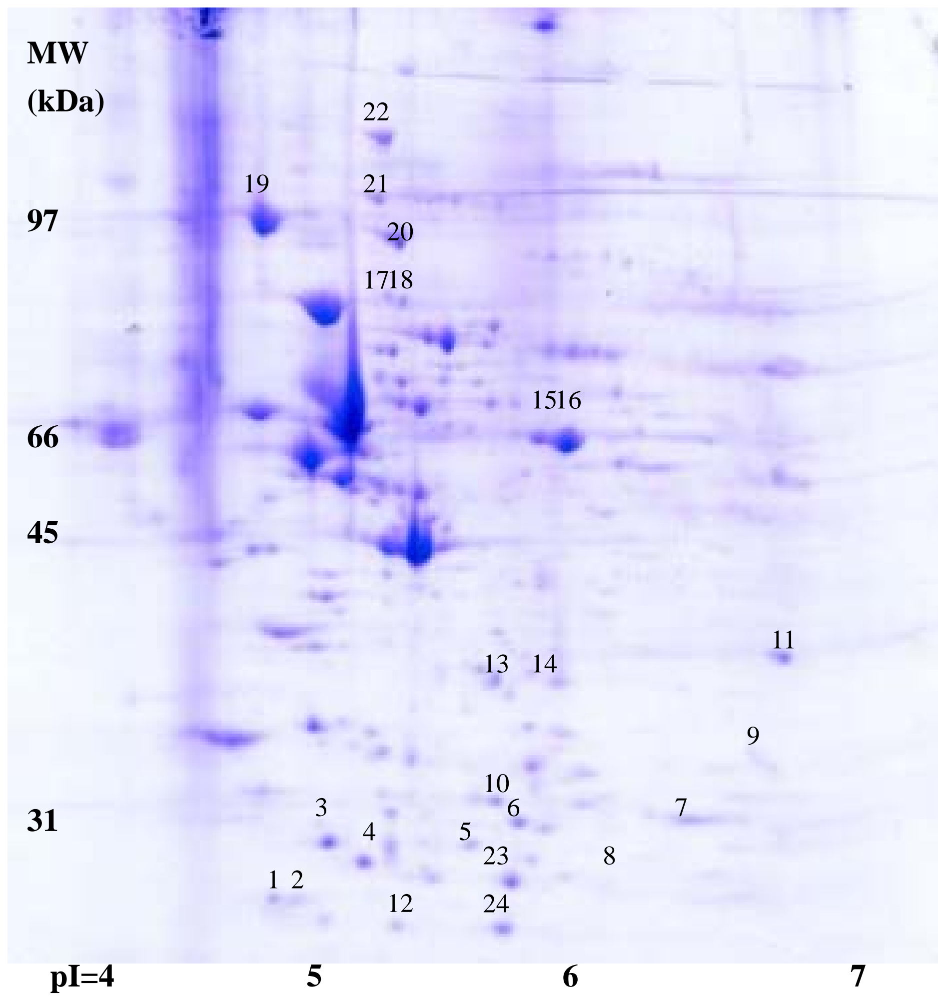

Figure 6 Protein profiles of HUVEC. Cytosolic proteins (1.2mg) was loaded on pI 4-7 linear IPG strips (11 cm), with 10% vertical SDS-PAGE as the second dimension. The gel was visualized by comassie blue staining.

Table 1. Formations of conjugated dienes and TBARS of different oxidative grades of LDL by copper and LDL circulating subfraction.

	Diene (OD=234nm)	MDA* (nmol/mg protein)
Native LDL	5.19	0.282
Copper oxidation		
1 hr-oxidized LDL	5.57	0.287
1.5 hr-oxidized LDL	5.54	0.361
2 hr-oxidized LDL	5.75	0.722
24 hr-oxidized LDL	21.20	-
Circulating subfraction		
L1	7.68	
L3	7.22	
L5	7.36	

*MDA: malondialdehye (MDA) was the product of TBARS test.

Table 2. Dose responses of HUVEC treated with 24 hr-oxidized LDL.

	Cell no (x 10^5)	MCP-1 (pg/10^5 cells)
10 ug/mL	7.5	58.4
50 ug/mL	15.0	49.4
100 ug/mL	7.9	99.0

Table 3. Cell viability and inflammatory reaction of HUVEC treated with oxidized LDL (100ug/mL) (n=2).

	Cell no (x 10^6)	MCP-1 (ng/10^6 cells)
M199 only	3.8	2.04
Native LDL	2.8	1.92
2 hr-oxidized LDL	2.9	2.49
24 hr-oxidized LDL	3.0	2.37

Table 4. Cell viability and inflammatory reaction of HUVEC treated with LDL circulating subfraction.

(50 ug/mL)	Cell no (x 10^6)	MCP-1 (ng/10^6 cells)
Native LDL	2.9	1.35
L1	1.8	2.21
L3	2.7	2.19
L5	1.8	3.56

Table 5. Lists of protein identified by mass spectrometer.

NO	Protein ID	Accession no	MW (kDa)	pI	Function	Reference
3	GDI α	gi30582607	23.2	5.02	G protein inhibitor	-
4	GDI β	gi14327952	23.0	5.10	G protein inhibitor	-
6	HSP27	gi4504517	22.7	5.98	Biomarker of atherosclerosis	4, 5, 13
7	Prdx 3	gi32483377	25.8	7.04	Decrease lipid peroxide	1, 6
9	Prdx 6	gi4758683	25.0	6.00	Decrease lipid peroxide	8, 32
10	Prohibitin	gi4505773	29.8	5.57	Negative regulator of cell proliferation	-
11	Proteasome α1	gi30582133	29.6	6.15	Destabilize and rupture atherosclerotic plague	12, 14, 15
12	ATP synthase	gi5453559	18.5	5.21		-
13	CapZα1	gi12652785	32.9	5.45	Regulate actin filament assembly and organization	19
14	Lactate dehydrogenase	gi49259212	36.5	5.86	Index of cell membrane damage	-
15	ER60	gi1085373	56.6	5.88	Cys protease of ER	20
16	ER60	gi2245365	56.7	6.10	Cys protease of ER	20
19	GP96	gi4507677	92.4	4.76	Activate tumor-specific CTL response	24
20	VCP	gi6005942	89.3	5.14	Associate to cancer prognosis and metastasis	33
21	HSP70RY	gi38327039	94.3	5.11	Heat and osmotic stress proteins	9
22	ORP150	gi5453832	111.3	5.16	1. Suppress cell death 2. Overexpression damage myocyte	2, 17, 30
23	Glutathione transferase	gi87564	23.2	5.42	1. Antioxidant 2. Detoxicfy carcinogen agent	-
24	Prdx 2	gi1617118	18.3	5.19	Decrease lipid peroxide	26

Table 6 Quantity of protein expression. Data were shown as the ratio of native LDL.

NO	Protein ID	native LDL	2hr-oxLDL	24hr-oxLDL	L1	L3	L5
3	GDI alpha	1.00	0.78	0.68	0.71	1.18	0.55
4	GDI beta	1.00	0.62	0.68	1.00	0.94	0.82
6	HSP27	1.00	0.55	0.61	1.32	0.93	1.01
7	Prdx 3	1.00	1.37	0.60	1.12	2.78	1.16
9	Prdx6	1.00	1.21	1.33	1.01	1.43	1.01
10	prohibitin	1.00	0.58	0.60	0.96	1.58	0.79
11	proteasome	1.00	0.37	0.50	0.48	0.76	0.75
12	ATP synthase	1.00	0.81	0.67	0.75	0.69	0.98
13	CapZa1	1.00	0.96	0.83	1.39	0.90	0.65
14	lactate dehydrogenase	1.00	0.81	1.00	1.33	1.51	0.99
15	ER60	1.00	0.70	1.21	1.16	0.76	0.86
16	ER60	1.00	1.32	1.45	1.71	1.73	1.64
19	GP96	1.00	0.75	1.21	0.20	0.74	0.94
20	VCP	1.00	1.89	1.12	1.45	1.35	1.55
21	HSP70RY	1.00	0.73	0.74	1.22	1.17	2.52
22	ORP150	1.00	0.70	0.90	1.10	1.36	1.82
23	glutathione transferase	1.00	1.08	0.72	1.13	0.85	0.53
24	Prdx2	1.00	0.98	0.66	0.95	0.79	0.45