Protein expressions in macrophage-derived foam cells: comparative analysis by two-dimensional gel electrophoresis[1]

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ABSTRACT

AIM: To study the overall protein expression changes induced by oxidized low-density lipoprotein (ox-LDL) in the U937 foam cells. **METHODS:** Foam cell model was established by incubating the human monoblastic leukemia (U937) cells with ox-LDL. Each protein samples in U937 control cells and U937 foam cells were separated by two-dimensional gel electrophoresis (2-DE). After the gels were stained by silver staining method, the images were analyzed by PDquest 2D-image-analysis software (Bio-Rad). Some of the spots were available via the Internet with links to the U937 proteomic map provided from the ExPASy Proteomics server. **RESULTS:** Using 2-DE, the overall protein map was obtained, in which 150 spots were matched with the control gel (match ratio: 75 %). Compared with U937 cells, 37 spots significantly changed in the foam cells ($P<0.05$), among which the expression levels in 28 spots increased and those in 9 spots decreased. Especially, 8 spots in U937 cells were absent in the foam cells, while 11 spots in the foam cells were absent in the control cells. **CONCLUSION:** The changed protein profiles induced by ox-LDL in U937 foam cells were established to support the functional studies on the macrophage-derived foam cells in atherosclerotic pathological states.

INTRODUCTION

The term proteome was recently coined to describe all the proteins encoded from a specific genome[1]. Proteomic analysis involves the qualitative alterations in proteins along with the quantitative changes in protein expression levels that occur in response to a given set of conditions. Despite the fact that proteomics-based research has only recently begun to be used, it is fast approaching a crossroad with respect to future utilization. The use of two dimensional gel electrophoresis (2-DE) is the best technique to separate complex protein mixtures[2]. Using 2-DE, the investigation of the cellular or molecular "big picture" with the use of proteomics may be one of the best approaches to elucidate the complex and multi-factorial basis for many aspects of cardiovascular biology, especially disease processes.

Atherosclerosis is not merely a disease in its own right, but a process that contributes principally to the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function in the extremities. During the process of atherosclerosis, monocytes seem to play a central role. Once monocytes adhere to the suben-

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dothelial space and enter into the intima of the artery, oxidized low-density lipoprotein (ox-LDL) and other substances associated with atherosclerosis may participate in transformation of the monocytes in macrophage. Uptake of ox-LDL by the macrophage through scavenger receptors will lead to foam cell formation[3,4].

The macrophage-derived foam cells not only result in formation of fatty streaks, which are believed to represent the earliest type of atherosclerotic plaque, but also play roles in the fibroproliferative process by their capacity to form numerous growth factors in particular platelet-derived growth factor (PDGF)[5], vascular endothelial growth factor (VEGF)[6,7], interleukin-1 (IL-1)[8], as well as tumour necrosis factor α (TNFα)[9]. Therefore, a further study on the global changes in protein expression accompanying the formation of foam cells *in vivo* becomes an urgent mission to be investigated.

In our previous studies, we established a macrophage-derived foam cell model through incubating the human monoblastic leukemia (U937) cells with ox-LDL[10,11]. The ICAM-1 and VEGF expression levels have been determined to be the same as the foam cells in the atherosclerotic plaques, which confirm the successful establishment of the pathological model. The aim of this study was to further investigate the global changes in protein expressions between the U937 foam cells and the control U937 cells using 2-DE.

MATERIALS AND METHODS

Reagents $CuSO_4$ and edetic acid were purchased from Sigma Chemical Co; bisacrylamide (Bis), hydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), glycine, N,N,N',N'-tetramethylethyl-diamide (TEMED), ammonium persulfate (APS), glycerol, ultrapure urea, and bromophenol blue were purchased from Pharmacia Biotech; acrylamide, dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Fluka BioChemika; All the water used in the experiments was 18-MΩ deionized from a MilliQ water purification system.

Cell culture LDL (d=1.019 to 1.063 kg/L, purchased from Sigma Co, USA) was sterilized by filtration through 0.45-μm Millipore membranes, and stored at 4 ºC as described previously[12]. After edetic acid was removed by dialysis, LDL was oxidized by incubation in $CuSO_4$ 10 μmol/L at 37 ºC for 16 h, and then dialyzed in phosphate buffer saline (PBS) containing edetic acid 0.1 mmol/L at 4 ºC for 24 h.

The human monocyte line U937 was obtained from Cell Bank in Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. U937 cells were cultured in RPMI-1640 containing 10 % fetal bovine serum. Then the cells were incubated with ox-LDL 80 mg/L for 48 h, and the control group was the U937 cells which were not treated with ox-LDL.

Protein extraction and quantification The cells were disrupted by ultrasonication (Sonics & Materials Inc, Vibra Cell™) twice, each for 20 s, on ice in extraction solution which containing urea 8 mol/L, 4 % 3-[3 (cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAP), and Tris 40 mmol/L. The resulting homogenate was centrifuged at 12 000×*g* for 10 min. The supernatant was fractioned in aliquots and stored at -20 ºC till further analyzed.

Protein quantitation was performed similarly to the Bradford method with some modifications. Briefly, using bovine serum albumin (BSA) in Tris solution as standard protein solution, furthermore, a small quantity of HCl (0.1 mol/L) was added in the reaction system with the reagent blank experiment to calibrate. The standard and samples were prepared in duplicates to minimize some occasional error during the course.

Two-dimensional gel electrophoresis Each sample of 40 μg protein was added to a rehydration solution containing urea 8 mol/L, 4 % CHAPs, Tris40 mmol/L, DTT 50 mmol/L, 0.2 % ampholyte (pH=3-10), and trace bromophenol blue. Immobilized dry strips (Amersham Pharmacia), 7 cm, linearly covering a pH range of 3-10, were allowed to rehydrate in 130 μL the above solution for 12 h under mineral oil. The following voltage/time profile was used: increasing voltage by gradient from 0-250 V for 30 min, increasing voltage by step-n-hold procedure from 250-4000 V for 3 h, and a final phase of 4000 V for 8000 Vh.

After the first dimensional run, the individual strip was equilibrated, and then put on the top of the second dimensional gel (12 %). Each gel was run at 10 mA at the beginning and 15 mA per gel was used till all the proteins being transferred from strip into gel. The run was completed until the bromophenol blue front reaching the bottom of the gel.

Silver staining and image analysis The gels were stained by silver staining method similarly to the method described[13] with modifications. Images were captured by scanning the silver-stained gels using Molecular Imager Fx (Bio-Rad), and then processed by

PDquest 2D-image-analysis software (Bio-Rad).

Protein bioinformatic analysis In addition to the PDquest software packages for analyzing the electrophoretic separation, bioinformatic tools were used. Some of the spots were available via the Internet with links to the U937 proteomic map provided from the ExPASy Proteomics server (http://us.expasy.org).

Statistics All results were shown as mean±SD. Statistical analysis was performed with *t*-test in the PDquest software.

RESULTS

Two-dimensional protein maps of the control U937 cells and foam cells All the gels were normalized against one in the U937 cell group as the control. A typical analytical 2-D gel from U937 cell line was shown in Fig 1A. Through image processing by PDquest, more than 1000 spots were detected, among which 200 spots were accorded with Gaussian distribution. Most of the spots were centralized in the area of acidic region (pI: 4-7). Through the 2-DE protein database of the U937 in the ExPASy Proteomics server, 6 spots were identified.

The foam cells were incubated four times in the same condition (n=4). The 2-D patterns obtained after 2-DE separation of 40 μg protein sample from U937 foam cells (Fig 1B). Through the PDquest software, 150 spots were matched with the spots in the control gel (match ratio: 75 %).

Proteomic comparison analysis on the U937 foam cells Compared with control U937 cells, 37 spots were found significantly changed in the foam cells (P<0.05, Tab 1). Among them, 8 spots were absent in the U937 foam cells compared with the normal U937 cells. However, 11 spots found only in the U937 foam cells showed no expression in control U937 cells.

Tab 1. The significant changed protein spots in the U937 foam cells compared with U937 control cells analyzed by 2-DE. n=4. Mean±SD. ^bP<0.05 *vs* U937control cells.

SSP	M_r (±1000)	pI (±0.15)	Average normalized quantity	
			U937 control cells	U937 foam cells
0105	26620	4.48	0	23917±88^b
0608	74800	3.95	6338±85	0^b
0703	79190	4.49	1548±35	6565±35^b
0707	101220	4.58	0	24478±84^b
1110	29120	4.62	0	24768±82^b
1202	31680	4.86	1883±40	0^b
1308	38700	4.85	2463±17	4811±20^b
1801	114300	4.63	3686±29	5674±14^b
2104	29230	4.96	0	24575±83^b
2509	47600	4.93	4361±33	0^b
2609	76780	5.02	1604±52	44346±63^b
2710	90370	5.21	1861±55	0^b
2802	148400	4.99	501±416	3742±37^b
2809	115810	5.20	1449±27	0^b
3711	91230	5.34	0	1262±39^b
4104	26860	6.09	14521±14	17947±6^b
4108	29620	6.14	1134±51	0^b
4306	37450	6.04	0	1736±24^b
4601	62960	5.75	2938±35	4722±10^b
4602	69250	6.02	1551±19	2926±10^b
4603	54420	6.12	5609±9	7962±6^b
4706	100500	5.87	0	1550±12^b
5215	32400	6.16	0	25862±74^b
5304	36040	6.40	881±38	1954±6^b
5305	37820	6.40	935±33.26	0^b
5307	39940	6.25	0	2701±10^b
5504	51390	6.31	1553±8	2210±11^b
5605	54980	6.37	6596±18	9930±19^b
5702	82990	6.30	1881±35	3325±16^b
5707	83300	6.23	848±39	2260±41^b
6208	31200	6.78	0	8159±78^b
6303	40310	6.59	2441±28	8104±32^b
6503	46690	6.62	1736±24	3312±28^b
6607	57250	6.66	0	24139±87^b
7207	31110	6.89	11125±30	3122±46^b
7503	51700	7.05	1719±7	0^b
8002	24580	7.60	10472±9	13380±11^b

DISCUSSION

Foam cells are the characteristic pathological cells in the atherosclerotic lesions, which represent the inflammatory reactions in atherosclerosis. The U937 foam cells were well established as a macrophage-derived foam cell model, which showed that U937 cells internalized ox-LDL through scavenger receptor and became foam formation.

2-DE is considered to be the key separation technique in proteome analysis due to its advantages of simultaneously separation of thousands of proteins at a time and excellent reproducibility. In the procedure of 2-DE analysis, we used silver staining method proposed by Andrej S *et al* in 1996 because of its high sensitivity together with its compatibility with mass spectrometric identification further. During the staining course as described previously[13], we found that the removal of acetic acid used for fixing proteins was very important

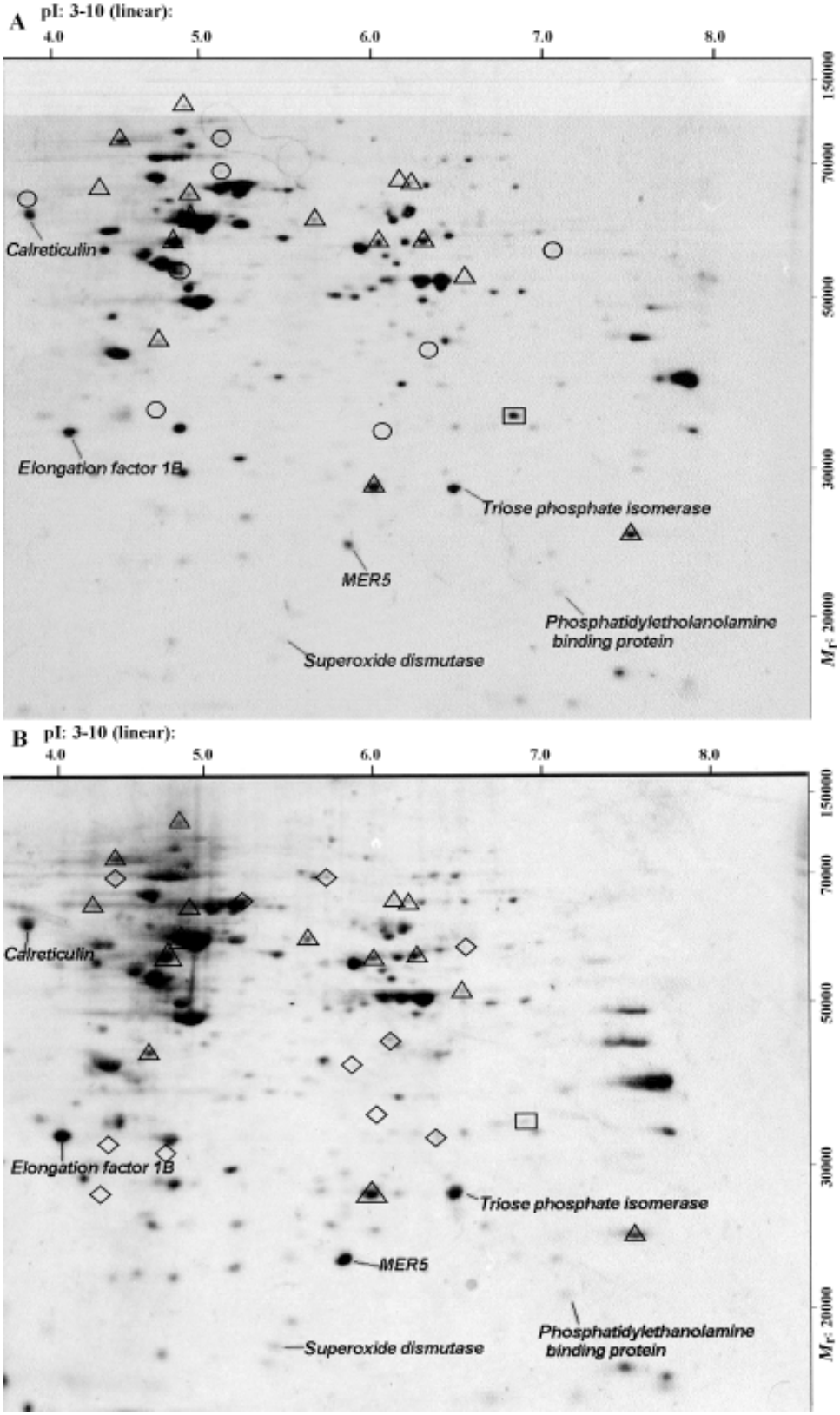

Fig 1. The two-dimensional protein maps of the U937 cells and foam cells. Protein extractions in U937 cells were separated by 2-DE and analyzed by the PDquest software (*n*=4). The annotated protein spots were provided from ExPASy Proteomics server. A) U937 control cells; B) U937 foam cells. ¡ð the protein spots only found in U937 control cells; ¡ó the spots only found in U937 foam cells; ¡÷ the expression levels increased in foam cells compared with control cells; ¡õ the levels decreased in foam cells compared with control.

to reduce the background and increase the contrast between the stained protein spots and grounding. So we increased washing times for the acid removal, but decreased the period of each washing turn to balance the overall time used.

The annotated proteins and 2-DE databases are the bioinformatics core of proteome research. The information from these database is invaluable to both researchers, novice, and expert alike, wishing to obtain indirect protein identification in a comparable 2D image of validation of a protein previously identified through analytical analysis. However, the available spots provided from the ExPASy Proteomics server are not significant changed during the formation of the foam cells. Since most of the annotated proteins in the 2-DE database are structure proteins, ox-LDL may have few effects on the expressions of the structure proteins during the foam cell formation. Subsequent studies should succeed in identifying additional proteins, thereby enhancing the list of already characterized proteins found in respective database.

The importance of 2-deprotein database has led to a major initiative in our laboratory to establish several databases to support several ongoing functional studies. The present studies provided a 2-DE protein database of the foam cells, which gave a further analysis on the complete complement of proteins expressed by a biological system in response to atherosclerotic pathophysiological conditions. Therefore, this finding develops a novel way to elucidate the cellular and molecular mechanisms associated with atherosclerotic pathological states.

Proteomic method has been proven to be an efficient way to investigate different pathological processes resulted from different conditions. Analysis by 2-DE just gave us the primary screening to find variant expressions during the formation of foam cells. The target proteins (Tab 1) remain to be identified with mass spectrometry (MS) techniques. Further studies are focusing on both the target protein identifications and their corresponding functional characteristics.

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