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Proteomic analysis of human macrophages exposed to hypochlorite-oxidized low-density lipoprotein

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ABSTRACT

The invasion of monocytes through the endothelial wall of arteries and their transformation from macrophage into form cells has been implicated as a critical initiating event in atherogenesis. Human THP-1 monocytic cells can be induced to differentiate into macrophages by phorbol myristate acetate (PMA) treatment, and can be converted into foam cells by exposure to oxidized low-density lipoprotein (oxLDL). To identify proteins potentially involved in atherosclerotic processes, we performed a proteomic analysis of THP-1 macrophages exposed to oxLDL generated by treatment with native LDL with hypochlorous acid/hypochlorite ($HOCl/OCl^-$). We detected more than a thousand proteins, of which 104 differentially expressed proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF) and the NCBI database. The largest differences in expression were observed for bifunctional purine biosynthesis protein, vacuolar protein sorting 33A, breast carcinoma amplified sequence, adenine phosphoribosyltransferase, and tropomyosin alpha 3 chain. Interestingly, many apoptotic proteins such as lamin B1, poly (ADP-ribose) polymerase, Bcl-2 related protein A1 and vimentin were identified by MALDI–TOF analysis. Identities were confirmed by matching the sequence of several tryptic peptides using MALDI–TOF/TOF MS, Western blot analyses and immunofluorescent microscopy. The data described here will contribute to establishing a functional profile of the human macrophage proteome. Furthermore, the proteins identified in this study are attractive candidates for further biomarkers involved in the pathogenesis of atherosclerosis.

1. Introduction

The oxidative modification of low-density lipoprotein (LDL) and subsequent alteration of endothelial cell function are generally accepted as important early events in the pathogenesis of atherosclerosis [1,2]. The upregulation of cell-adhesion molecules greatly increases the adherence of blood monocytes to the endothelium. After adhesion, monocytes migrate into the subendothelial space where they differentiate into macrophages. Uptake of oxidized low-density lipoprotein (oxLDL) by the macrophage through scavenger receptors will lead to foam cell formation [3]. The THP-1 human monocytic cell line is a well-characterized model system with which to study the transformation of macrophages to foam cells [4]. THP-1 monocytes can be induced with phorbol 12-myristate 13-acetate (PMA) to undergo differentiation into a macrophage-like phenotype, and the resulting macrophages can then be converted to foam cells following treatment with oxLDL [5].

The mechanisms for the generation of oxLDL *in vivo* are not well defined. Experimentally, LDL oxidation *in vitro* is usually performed by incubating the lipoprotein with high concentrations of free metal ions such as copper and iron. Oxidation of the LDL-lipid moiety leads to the generation of lipid peroxidation products. Although copper has been shown to be present in atherosclerotic lesions [6], the significance of trace metal mediated LDL oxidation *in vivo* has been called into question [7]. There is little increase in markers of copper- and hydroxyl radical-mediated protein damage in either lesion LDL fatty streaks, suggesting that free metal ions are unlikely to be involved early in atherogenesis. In contrast, the involvement of chlorinated oxidants which produce little modifications in the LDL–lipid moiety and preferentially affect its protein moiety, notably apolipoprotein B, appears more likely to occur *in vivo*. This pathway involves myeloperoxidase, which catalyses the production of hypochlorous acid/hypochlorite ($HOCl/OCl^-$) from hydrogen peroxide and chloride ions in activated neutrophils and monocytes [8,9]. Hypochlorous acid generated by myeloperoxidase converts tyrosine to 3-chloroutyrosine and cholesterol to chlorinated compounds. An increasing number of

Abbreviations: PMA, phorbol 12-myristate 13-acetate; oxLDL, oxidized low-density lipoprotein; DAPI, 4′-6-Diamidino-2-phenylindole; DIC images, Differential Interference Contrast image

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studies have demonstrated that LDL oxidized by hypochlorite *in vitro* is able to mimic fundamental atherogenic processes including adhesion to endothelial cells, activation of phagocytes with increased formation of reactive oxygen species, and formation of foam cells [10–12]. The products of myeloperoxidase were detected at elevated levels in a lesion of LDL and all stages of atherosclerosis, suggesting that reactive tyrosyl radicals generated by myeloperoxidase represented one pathway of LDL oxidation *in vivo*.

Despite studies of the critical roles of oxLDL in foam cell formation and atherogenesis, relatively little is known about the mechanisms by which oxLDL activates macrophages. In an attempt to increase our understanding of these mechanisms, we initiated a search for proteins that are specifically up- or downregulated in oxLDL activated human THP-1 macrophages compared with nonstimulated cells. Gene expression studies have identified many genes to be either upregulated or downregulated in human atherosclerosis. However, protein expression patterns do not always reflect differential gene expression patterns. Furthermore, protein functions can also be influenced by post-translational modifications. Proteomics offers a unique means for analyzing the expressed genome, to provide important clues to the mechanisms involved in this complex process [13]. Recently, Conway and Kinter [14] reported a proteomics study to identify proteins associated with the foam cell formation. They used murine macrophages that had been chronically exposed to copper-oxLDL. However, copper-mediated LDL oxidation seems not to be pathologically relevant. Therefore, in the present study, we investigated the effects of oxLDL generated by treatment with HOCl on the transition of macrophages into foam cells at the protein levels. The associations of two-dimensional electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) and database interrogations allowed us to identify various proteins differentially expressed in monocyte-derived macrophage THP-1 cells. Thus, we identified various proteins more especially implicated in regulation of apoptosis, growth, cell mobility, and signal transduction.

2. Materials and methods

2.1. Isolation and oxidation of LDL

LDL (density = 1.020–1.063 g/ml) were isolated by sequential flotation ultracentrifugation from human plasma, as described previously [15]. Before oxidative modification, the LDL was dialyzed against phosphate-buffered saline (PBS), filtered through a 0.2 μm Millipore membrane (Millipore, Bedford, MA, USA), and stored in PBS containing 1 mM EDTA at 4 °C. Hypochlorite modification of LDL was essentially performed according to Vicca et al. [16]. LDL oxidation was induced for 30 min at 37 °C with 40 mM HOCl (i.e. corresponding to oxidant/protein molar ratios (R) of 2000/1). OxLDL was dialyzed overnight against 10 mM phosphate-buffered saline (PBS), at pH 7.4.

2.2. Cell culture and treatment

Human THP-1 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (v/v), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.15% sodium bicarbonate (w/v), 0.45% glucose (w/v), 1×10^{-5} M β-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml), and kept at 37 °C in an atmosphere of 5% CO_2. The cell medium was replaced every 2 days for the duration of the culture before the beginning of cell treatment. Cell were seeded at a density of 5×10^5 cells/ml in medium containing 100 nM PMA (Sigma, St. Louis, MO for 48 h. The medium was then replaced by culture medium with 100 μg/ml of vehicle (50 mM Tris, pH 7, 150 mM NaCl, 0.3 mM EDTA), and LDL(50 μg/ml), which was oxidized with HOCl. Macrophages were transformed into foam cells by incubation with the presence or absence of oxLDL in culture medium for 24 h. Cells were washed three times with PBS, fixed for 30 min with 5% formalin solution in PBS, stained with Oil Red O for 30 min, and counterstained with DiffQuick staining kit (IMEB, Inc.). Finally, the cells were examined by light microscopy. Human monocyte-derived macrophage (HMDM) were kindly provided from Dr. In-San Kim (School of Medicine, Kyungpook National University, Korea) [17].

2.3. Extraction of proteins from monocyte-derived macrophages

Cells were washed three times with 25 mM Tris, pH 7.4 and scraped in buffer containing 50 mM Tris pH 8.6, 10 mM EDTA, 65 mM dithiothreitol (DTT) and a protein-inhibitor cocktail tablet (complete TM; Roche Diagnostics, Meylan, France). Cells were harvested at 1400 ×*g* for 10 min. Harvested cells were mixed with lysis buffer containing 9.5 M urea, 2% CHAPS, 0.8% carrier ampholytes (Pharmalyte; Amersham Pharmacia Biotech, Piscataway, NJ), and 1% DTT and a protein-inhibitor cocktail tablet. After incubation at room temperature for 1 h and sonication for 3 min, the samples were centrifuged for 15 min at 10,000 ×*g*, and the supernatants were directly applied to a 180 mm immobilized pH gradient (IPG) strip for isoelectric focusing (IEF). Protein concentrations were determined with commercial Bradford reagent (Bio-Rad, Hercules, CA), and the samples were stored at −70 °C until use.

2.4. Two-dimensional gel electrophoresis (2-DE)

2-DE was performed in an Ettan IPGphor IEF System (Amersham Pharmacia Biotech) using 180 mm pH 3–10 and pH 4–7 Immobiline DryStrips (180 mm × 3 mm × 0.5 mm) for the first dimension, and 12% SDS–polyacrylamide gels for the second dimension. SDS–PAGE was performed in an Ettan DALTsix Larger Vertical System (Amersham Pharmacia Biotech).

IPG–IEF can be simplified by the use of the integrated IPGphor system, in which rehydration with sample solution and IEF are performed in a one-step procedure. Initial rehydration was for 12 h at 20 °C. IEF was then carried out using the following voltage program: 500 V (gradient over 1 h), 1000 V (gradient over 1 h), 8000 V (fixed for 4 h) at 50 μA/strip. The IPG strips were equilibrated for 30 min in 125 mM Tris (pH 6.8) containing 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), and 65 mM DTT and then for a further 30 min in the same buffer, except that DTT was replaced with 260 mM iodoacetamide. The IPG strips were then sealed with 0.5% agarose in SDS running buffer at the top of slab gels (260 mm × 200 mm × 1.5 mm).

The second-dimension electrophoresis was performed in the Ettan DALTsix Larger Vertical System at 180 V/gel and room temperature for 8 h. The gels were then fixed with 50% ethanol containing 3% phosphoric acid for 30 min and stained with 0.02% Coomassie Brilliant Blue (CBB)–G250, 3% phosphoric acid, 17% ammonium sulfate, 34% ethanol. Relative molecular weights were determined by simultaneously running standard protein markers (MBI Fermentas, Hanover, MA) in the range 10–170 kDa. The p*I* values used were those given by the supplier of the immobilized pH gradient strips. Excess dye was washed from the gels with distilled water and the gels were scanned with a UMAX PowerLook 1120 scanner (UMAX Technologies, Dallas, TX). Protein spots were outlined (first automatically and then manually) and quantified using PDQUEST software (Bio-Rad). Three batches of cell proteins extracted from untreated cells and oxLDL-treated cells were subjected to 2-DE. Data of 2-DE experiments are shown as means ± standard deviation (SD). For the differential analysis, statistical significance was estimated with Student's *t*-test. Values of $p < 0.05$ were considered significant.

2.5. MALDI–TOF MS and MALDI–TOF/TOF MS

To identify the protein spots on gel pieces, they were excised from preparative 2-DE gels, and cut into 1–2 mm pieces. These were added to 100 μl of 25 mM NH_4HCO_3/50% acetonitrile, incubated for