Proteomic analysis of differential protein expression in atherosclerosis and regulatory role of human LZIP in development of atherosclerosis

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Dedicated to my parents and my wife, who have encouraged me.

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ABBREVIATIONS

2 DE	: 2 Dimensional Electrophoresis
CCR2	: CC chemokine Receptor2
C/EBP	: CCAAT/Enhancer Binding Proteins
CRP	: C-Reactive Protein
DcR1	: Decoy Receptor1
DTT	: 2,3-dihydroxy-1,4-dithiobutane; 1,4-dithiothreitol
EDTA	: Ethylenediaminetetraacetic Acid
EMSA	: Electrophoretic Mobility Shift Assay
FACS	: Fluorescence-Activated Cell Sorter
Lkn-1	: Leukotactin-1
MALDI-TOF	: Matrix-Assisted Laser Desorption Ionization Time Of Flight
MCP-1	: Monocyte Chemoattractant Protein-1
oxLDL	: Oxidized Low Density Lipoprotein
PMA	: Phorbol 12-Myristate 13-Acetate
PMSF	: Phenylmethylsulphonylfluoride
RPA	: RNase Protection Assay
RT-PCR	: Reverse Transcription-Polymerase Chain Reaction
siLZIP	: Small Interfering LZIP
TFA	: Trifluoroacetic Acid

CHAPTER I

Proteomic analysis of differential protein expression in atherosclerosis

ABSTRACT

Although recent studies have shown that several pro-inflammatory proteins can be used as biomarkers for atherosclerosis, the mechanism of atherogenesis is not clear and little information is available regarding proteins that are involved in development of the disease. Atherosclerotic tissue samples were collected from patients in order to identify the proteins involved in atherogenesis. The protein expression profile of atherosclerosis patients was analyzed using two dimensional electrophoresis-based proteomics. Thirty-nine proteins that were differentially expressed in the atherosclerotic aorta compared with the normal aorta were detected. Twenty-seven of these proteins were identified in the MS-FIT database. They are involved in a number of biological processes, including calcium-mediated processes, migration of vascular smooth muscle cells, matrix metalloproteinase activation, and regulation of pro-inflammatory cytokines. Confirmation of differential protein expression was performed by Western blot analysis.

Potential applications of these results include identification and characterization of signaling pathways involved in atherogenesis, and further exploration of the role of selected identified proteins in atherosclerosis.

Keywords : atherosclerosis, cardiovascular disease, biomarker, inflammation, two dimensional electrophoresis

I. INTRODUCTION

Atherosclerosis is a major cause of sudden cardiac death, acute myocardial infarction, and unstable angina pectoris (Tousoulis *et al*., 2003). There is now a general agreement that atherosclerosis is an inflammatory vascular disease characterized by endothelial activation, cellular influx, and production of mediators and cytokines (Ross, 1999; Vorchheimer *et al*., 2001; Elgharib *et al*., 2003). This process leads to formation of foamy macrophages and atheromatous plaques, atheroma instability and plaque disruption followed by local thrombosis, that underlies the clinical presentation of acute coronary symdromes (Ross, 1999; Vorchheimer *et al*., 2001).

There have been efforts to understand the mechanism of atherosclerosis and identify biomarkers in cardiovascular disease, and evidence from recent studies indicates that many proteins are involved in atherogenesis (Blann *et al*., 2003; Elgharib *et al*., 2003; Nomoto *et al*., 2003). Since inflammation plays a pivotal role in all stages of atherogenesis, insight gained from recent

basic and clinical data linking inflammation to atherosclerosis has yielded important diagnostic and prognostic information (Ross, 1999; Vorchheimer *et al*., 2001; Elgharib *et al*., 2003). Although measurement of lipid levels, stress testing, and coronary angiography are effective indicators of the extent and severity of the disease, biomarkers that can be easily measured would be powerful tools to diagnose, monitor, and intervene in this disease process.

As a marker of low-grade chronic inflammation, C-reactive protein (CRP) expression has been widely used to predict the future risk of acute coronary syndrome independent of traditional cardiovascular risk factors (Abrams, 2003). CRP is a major acute phase response protein synthesized in the liver in response to elaboration of the acute phase response cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) (Elgharib *et al*., 2003). However, CRP is a relatively non-specific marker of inflammation. Serum levels of CRP have been used as an inflammatory marker for other diseases, such as rheumatoid arthritis (Abrams, 2003).

Other associated acute phase proteins include serum amyloid A protein (Fyfe *et al*., 1997), fibrinogen (Danesh *et al*., 1998), and plasminogen activator inhibitor (PAI-1) (Ridker *et al*., 1993). Various cytokines, such as IL-6 and TNF-α, and adhesion molecules, such as intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1), have been found to be independently associated with cardiovascular end points (Reape *et al*., 1999; Fassbender *et al*., 1999). The adhesion molecule P-selectin is of interest because of its role in modulating interactions between blood cells and the endothelium, and also due to possible use of the soluble form as a plasma predictor of adverse cardiovascular events (Blann *et al*., 2003). Matrix metalloproteinases (MMPs) and interferon-gamma (IFN-γ), which participate in collagen degradation, are pathological factors in plaque vulnerability as an important mechanism underlying acute coronary syndrome (Nomoto *et al*., 2003).

Although these proteins are known to be involved in atherosclerosis, how these proteins are related to each other and the physiological roles of these

proteins in atherogenesis still remain to be characterized. Also, atherosclerosis specific proteins that function as a driving force for the atherogenic process have not been identified. In an effort to identify proteins involved in atherogenesis or specific biological markers for atherosclerosis, protein profiles that show differential expression in atherosclerosis were analyzed using the MALDI-TOF and MS-FIT database.

II. MATERIALS AND METHODS

Materials

Hematoxylin and eosin solution were obtained from Sigma (St. Louis, MO, USA). Acrylamide and DTT were purchased from Amresco (Solon, OH, USA) and immobiline dry strips were obtained from Amersham Biosciences (Uppsala, Sweden). Ultrapure electrophoretic reagents and silver stain reagents were purchased from Bio-Rad (Richmond, CA, USA). Sequence grade trypsin was obtained from Promega (Madison, WI, USA) and in-gel digestion reagents were purchased from Sigma (St. Louis, MO, USA). SelfPack POROS 10 R2 for ZipTip process was purchased from Applied Biosystems (Foster city, CA, USA). Goat polyclonal anti-decoy receptor 1 (DcR1), rabbit polyclonal anti-14-3-3γ, and goat polyclonal anti-annexin-5 (ANX-5) antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma.

Tissue sample preparation

Atherosclerotic specimens were obtained from seven patients undergoing aorta bypass surgery. Atherosclerotic aorta tissues were obtained from the ascending aorta during the bypass procedure, and biopsies of the normal aorta tissues were also obtained from the same patients. Tissue samples were immediately stored at -80°C until use. All samples were gathered with the informed consent of the patients after permission was obtained from the institutional ethics committee.

Tissue staining

Normal and atherosclerotic aorta tissues were fixed in 10% formalin and paraffin-embedded. The sections (4 μm thick) of normal and atherosclerotic tissues were routinely stained by hematoxylin and eosin. The sections were cleared with xylene (two changes, 5 minutes each) and rehydrated through decreasing grades of ethanol (100% to 70%, 1 minute each). After staining with hematoxylin and eosin, the sections were dehydrated and mounted with Canada balsam. The sections were photographed on an Olympus photomicroscope (Inha, Japan).

Sample preparation for 2 dimensional electrophoresis (2-DE) analysis

Frozen tissues were homogenized at 3,000 rpm with lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris, 20 mM DTT and 100 mM PMSF) on ice until completely lysed. The debris was removed by centrifugation at 15,000 × g for 30 min at 4°C. Before isoelectric focusing (IEF), 700 μg of total sample proteins were mixed with rehydration buffer (4 M urea, 2% CHAPS, 20 mM DTT and 1% v/v carrier ampholytes 3-10 and 5-8). IEF was carried out on linear, wide-range immobilized pH gradients (pH 4-7, 24 cm long) by using the IPGphor system (Amersham Biosciences) for a total run of 69.5 kV. Following IEF, the gel strips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8) buffer containing 6 M urea, 30% glycerol, 2% SDS, 1% DTT and a few grain of bromophenol blue. After the first-dimensional separation, the IPG strips were loaded on the top of vertical 10% SDS-polyacrylamide gels with a run of 2.5 W per gel for 30 min followed by 17 W per gel for 4 to 5 h

at 10 °C. For analytical purpose, the gels were silver stained as described (Gevaert *et al*., 2000).

Image acquisition and data analysis

Spot signals were measured by densitometric scanning. Matching and quantification of spots were performed with PDQuest™ software (Bio-Rad, V7.1) package.

In-gel digestion of proteins

The protein spots of interest were excised from the gel, and placed in a 0.5 ml siliconized centrifuge tube. The gel pieces were destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. The dehydration was performed with acetonitrile and dried in a vacuum centrifuge. For trypsin digestion, the gel pieces were re-swollen in 50 mM ammonium bicarbonate buffer (pH 7.8), 1 μg of trypsin solution was added, and the mixture was incubated 45 min on ice. After incubation, the supernatants were discarded and 50 mM ammonium bicarbonate buffer (pH 7.8) was added. For complete

digestion, the gel pieces were incubated overnight at 37°C. The digested gel pieces were purified with ZipTip process. The peptides were eluted from the tip directly onto the MALDI plate with a solution of α-cyano-4-hydroxycinnamic acid (CHCA) (6 mg/ml in acetonitrile/0.6% TFA).

MALDI-TOF mass spectrometry

Analysis was performed on a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) Voyager-DE STR mass spectrometer (Applied Biosystems) operated in delayed extraction mode. Samples (0.5 μl) were spotted onto a sample plate to which matrix was added. The sample-matrix mixture was dried at room temperature and analyzed in reflector mode. The time of flight was measured using following parameter: 20 kV accelerating voltage, 66% grid voltage, 0% guide wire voltage, 200 nsec delay and low mass gate 800 Da. External calibration was performed using angiotensin I (m/z 1296.8585), fibropeptide B (m/z 1570.8669), adrenocorticotropic hormone fragment 1-17 (m/z 2094.3471), 18-39 (m/z 2466.6449). Spectra were the sum of 200 laser shots, and those peaks with a signal-to-noise ratio

of greater than 4:1 were selected for data base searching.

Protein database search

The monoisotopic masses for each peptide were entered into the program MS-FIT (available at prospector.ucsf.edu) for searches against the Swiss-Prot and NCBI. For MS-FIT searches, masses derived from trypsin, CHCA, and keratin were excluded. Typically, the initial searching parameter were (a) search species: *Homo sapiens*; (b) pI range: 4-7; (c) mass tolerance: ±100 ppm; (d) a minimum of four peptide 'hits' required for a match; (e) cysteine as carboxylamidomethyl cysteine; and (f) methionine in an oxidized form.

Western blot analysis

Normal and atherosclerotic tissue samples were homogenized in phosphate buffered saline at 3000 rpm on ice until completely lysed. The debris was removed by centrifugation at 15,000 × g for 30 min at 4°C. Protein samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters. The blots were incubated with anti-DcR1, anti-ANX-5

or anti-14-3-3γ antibodies for 2 h. After washing three times with Tris buffered saline (TBS) containing 0.1% Tween 20, the blots were incubated with anti-goat antibody conjugated with horse radish peroxidase for 90 min and washed three times with TBS containing 0.1% Tween 20, then developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech., Piscataway, NJ, USA). The same blot was stripped and reprobed with anti-β-actin antibody for use as an internal control.

III. RESULTS

Histological examination of normal and atherosclerotic aorta tissues

To examine the morphological and histological changes in normal and atherosclerotic regions of aorta, hematoxylin and eosin staining was performed. Results from tissue staining showed that atherosclerotic aorta appeared to be much thicker than normal aorta, indicating the plaque formation on the endothelial vessel wall of atherosclerotic tissues (Figure 1).

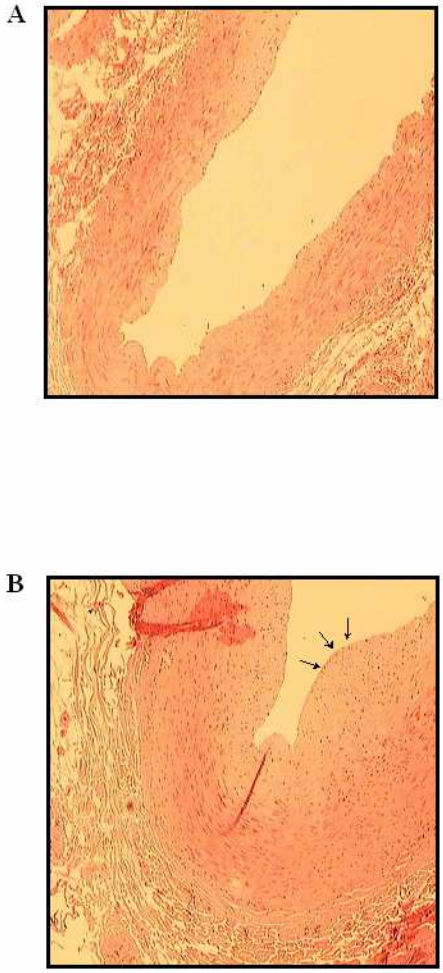

Figure I-1. Hematoxylin and eosin staining of tissues. Paraffin-embedded blocks were sectioned and stained with hematoxylin and eosin (original magnification × 100). Atherosclerotic aorta (B) shows the thicker endothelial vessel than normal aorta (A).

Proteomic analysis of differentially expressed proteins

To identify newly expressed proteins in atherosclerosis that might serve as biomarkers or help elucidate the development of atherosclerosis, protein profiles were investigated using proteomic analysis in atherosclerotic aorta and normal aorta tissues as a control. Protein extracts were prepared from atherosclerotic aorta tissues of seven atherosclerosis patients and normal aorta tissues obtained from the same patients. Protein expression patterns of normal and atherosclerotic tissues were examined using 2-DE (pH 4-7, 24 cm long), and proteins that were differentially expressed in each sample were analyzed. To avoid experimental variation, 2-DE separation for each sample was repeated more than four times. Figure 2 shows the results of 2-DE separation of protein extracts from atherosclerotic tissues. To obtain comparable staining intensities, equal amounts of protein were separated and proteins were visualized by silver staining. 2-D gel image scanning and densitometric scanning of spot signals were performed using the PDQuest^{TM} software package. The expression patterns of more than 900 protein spots were

analyzed from normal and atherosclerotic tissues. Results from 2-DE image analyses showed that the protein profile was significantly different between normal and atherosclerotic tissues. Thirty-nine differently expressed proteins were detected in atherosclerotic tissues, of which 20 proteins showed increased levels in all seven atherosclerotic tissues and 19 proteins showed increased levels in one or two atherosclerotic tissues.

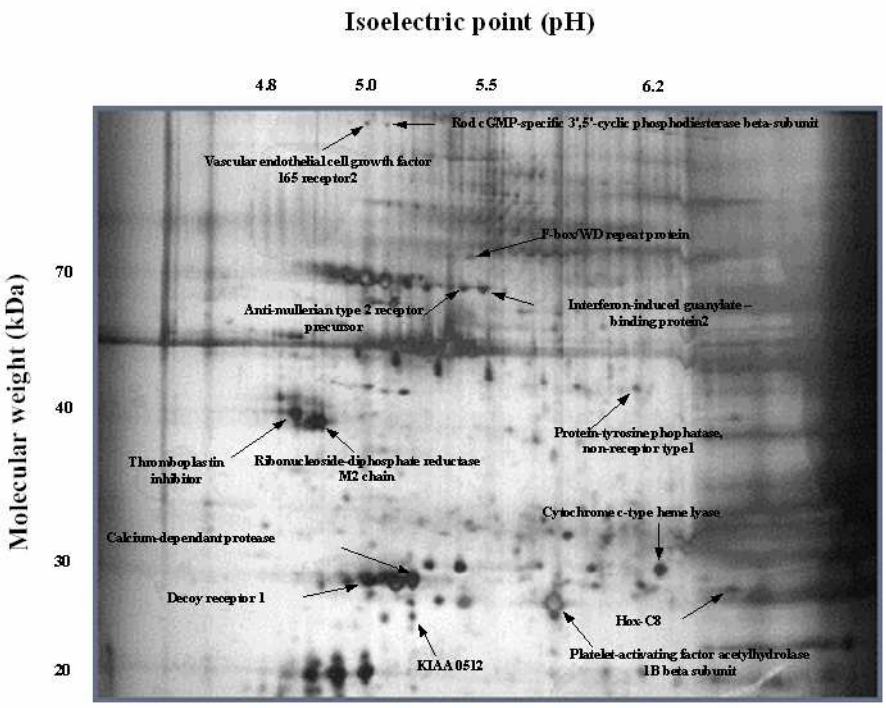

Figure I-2. 2-DE expression patterns of atherosclerosis tissue. This gel was separated on a 25.5 x 20.5 plate, and stained with silver stain as described in section 2.3. The horizontal axis is the IEF dimension, which stretches from pH 4 to 7 and the vertical represents a 10 % SDS-PAGE gel. Seven hundred μg of total sample proteins were loaded. Proteins that were highly expressed in all seven atherosclerotic tissues were indicated.

Identification of proteins specifically expressed in atherosclerosis

Since protein profiles from the seven atherosclerotic tissues were not identical, I focused on identification of proteins that showed increased expression levels in all seven atherosclerotic samples, but not or less expressed in normal aorta. Figure 3 shows the results of PDQuest™ image analysis. Images of the identified protein spots are presented with a histogram of the expression patterns (Figure 3). All protein spots from 2-D gels of each tissue sample were excised and trypsinized before MALDI-TOF mass spectrometric analysis. Figure 4 shows representative peptide peaks from the MALDI-TOF analysis. Of the 39 proteins that were highly expressed in atherosclerosis, 27 proteins were identified from the MS-FIT database. Twelve protein spots were unidentified. Table I and II show the complete results of the database search. Twenty proteins were highly expressed in all seven atherosclerotic tissues and 19 proteins were expressed in only one or two tissues. Of the 20 proteins that were highly expressed in all atherosclerotic tissues, 14 proteins were identified using the MALDI-TOF

mass and the MS-FIT database (Table I). These identified proteins are known to be involved in a number of biological processes, including calcium-mediated processes, migration of vascular smooth muscle cells (VSMCs), MMP activation and regulation of pro-inflammatory cytokines. Of the 19 proteins that were highly expressed in one or two atherosclerotic tissues, 13 proteins were identified (Table II). These proteins are known to be involved in smooth muscle regulation, signal transduction, and angiogenesis.

Confirmation of differential protein expression was performed by Western blot analysis. Among 27 proteins that I have focused on, the protein expression levels of selective 3 proteins were examined. Results from Western blotting showed that the protein expression levels of DcR1, ANX-5, and 14-3-3γ were increased in atherosclerotic samples compared to normal samples (Figure 5A). These results were also confirmed using densitometry. The expression levels of DcR1, ANX-5, and 14-3-3γ were increased approximately 2-fold in atherosclerotic samples (Figure 5B).

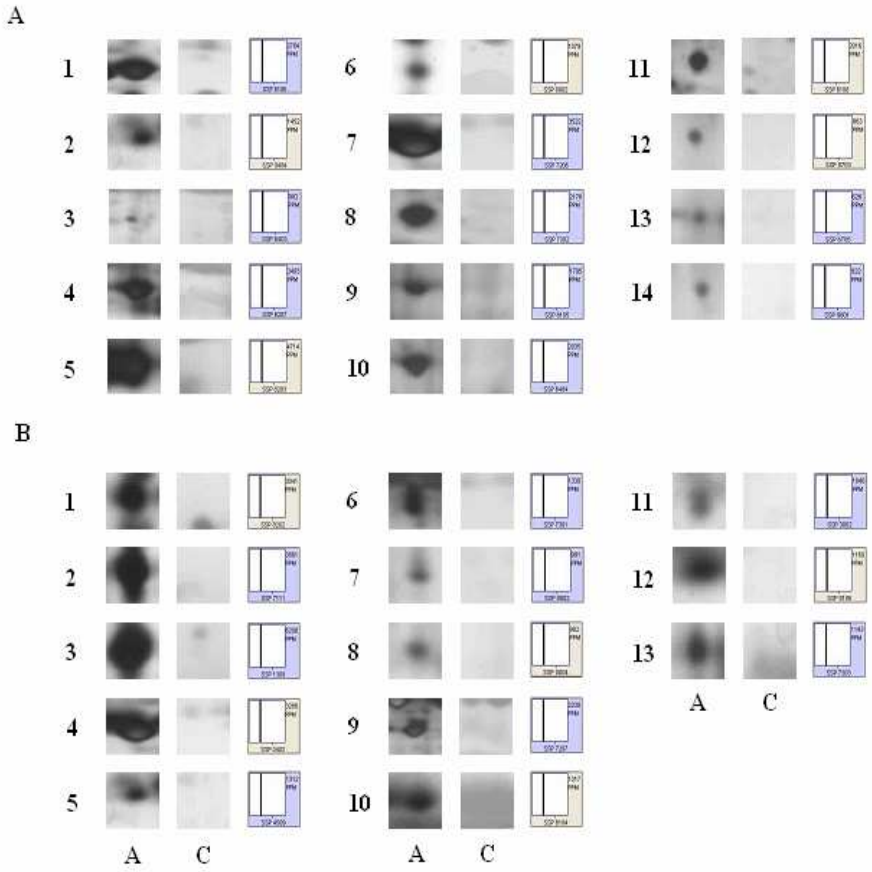

Figure I-3. Image analysis of differentially expressed proteins between atherosclerotic and normal tissues. The expression kinetics, determined by PDQuest^{TM} software, is depicted in histogram on right (A; atherosclerotic tissue, C; control aorta tissue). (A) Proteins highly expressed in all seven atherosclerotic tissues, (B) Proteins highly expressed in one or two atherosclerotic tissues.

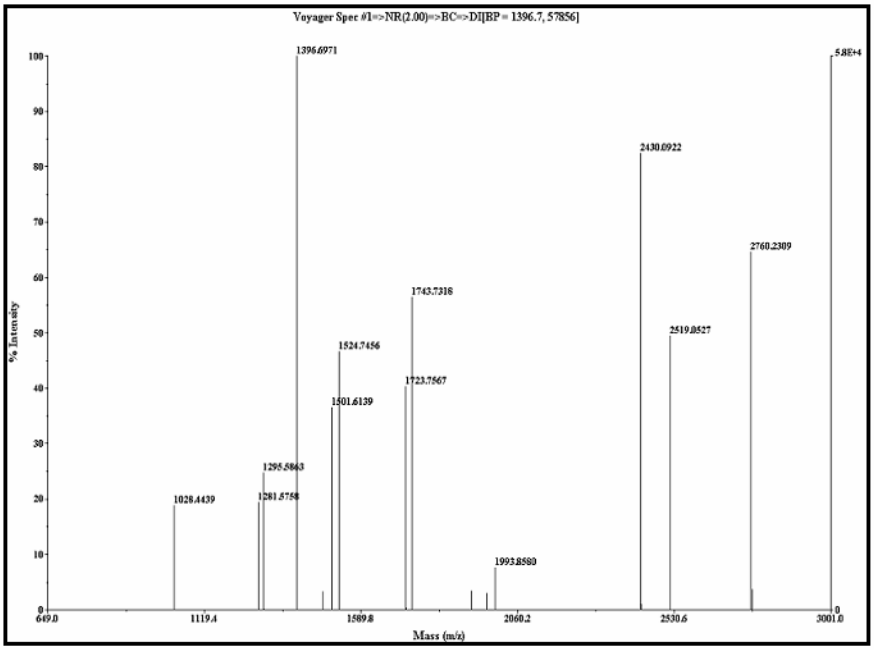

Figure I-4. MALDI-TOF spectrum of a representative protein spot (Calcium-dependant protease). The silver stained protein spot from a 2-DE gel was trypsin-digested as described. The spectrum was obtained by delayed extraction and reflector mode and the MS-FIT database search was performed. This spectrum is the one of 39 spectrums.

Table I-I. Proteins highly expressed in all seven atherosclerotic tissues

Spot ID	Protein name	MW	PI	Swiss-Prot. Access #.
1	Calcium-dependant protease	28316	5.0	P04632
2	Homeobox protein Hox-C8	27755	6.6	P31273
3	Protein-tyrosine phosphatase, non-receptor type 1	49967	5.9	P18031
4	Annexin A5 (thromboplastin inhibitor)	35937	4.9	P08758
5	Ribonucleoside-diphodphate reductase M2 chain	44878	5.3	P31350
6	Human hypothetical protein KIAA 0512	32234	5.3	Q14165
7	Tumor necrosis factor receptor superfamily member 10C precursor (decoy receptor 1)	27395	4.8	O14798
8	Platelet-activating factor acetylhydrolase 1B beta subunit	25569	5.6	Q29459
9	Anti-mullerian type 2 receptor precursor	62750	5.5	Q16671
10	Interferon-induced guanylate-binding protein 2 (guanine nucleotide-binding protein 2)	67184	5.5	P32456
11	Cytochrome c-type heme lyase	30602	6.2	P53701
12	Neuropilin-2 precursor (vascular endothelial cell growth factor 165 receptor 2)	104832	5.0	O60462
13	Rod Cgmp-specific 3',5'-cyclic phosphodiesterase beta-subunit	98408	5.1	P35913
14	F-box/WD repeat protein	67297	5.3	Q8N3Y1

Table I-II. Proteins highly expressed in one or two atherosclerotic tissues

Spot ID	Protein name	MW	PI	Swiss-Prot. Access #.
1	Myosin regulatory light chain2 (cardiac muscle isoform)	19827	4.8	P24844
2	Myosin regulatory light chain2 (cardiac muscle isoform)	19827	4.8	P24844
3	Myosin regulatory light chain2 (cardiac muscle isoform)	19827	4.8	P24844
4	14-3-3 protein gamma	28303	4.8	P35214
5	Calcineurin B subunit isoform 1 (protein phophatase 2B regulatory subunit 1)	19300	4.6	P06705
6	Methionine aminopeptidase 2	52892	5.6	P50579
7	SH3 domain-binding protein 5	47030	5.1	O60239
8	Guanine nucleotide-binding protein G (Y), alpha subunit	42124	5.5	P29992
9	ATP synthase beta chain, mitochondrial precursor	56560	5.3	P06576
10	Interferon regulatory factor 6	53130	5.2	O14896
11	Calcium-binding protein CaBP 5	19826	4.5	Q9NP86
12	Tropomyosin alpha 3 chain	32819	4.7	P06753
13	Serine/threonine protein phophatase 2A, 48kDa regulatory subunit B	47661	4.6	Q9Y5P8

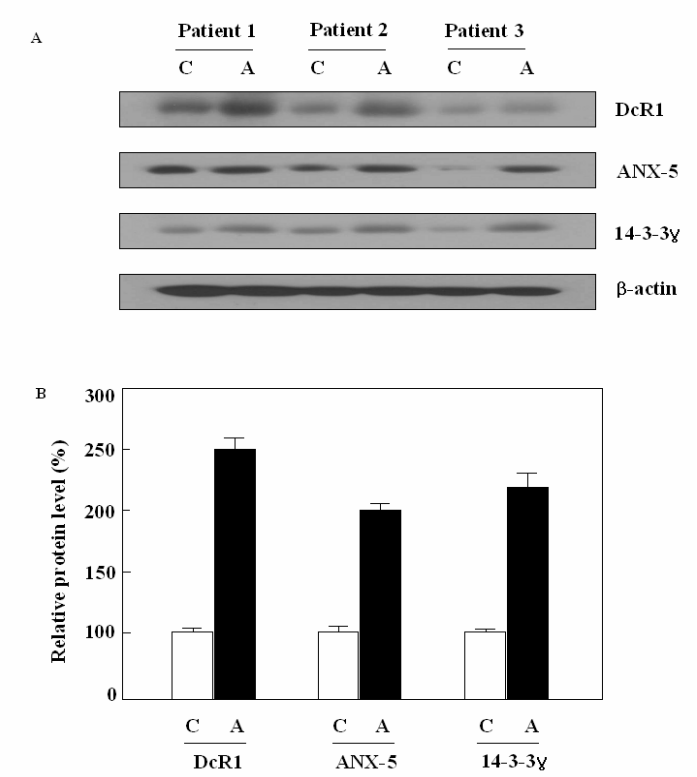

Figure I-5. Proteins highly expressed in atherosclerotic tissues. (A) Normal and atherosclerotic tissues from three patients were homogenized. Forty μg of total sample proteins were loaded on 10 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The protein expression levels of DcR1, ANX-5, and 14-3-3γ were determined by Western blotting. The same blots were stripped and reprobed with anti-β-actin antibody for use as an internal control (C: control aorta, A: atherosclerotic aorta). (B) Densitometric analysis was performed using Quantity One software (Bio-Rad). Data are expressed as the mean value ± standard deviation (the protein level of control aorta is set to 100%). Data represents three independent experiments

IV. DISCUSSION

In this study, a 2-DE-based proteomics was used to identify proteins that are differentially expressed in atherosclerotic tissues. A variety of proteins, many of which are involved in calcium-mediated processes, migration of VSMCs, MMP activation and regulation of pro-inflammatory cytokines in atherosclerosis progression, showed differential expression on 2-D gel. A few proteins showed decreased expression levels in atherosclerotic tissues compared to normal aorta tissues. However, I focused on proteins that showed increased expression levels in atherosclerosis in order to identify proteins responsible for atherogenesis or biomarkers for atherosclerosis.

Calcium-dependant protease, one of the 14 proteins identified in all seven atherosclerotic tissues, is important in intracellular calcium-mediated processes (Sorimachi *et al*., 1997) and is related to a variety of pathological conditions, such as cataracts (Andesson *et al*., 1996), oxidative stress (Andesson *et al*., 1998) and ischemia reperfusion injury (Yoshida *et al*., 1995).

In post-ischemic myocardium, the proteolytic activity of this protein is related to degradation of the sarcomeric proteins troponin 1 and desmin (Papp *et al*., 2000; Gao *et al*., 1996), indicating that intracellular calcium-mediated processes may control the pathological progression in atherosclerosis. Recent studies also show that arterial calcification is associated with atherosclerosis (Doherty *et al*., 2004). Protein-tyrosine phophatases are responsible for migation of VSMCs and MMP activation, which are important for atherosclerotic progression (Uzui *et al*., 2000). Protein-tyrosine phophatases also play a role in type 2 diabetes and obesitiy as a negative regulator of insulin (Ramachandran *et al*., 2003). Decoy receptor 1 is known to regulate the activities of primary pro-inflammatory cytokines and chemokines (Antovani *et al*., 2001), as well as apoptosis signaling (Ashkenazi *et al*., 1999). The soluble decoy receptors are involved in the antagonizing receptor-mediated function in atherosclerosis (Jalkanen *et al*., 2003). Platelete-activating factor acetylhydrolase 1B β subunit is involved in pro-inflammatory responses and has correlation with asthma, stroke, myocardial

infarction and non-familial cardiomypathy (Tjoelker *et al*., 2000). This protein is also concerned to one of the most potent lipid mediators. Other identified proteins are involved in vascular endothelial cell growth and inflammation via coagulation mechanism.

Of the 13 proteins identified in one or two atherosclerotic tissues, myosin regulatory light chain 2 is a major regulatory subunit of smooth muscle and a modulator of troponin controlled regulation of striated muscle contraction (Danuta *et al*., 2004). This protein is one of the sarcomeric proteins associated with familial hypertrophic cardiomyopathy (Poetter *et al*., 1996; Flavigny *et al*., 1998; Anderen *et al*., 2001). Changes in myosin subunit isoform expression are also involved in intracellular calcium homeostasis and metabolism (Marcus *et al*., 1998). The 14-3-3 protein family is believed to be an important regulator of multiple signal transduction processes (Wheeler-Jones *et al*., 1996) and it has been suggested that 14-3-3 is involved in control of platelet secretion, in addition to the role recently assigned to 14-3-3 in secretion/exocytosis from the adrenal medulla (Morgan *et al*., 1992). 14-3-3γ

is especially induced in injured vessels and plays an important role in cellular proliferation by binding to VSMCs and activating the protein kinase Raf-1 (Autieri *et al*., 1996). In VSMCs treated with platelet-derived growth factor (PDGF), 14-3-3γ was expressed and phosphorylated in an activation-dependent manner (Autieri *et al*., 1999). Methionine aminopeptidase 2 (MetAP 2) is up-regulated during cell proliferation and has been studied as a target molecule in cancer therapy (Wang *et al*., 2003). MetAP2 is also known to regulate both N-terminal modification of proteins and the peptides required in processes such as maturation, activation, and degradation, and is involved in angiogenesis (Sato, 2003). Although cytomegalovirus and *Chlamydia pneumoniae* were known as important factors in atherosclerosis development at the transcription level, microorganism-originated molecules were not detected in the MS-FIT database.

In this study, differentially expressed proteins in atherosclerotic tissues have been isolated and identified using proteomics. Although the role of each protein involved in atherosclerosis has not been characterized, these data

indicate that proteins involved in intracellular calcium-mediated processes, migration of VSMCs, MMP activation and regulation of pro-inflammatory cytokines are important in atherosclerosis progression. These results indicate that cellular inflammatory processes play important roles in atherogenesis. Since the atherosclerotic tissues instead of plasma samples were analyzed, well-known circulating biomarkers for inflammation, such as CRP, IL-6, and TNF-α were not detected. Also thirty percent of the proteins that were analyzed were either unidentified or functionally uncharacterized.

Further studies will be required to establish a database of specific proteins that are involved in development of atherosclerosis, and clinical confirmation tests will be required to use candidate proteins as biomarkers or therapeutic targets. However, to my knowledge, this is the first attempt at analysis of proteins that are differentially expressed in atherosclerotic tissues. We can now begin to understand the molecular mechanisms of atherosclerosis, which are important pathological processes. Potential applications of these data include identification and characterization of signaling pathways involved in

atherogenesis, and further exploration of the role of selected identified proteins in clinical samples.

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CHAPTER II

Human LZIP induces monocyte CC chemokine MCP-1 and its receptor CCR2 expression and is involved in atherosclerosis development

ABSTRACT

Monocyte chemoattractant protein (MCP)-1 and CC chemokine receptor (CCR) 2 play major roles in atherosclerosis development including promoting migration of circulating monocytes to the arterial wall during atherogenesis. The role of the novel transcription factor LZIP in foam cell formation and in expression of MCP-1 and CCR2 during the process of atherosclerosis was investigated. RNase protection analysis showed that LZIP increased mRNA expression of MCP-1, leukotactin-1 (Lkn-1), CCR1, and CCR2 in THP-1 cells. Flow cytometric data showed that surface expressions of both CCR1 and CCR2 were increased by LZIP, which also enhanced the chemotactic activities of MCP-1 and Lkn-1. During the formation of foam cells, LZIP expression was significantly elevated in foam cells and siLZIP inhibited foam cell formation. Results from an electrophoretic mobility shift assay showed that LZIP binds to the C/EBP element in the CCR2 promoter. Western blot data showed that the LZIP level was highly elevated in serum from coronary

atherosclerosis patients compared to normal healthy people. These results suggest that LZIP regulates expression of chemokines and chemokine receptors that are involved in development of atherosclerosis and plays an important role in foam cell formation. LZIP is probably a key modulator in atherogenesis and is a putative biomarker for atherosclerosis.

Keywords : atherosclerosis, inflammation, leukocytes, receptors, signal transduction

I. INTRODUCTION

Atherosclerosis is a complex chronic inflammatory disease that is a cause of death in western countries and Asia (Ross, 1995). In the early stage of atherosclerosis, monocyte-derived macrophages that have left circulation penetrate and accumulate in the arterial wall (Ross, 1993). Foam cell formation occurs in this process caused by migrated macrophages that ingest oxidized low-density lipoproteins (oxLDL) (Ross, 1993). The accumulation of foam cells in the intima is a feature of the early stage of atherosclerosis and is a primary component of fatty streak (Ross, 1993). Various molecules, including adhesion molecules, chemokines, and chemokine receptors are involved in development of atherosclerosis (Dessein *et al*., 2005). Elevated circulating adhesion molecules are associated with cardiovascular risk factors (Ponthieux *et al*., 2004) and can be used to predict atherosclerosis and cardiovascular events (Hwang *et al*., 1997; O'Malley *et al*., 2001). Chemokines are a superfamily of chemotactic cytokines that are categorized

into four groups on the basis of their conservation of four cysteine residues and on their ability to induce leukocyte trafficking into sites of inflammation (Gerard *et al*., 2001). The action of chemokines is mediated by their receptors, a family of seven transmembrane G-protein-coupled receptors (Gerard *et al*., 2001). Evidence suggests that chemokines and their receptors are involved in inflammation development including atherosclerosis (Reape *et al*., 1999). Recruitment of monocytes into the arterial subendothelium is one of the earliest steps in development of atherosclerosis (Gu *et al*., 1998). CC chemokine monocyte chemoattractant protein (MCP)-1, which is a chemoattractant for monocytes, is an important chemokine for formation of atherosclerotic plaque (Nelken *et al*., 1991). MCP-1 is induced by various stimuli, such as cytokines and oxidized adducts, and is secreted from endothelial cells, smooth muscle cells and macrophages (Myers *et al*., 1996). CC chemokine receptor (CCR) 2 is highly expressed in peripheral blood monocytes, macrophages, and T lymphocytes and it is the only established functional receptor for MCP-1 on hematopoietic cells (Boring *et al*., 1998).

Although MCP-1 and CCR2 are believed to play an important role in leukocyte trafficking and the development of atherosclerosis, little information is available regarding the key modulator in the atherosclerosis process and the mechanism of intracellular signaling events that regulates the formation of foam cells.

The human leucine zipper protein LZIP is a ubiquitous transcription factor that belongs to the bZIP superfamily (Burbelo *et al*., 1994; Lu *et al*., 1997; Lu *et al*., 1998). LZIP is known to be involved in regulation of cell growth by binding to human host cell factor, which is involved in cell proliferation (Jin *et al*., 2000). It has also been suggested that LZIP serves a novel cellular tumor suppressor function that is targeted by the hepatitis C virus core (Jin *et al*., 2000). However, the exact biological role and the natural target genes of LZIP have not been characterized. Although there is controversy regarding the localization of endogenous LZIP, it has been reported that LZIP physically interacts with CCR1 and participates in regulation of leukotactin-1 (Lkn-1)-dependent cell migration without affecting the chemotactic activities of other

CCR1-binding chemokines (Ko *et al*., 2004). These results indicate that LZIP probably functions as a cellular modulator as well as a transcription factor. Since LZIP regulates Lkn-1-induced chemotaxis and Lkn-1 is involved in atherosclerosis, I investigated whether LZIP plays a role in the development of atherosclerosis and whether it affects regulation of CCR2 and MCP-1 expression.

II. MATERIALS AND METHODS

Materials

Human THP-1 cells were purchased from American Type Culture Collection (ATCC). RPMI 1640, fetal bovine serum (FBS) and Trizol reagent were obtained from Invitrogen corp. (Gaithersburg, MD, USA). Human oxLDL was purchased from INTRACEL (Frederick, MD, USA). RNase protection assay kit was obtained from BD bioscience (San Jose, CA, USA). Recombinant human MCP-1, Lkn-1, macrophage inflammatory protein (MIP)-1α, and anti-CCR1 and anti-CCR2 antibodies were products of R&D systems (Minneapolis, MN, USA). FITC-conjugated goat anti-mouse IgG and rabbit polyclonal IgG-HA probe were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [α-^{32}P]UTP, [γ-^{32}P]ATP, albumin and IgG removal kit were from Amersham Pharmacia Biotech. (Piscataway, NJ, USA). FITC-conjugated rabbit anti-rat IgG, monoclonal anti-CRP antibody and other ultra pure chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-LZIP antibody was raised

against an N-terminal peptide (amino acids 2-29) conjugated to keyhole limpet hemocyanin.

Cell culture and transient transfection

THP-1 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). THP-1 cells were seeded into 6-well plates at a density of 2×10^6 cells/well. Cells were transfected with 4 μg of DNA using lipofectamine reagents (Invitrogen) according to the manufacture's instructions.

FACS analysis

Transfected cells were harvested and washed with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Blocked cells with normal rabbit IgG were separated into new tubes. Each tube was added

with PBS containing anti-HA, anti-CCR2, or anti-CCR1 antibodies. Baseline fluorescence was obtained from cells transfected with the mock vector alone. Following incubation and washings, cells were incubated for 30 min at 4℃ with FITC-conjugated goat anti-mouse IgG or rabbit anti-rat IgG. Then cells were washed and analyzed on a FACSort cytofluorimeter (Becton Dickinson, Franklin Lakes, NJ, USA).

Chemotaxis assay

Migration of cells was monitored using a 48-well microchamber (Neuroprobe, Cabin John, MD, USA) as previously described (Ko *et al*., 2002). Briefly, the lower wells were filled with 28 μl buffer alone or with buffer containing MCP-1 or Lkn-1 and the upper wells were filled with 50 μl of THP-1 cells at 1×10^6 cells/ml in RPMI 1640 containing 1% BSA and 30 mM HEPES. The two compartments were separated by a polyvinylpyrrolidone-free filter (Neuroprobe) with 5 μm pores that was precoated with RPMI 1640 containing rat tail collagen type I at 4℃ overnight. After incubation for 6 h at 37℃, the filters were removed from the chamber,

washed, fixed, and stained with Diff-Quick (Baxter, Deerfield, IL, USA). The cells of two randomly selected oil-immersed fields were counted using Axiovert 25 (Carl Zeiss, Jena, Germany) and Visus Image Analysis System (Foresthill Products, Foresthill, CA, USA). The chemotactic index (CI) was calculated from the number of cells that migrated to the control.

RNase protection assay

THP-1 cells were seeded into 6-well plates at 2×10^6 cells/well and cultured in RPMI 1640. After transfection, the cells were harvested and total RNA was extracted using Trizol reagent (Invitrogen) as described by manufacturer's instruction. The hCR5 template set containing DNA templates for CCR1, CCR3, CCR4, CCR5, CCR8, CCR2a+b, L32, and GAPDH was purchased from BD bioscience. [α-^{32}P]UTP-labeled RNA probes were synthesized from the hCR5 template set by T7 RNA polymerase. The hCK5 template set contains RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8, I-309, L32 and GAPDH. Probes were hybridized overnight with target RNA. Free probes and other single-stranded RNA were digested with RNase, followed by

proteinase K treatment and phenol chloroform extraction. After ethanol precipitation with 4 M ammonium acetate, the RNase-protected probes were dissolved in loading buffer. Denaturing polyacrylamide gel (4.75%) was pre-electrophoresed in 0.5 × Tris-borate EDTA (TBE) buffer for 30 min prior to loading and electrophoresis continued at 55 W for 2 h. Dried gel was visualized by autoradiography. Specific bands were identified by their individual migration patterns in comparison with the undigested probes. The bands were normalized with GAPDH band.

Foam cell formation and lipid staining

THP-1 cells were treated with 100 nM of phorbol 12-myristate 13-acetate (PMA) for 48 h and then oxLDL (25 μg/ml) for 4 days. To detect lipids accumulated in THP-1 derived macrophages, oil red-O staining was performed as previously described (Yu *et al*., 2004).

Semi-quantitative RT-PCR

Total RNA was extracted from cells using Trizol reagent according to

manufacture's instruction. The cDNA was synthesized from 2 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). The reaction was carried out for 50 min at 42°C and for 15 min at 70°C. The resulting cDNA samples were amplified by 25 cycles (denaturing at 94℃ for 30 s, annealing at 60℃ for 40 s, and elongating at 72℃ for 40 s) using CCR2a primers (sense primer 5- ATGCTGTCCACATCTCGTTCTCG-3 and antisense primer 5-CTAGGCTCCTTCTTTGTCCTGAAGA-3) and MCP-1 primer (sense primer 5-CAGCCAGATGCAATCAATGC-3), Lkn-1 primer (sense 5-CTCCTACACCCCACGAAGCAT-3). GAPDH was amplified as an internal control. The PCR products were electrophoresed on a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide, and the sizes of the products were determined by comparison with 1 kb DNA ladder marker (Invitrogen). All the PCR reactions were repeated at least three times. The intensity of each band amplified by RT-PCR was analyzed using MultiImage^{TM} Light Cabinet (Alpha Innotech Corp., San Leandro, CA, USA), and normalized to that of GAPDH mRNA in corresponding samples.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from confluent flasks of the mock vector or HA-LZIP transfected HEK 293 cells. The oligonucleotides of CCAAT/enhancer binding proteins (C/EBP) binding site in CCR2 promoter (-324~-298) were synthesized. The oligonucleotide was 5-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega, Madison, WI, USA). Unincorporated nucleotide was removed by passage over a Bio-Gel P-6 spin column (Bio-Rad, Hercules, CA, USA). Nuclear extracts (15 μg of total protein) were incubated with radiolabeled probe for 20 min at room temperature, and protein-DNA complexes were separated from free probe by electrophoresis on a 4% native polyacrylamide gel in 0.5 × Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 50 μg/ml of poly(dI-dC)•poly(dI-dC). Gels were pre-electrophoresed in 0.5 × TBE for 30 min prior to loading and electrophoresis continued for approximately 3 h until the bromophenol blue dye approached the bottom of the gel. Dried gels were visualized by autoradiography. For competition

experiments, binding reactions were incubated with 20-fold molar excess of unlabeled C/EBP binding oligonucleotide for 20 min before addition of the radiolabeled oligonucleotide. For supershift assays, the antibodies were added to the reaction mixture for an additional 30 min. After incubation, the reaction mixture was then separated by electrophoresis through a 4% native polyacrylamide gel, and the results were recorded by autoradiography.

Western blot analysis

Serum samples were donated from healthy donors and coronary atherosclerosis patients. Each patient serum was collected from vein and aorta. Serum IgG and albumin were discarded using albumin and IgG removal kit (Amersham Pharmacia biotech., Piscataway, NJ, USA) according to manufacture's instruction. Protein samples (30 μg of each lane) were separated by a 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. The blots were incubated with anti-LZIP-antibody and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia biotech.).

III. RESULTS

LZIP increases mRNA expressions of MCP-1 and CCR2 in THP-1 cells

To investigate the effect of LZIP on mRNA expression of selected chemokines and CC chemokine receptors, an RNase protection assay in THP-1 cells transfected with the mock vector or LZIP was performed. CCR1 and CCR2 mRNA expressions were increased in cells transfected with LZIP compared to cells transfected with the mock vector (Figure 1A). CCR3 and CCR4 mRNA expressions were slightly increased by LZIP (Figure 1A). Although mRNA expressions of CCR1 and CCR2a were significantly enhanced by LZIP, CCR2b mRNA expression was slightly decreased in LZIP transfected cells (Figure 1A). Figure 1B shows that mRNA expression of selected chemokines is increased in cells transfected with LZIP. Especially, MCP-1 mRNA was highly expressed in cells transfected with LZIP (Figure 1B). mRNA expression of IP-10, MIP-1α, MIP-1β, and IL-8 were also increased in LZIP transfected cells. Yeast tRNA was used as a negative

control and GAPDH was used for normalization of samples. Figure 1C shows the transfection efficiency of THP-1 cells analyzed using a FACSorter cytofluorimeter. These results indicate that LZIP up-regulates mRNA expression of chemokines and chemokine receptors, including MCP-1 and CCR2.

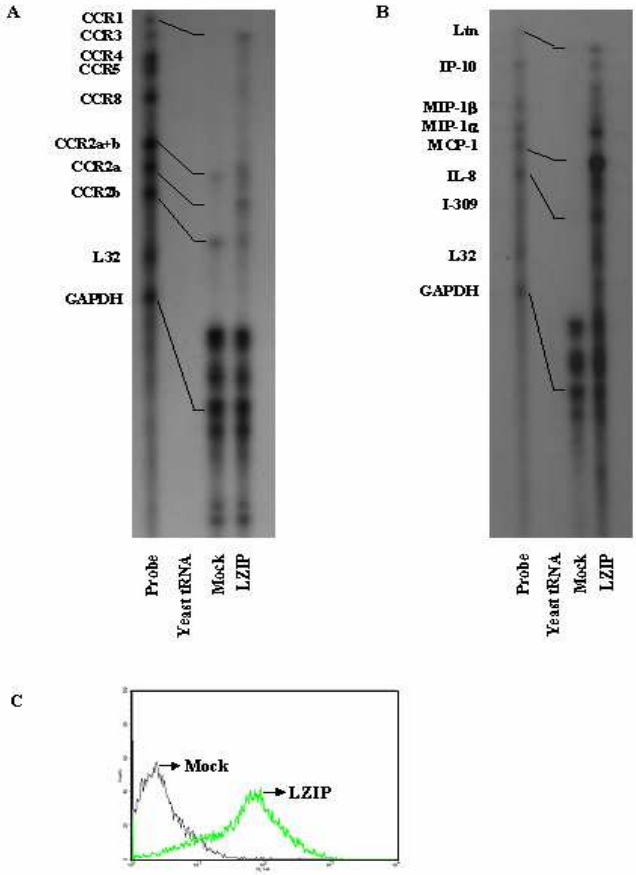


Figure II-1. LZIP increases mRNA expressions of MCP-1 and CCR2 in THP-1 cells. THP-1 cells were transfected with the mock vector or HA-LZIP and total RNA was extracted from transfected cells. The mRNA levels of CCRs (A) and chemokines (B) were analyzed by RNase protection assay using template sets as described in methods. Specific bands were detected by comparing with the undigested probes. The bands were normalized with GAPDH and L32 bands. Data are expressed as representative of three individual experiments. C, Expression of transfected LZIP was confirmed by fluorescence-activated cells sorter using polyclonal HA probe.

Surface expressions of CCR1 and CCR2 are increased by LZIP

Since LZIP increased mRNA expressions of CCR1 and CCR2, I examined whether surface protein expressions of CCR1 and CCR2 are affected by LZIP. Strong CCR1 and CCR2 surface expressions were detected by flow cytometry in LZIP transfected THP-1 cells compared to cells transfected with the mock vector (Figure 2). CCR1 and CCR2 surface expressions were increased by LZIP 2.1- and 1.9-fold, respectively. These results correlate with CCR1 and CCR2 mRNA expressions due to LZIP. These data indicate that LZIP up-regulates mRNA and protein expressions of both CCR1 and CCR2.

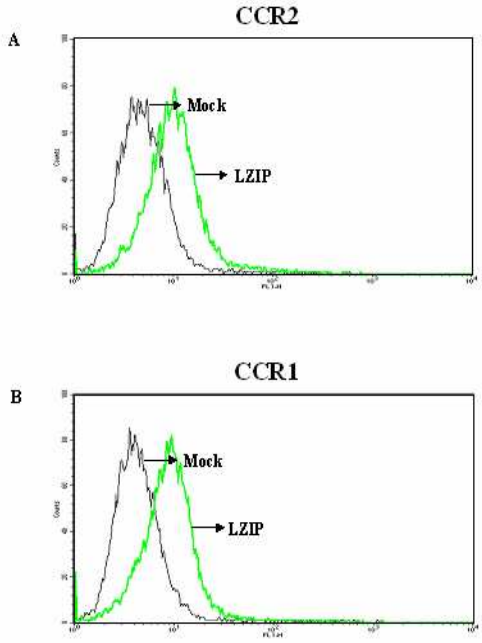


Figure II-2. Surface expressions of CCR1 and CCR2 are increased by LZIP. THP-1 cells were transfected with the mock vector or HA-LZIP. Surface expressions of CCR2 (A) and CCR1 (B) were analyzed by fluorescence-activated cells sorter using monoclonal anti-CCR1 and anti-CCR2 antibodies. Data are expressed as representative of three individual experiments.

LZIP enhances THP-1 cell migration in response to MCP-1 and Lkn-1

Cell migration is the primary cellular event initiated by the chemokine receptor interacting with chemokine. Therefore, I investigated whether LZIP affects the chemotactic activities of MCP-1 and Lkn-1 in THP-1 cells. To examine the chemotactic activity in LZIP transfected THP-1 cells, a cell migration assay was performed in a 48 well microchamber. MCP-1 showed the typical bell-shape curve in THP-1 chemoattraction with the peak of the curve at 10 ng/ml (Figure 3A). THP-1 cell migration in response to MCP-1 was increased by 2-fold based on the chemotactic index in cells transfected with LZIP (Figure 3A). The chemotactic activity of Lkn-1 was increased by 5-fold at 100 ng/ml in LZIP transfected cells compared with cells transfected with the mock vector (Figure 3B). However, the chemotactic activity of MIP-1α, used as a negative control in LZIP transfected cells, was comparable with cells transfected with the mock vector (Figure 3B). These data indicate that LZIP up-regulates CCR1 and CCR2 expressions at both the mRNA and protein levels in THP-1 cells and enhances the chemotactic activities of MCP-

1 and Lkn-1.

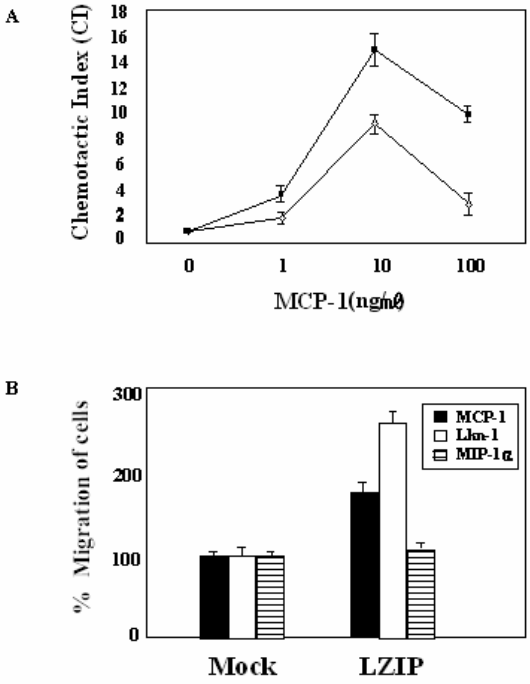
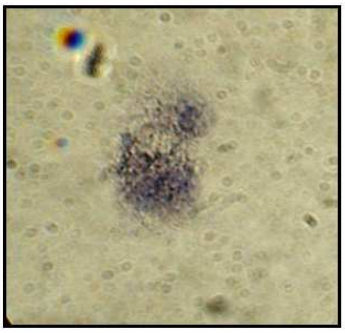


Figure II-3. LZIP enhances THP-1 cell migration in response to MCP-1 and Lkn-1. A, THP-1 cells transfected with the mock vector (◇) or HA-LZIP (■) were incubated with the indicated concentrations of MCP-1. B, THP-1 cells transfected with the mock vector or HA-LZIP were incubated with MCP-1 (10 ng/ml), Lkn-1 (100 ng/ml), and MIP-1α (1 ng/ml). After incubation for 5 h, filters were stained with Diff-Quick (Sigma). The number of cells that migrated was counted microscopically in four randomly selected fields per well. The chemotactic index (CI) was calculated from the number of cells migrating to the test chemokines divided by that migrating to the controls. Data are expressed as mean CI ± SEM of three independent experiments.

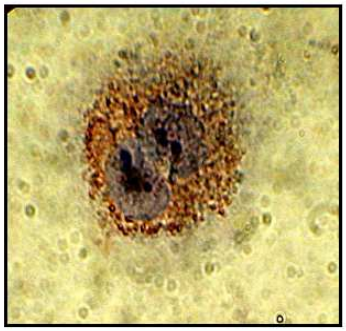
LZIP is involved in formation of foam cells

Since LZIP increased the chemotactic activities of MCP-1 and Lkn-1 that are known to be involved in atherosclerosis, I examined whether LZIP is involved in the process of foam cell formation. THP-1 cells were treated with PMA for 48 h, then with oxLDL for 4 days in order to induce the formation of foam cells. Cells were stained with oil red-O to detect foam cell formation. Cells treated with PMA and oxLDL showed accumulations of lipid aggregates inside of the cell, indicating typical formation of foam cells (Figure 4). Total RNA was isolated and semi-quantitative RT-PCR was performed to examine the mRNA expression of LZIP. LZIP mRNA expression was low in control THP-1 cells and was increased 2-fold by PMA treatment (Figure 5A and B). LZIP mRNA expression was increased by 6-fold in foam cells treated with PMA and oxLDL compared to control THP-1 cells (Figure 5A and B). Since LZIP mRNA expression was increased in foam cells, I determined whether LZIP affects the formation of foam cells using siRNA-LZIP (siLZIP) in THP-1 cells. siLZIP that can reduce the expression of LZIP to 0-50% of the

endogenous level was generated (Ko *et al*., 2004). After transfection, THP-1 cells were treated with PMA and oxLDL. Cells transfected with the mock vector formed foam cells while cells transfected with siLZIP failed to form foam cells (Figure 6). LZIP did not increase the formation of foam cells by the treatment of PMA and oxLDL (Figure 6). These data indicate that LZIP is involved in foam cell formation and may play an important role in the subsequent development of atherosclerosis.



Control



PMA+oxLDL

Figure II-4. *In vitro* foam cell formation. THP-1 cells were treated with 100 nM of PMA for 48 h, and the differentiated macrophages were incubated with 25 μg/ml of oxLDL for 4 days. Oil red-O staining was performed to determine foam cell formation and hematoxylin was used for counter-stain. Red color area indicates formation of foam cell.

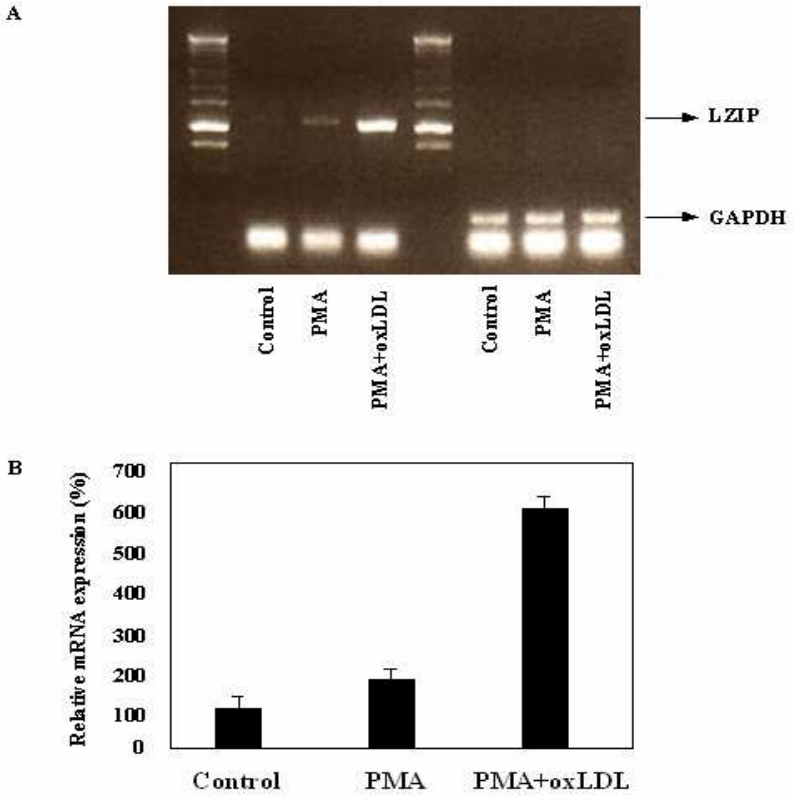
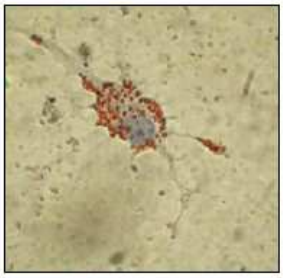
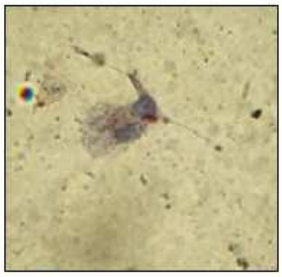


Figure II-5. LZIP mRNA expression is elevated in foam cells. A, Total RNA was extracted from THP-1 cells treated with or without PMA and oxLDL, and RT-PCR analysis was performed. B, Relative mRNA expression of LZIP was analyzed by densitometer and normalized with GAPDH. All experiments were performed three times. The bars represent the mean ± S.D.

Mock



LZIP



siLZIP

Figure II-6. LZIP is involved in formation of foam cells. THP-1 cells were transfected with the mock vector, LZIP or siLZIP. Transfected cells were treated with 100 nM of PMA for 48 h, and the differentiated macrophages were incubated with 25 μg/ml of oxLDL for 4 days. Oil red-O staining was performed to determine foam cell formation and hematoxylin was used for counter-stain. Original magnification, × 40.

LZIP increases mRNA expressions of MCP-1 and CCR2a in foam cells

Since LZIP is involved in foam cell formation, the effect of LZIP in MCP-1 and CCR2 mRNA expressions in foam cells was examined. THP-1 cells transfected with the mock vector, vectors encoding LZIP, or siLZIP were stimulated with PMA and oxLDL to induce foam cell formation, and then mRNA expression was analyzed by semi-quantitative RT-PCR. THP-1 cells lacking treatment with PMA and oxLDL were used as a negative control. Foam cells transfected with LZIP highly expressed the mRNA of MCP-1, CCR2a, and Lkn-1 compared to foam cells transfected with the mock vector (Figure 7A). However, mRNA expressions of MCP-1, CCR2a, and Lkn-1 were repressed in foam cells transfected with siLZIP compared to cells transfected with the mock vector (Figure 7A). Foam cells cotransfected with LZIP and siLZIP restored mRNA expressions of MCP-1, CCR2, and Lkn-1 to normal levels (Figure 7A). Results from densitometric analysis showed that relative mRNA expressions of MCP-1, CCR2a, and Lkn-1 were increased by approximately 2 to 3-fold due to LZIP in foam cells (Figure 7B). These data

indicate that LZIP increases mRNA expressions of MCP-1 and CCR2a in foam cells.

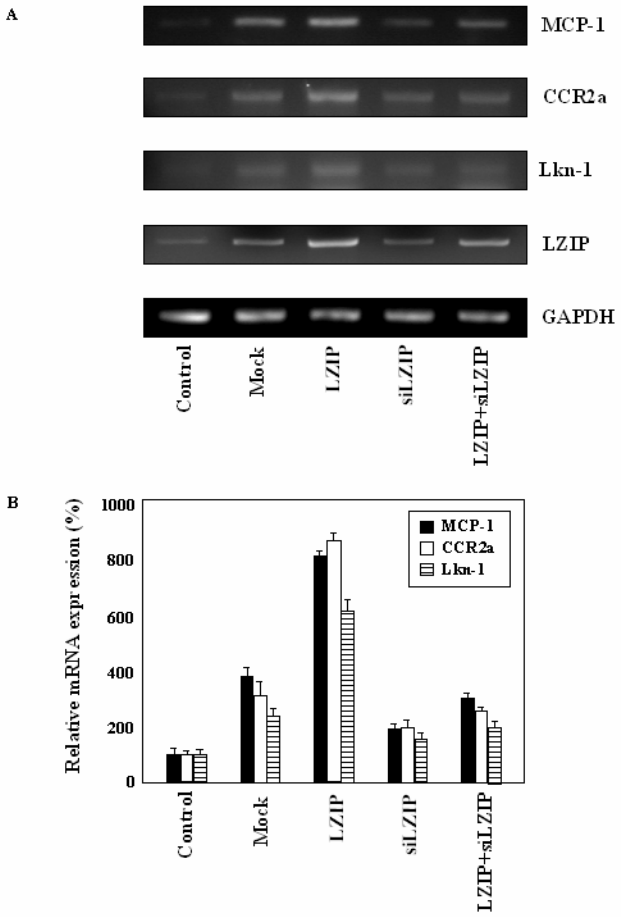


Figure II-7. LZIP increases mRNA expressions of MCP-1 and CCR2a in foam cells. A, THP-1 cells were transfected with the mock vector, LZIP or siLZIP. Total RNA was extracted from transfected cells treated with 100 nM of PMA for 48 h and 25 μg/ml of oxLDL for 4 days, and RT-PCR analysis was performed. B, Relative mRNA expression was analyzed by densitometer and normalized with GAPDH. All experiments were performed three times. The bars represent the mean ± S.D.

LZIP binds to the C/EBP element in CCR2 promoter

CCR2 promoter contains the CCAAT enhancer binding protein (C/EBP) element (Yamato *et al*., 1999), and LZIP binds to the C/EBP element and activates transcription from C/EBP-containing reporter genes (Lu *et al*., 1997). To investigate the mechanism of CCR2a mRNA expression in foam cells, an electrophoretic mobility shift assay (EMSA) was performed using a ^{32}P-labeled C/EBP element as a probe. Figure 8A shows a shifted LZIP nuclear complex bound to the probe. Cells transfected with LZIP showed an increase in the formation of this complex. To determine the specificity of LZIP binding activity, nuclear extracts were incubated with the labeled LZIP binding probes in the absence and presence of a 20-fold molar excess of unlabeled LZIP binding probes. Competition analysis showed that the LZIP binding complex competed with the unlabeled LZIP binding probes (Figure 8B), indicating that the LZIP binding activity is specific. The authenticity of the LZIP nuclear complex was confirmed using supershift EMSA by incubation of nuclear extracts with antibody against LZIP (Figure 8B). These data indicate that

LZIP binds to the C/EBP element and induces CCR2 mRNA and protein expression.

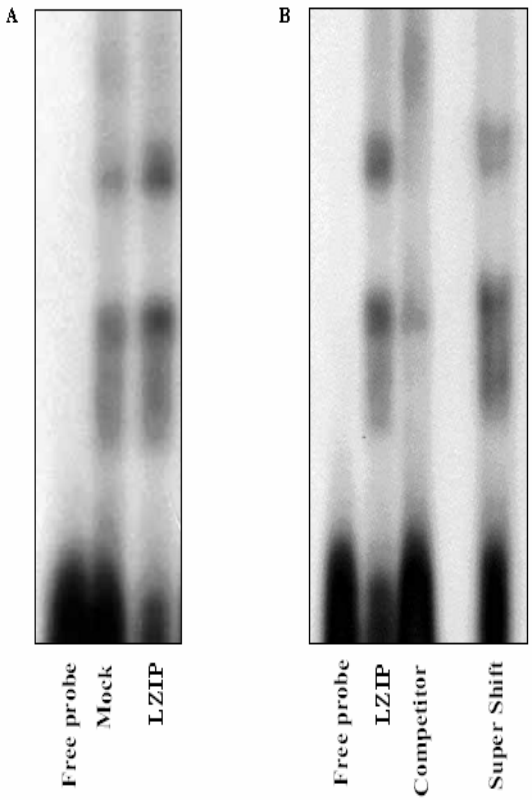


Figure II-8. LZIP binds to the C/EBP element in CCR2 promoter. Nuclear extracts (15 μg) from mock or HA-LZIP transfected HEK 293 cells were prepared and subjected to EMSA for the DNA binding activity of LZIP with ^{32}P-labeled oligonucleotides in the absence (A) and presence (B) of 20-fold molar excess of unlabeled competitors. Anti-HA antibody was added to the reaction mixture for an additional 30 min for super shift assay.

The LZIP level is elevated in serum from coronary atherosclerosis patients

I examined whether LZIP expression is affected by atherogenesis. My preliminary data indicated that LZIP is present in serum, suggesting that LZIP is secreted into the extracelluar region by a stimulus. Therefore, the expression level of LZIP in normal serum and in serum from atherosclerosis patients was examined. Normal serum was donated by 3 different healthy people and atherosclerotic serum was obtained from 3 different coronary atherosclerosis patients. LZIP was highly expressed in serum from atherosclerosis patients both in the vein and aorta compared to normal serum (Figure 9). Vein serum samples from atherosclerosis patients showed a higher LZIP level than aorta serum samples (Figure 9). C-reactive protein (CRP), a well known inflammatory disease marker for atherosclerosis (deFilippi *et al*., 2003; Elgharib *et al*., 2003), was also detected in the serum samples of atherosclerosis patients, however, LZIP expression was greater than CRP expression in atherosclerosis patients (Figure 9). These data indicate that LZIP probably plays an important role in atherosclerosis development and may be

used as a biomarker for atherosclerosis.

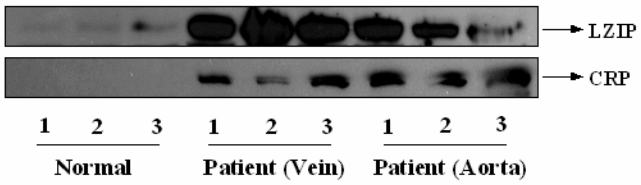


Figure II-9. The LZIP level is eleveated in serum from coronary atherosclerosis patients. Serum IgG and albumin were discarded as described in methods. Protein samples (30 μg) were separated by a 10% SDS-PAGE and transferred to nitrocellulose filters. The LZIP level was detected by Western blotting using anti-LZIP antibody. The same blot was stripped and reprobed with monoclonal anti-CRP antibody.

IV. DISCUSSION

CCR2 and MCP-1 are known to be important molecules in atherosclerosis. During the early stage of atherogenesis, CCR2 and MCP-1 play important roles in atherosclerotic plaque formation via accumulation of circulating monocytes in the arterial wall (Dawson *et al*., 1999). The role of uncharacterized transcription factor LZIP in atherosclerosis development was investigated.

Results from RPA analysis indicated that LZIP increases CCR2a mRNA expression, but slightly decreases CCR2b mRNA expression. It has been reported that CCR2a and CCR2b, the two isoforms of MCP-1 receptor (Charo *et al*., 1994), are differentially expressed in inflammatory myopathies (Bartoli *et al*., 2001). It has also been reported that CCR2a is up-regulated in inflammatory myopathies, such as vessel walls, is expressed by some mononuclear cells (Bartoli *et al*., 2001), and that CCR2b is down-regulated by oxLDL in THP-1 cells (Han *et al*., 2000). Although CCR2b is the

predominant isoform described in human monocytes, CCR2b is not increased in inflammatory myopathies (Bartoli *et al*., 2001). The function of CCR2a has not been clearly defined but CCR2a is strongly expressed in the heart endothelium (Berger *et al*., 1999). LZIP also increased CCR1 mRNA expression. The previous report suggests that LZIP functions as a cellular modulator of CCR1-dependent chemokines and is involved in Lkn-1-induced cell migration (Ko *et al*., 2004). CCR1 mRNA expression in THP-1 cells transfected with the mock vector was very low, however, cells transfected with LZIP showed an increase in CCR1 mRNA expression. Surface expressions of CCR1 and CCR2 were also increased by LZIP, indicating that LZIP plays a role in the expressions of CCR1 and CCR2 both at the mRNA and at the protein level, and probably functions as a positive regulator of CCR1 and CCR2 involved in atherosclerosis. Various chemokines, including MCP-1 and Lkn-1, which are related to inflammation and development of atherosclerosis, were significantly expressed in LZIP transfected cells. Especially, MCP-1 mRNA expression was greatly increased by LZIP.

Transmigration of monocytes through endothelial monolayers is induced by chemotaxis, which is an essential step for complete monocyte recruitment to the vessel wall. It was shown that LZIP modulates MCP-1-mediated monocyte chemotactic activity and Lkn-1-mediated chemotaxis. However, LZIP did not affect the chemotactic activity of MIP-1α, indicating that LZIP probably participates in plaque formation via an increase in CCR1, CCR2, Lkn-1, and MCP-1 expressions and subsequent induction of the chemotactic activities of MCP-1 and Lkn-1.

Human THP-1 monocytes differentiate to macrophages and are transformed to foam cells in response to PMA and oxLDL. MCP-1 participates in formation of foam cells and atherosclerotic plaque (Nelken *et al*., 1991). Lkn-1 is also involved in foam cell formation, and is significantly increased in human carotid atherosclerotic lesions and the plasma of atherosclerotic patients (Yu *et al*., 2004). According to the RT-PCR data, LZIP mRNA expression was elevated 6-fold in foam cells compared to naive THP-1 cells. Since LZIP increases the expressions of CCR1, CCR2, Lkn-1 and MCP-1

involved in foam cell formation, LZIP probably is the driving force to induce foam cells and atherosclerotic plaques by up-regulating the chemokines and chemokine receptors involved in atherogenesis. This hypothesis was confirmed using siLZIP. Although LZIP did not increase foam cell formation compared to THP-1 cells transfected with the mock vector, siLZIP dramatically inhibited the formation of foam cells induced by treatment with PMA and oxLDL. These data indicate that LZIP plays an important role in foam cell formation. It was also found that LZIP increased mRNA expressions of MCP-1, Lkn-1 and CCR2a in foam cells and that siLZIP inhibited the positive regulatory effects of LZIP in mRNA expression of these molecules. It can be proposed that LZIP is not only an important molecule in foam cell formation, but is also a key regulator of the process of atherosclerosis via regulation of chemokines and chemokine receptors.

To determine the regulatory mechanism of LZIP in CCR2 expression, the promoter region of CCR2 was analyzed. LZIP is a basic leucine zipper transcription factor that binds consensus DNA elements, such as CRE, AP-1

and C/EBP (DenBoer *et al*., 2005). Based on reports regarding the nucleotide sequence of the human CCR2 gene (Yamato *et al*., 1999), C/EBP elements exist in CCR2 promoter. Results from EMSA show that LZIP binds to the C/EBP element in CCR2 promoter, indicating that LZIP functions as a transcription factor that binds to the C/EBP element of CCR2 and accelerates the transcriptional activation of CCR2. Although further studies are required to characterize the exact regulatory mechanism of LZIP in expression of chemokines and chemokine receptors, these data are consistent with previous results.

Efforts to find biomarkers in inflammatory diseases, including atherosclerosis and rheumatoid arthritis, have been made. In atherosclerosis, CRP is a useful biomarker (deFilippi *et al*., 2003; Elgharib *et al*., 2003), and serum amyloid protein A is also a circulating inflammation marker that is related to carotid artery atherosclerosis (Larsson *et al*., 2005). Although LZIP is a transcription factor, the preliminary data show that LZIP was secreted into the extracellular region. Therefore, the possibility that LZIP can be a putative

biomarker for atherosclerosis was investigated. Western blot analysis using sera from normal healthy people and atherosclerosis patients showed that LZIP was highly elevated in the serum of atherosclerosis patients compared to normal serum. The secretory mechanism of LZIP into the serum has not been known. However, LZIP is cleaved by proteases (Chen *at al*., 2002). Therefore, the possibility that a cleaved fragment of LZIP is secreted into the serum is under investigation. The level of LZIP in serum from atherosclerosis patients was much higher than the well known inflammatory marker CRP. To validate the possible use of LZIP as a biomarker for atherosclerosis, I am screening atherosclerosis patients according to clinical history.

Herein, I report important roles for LZIP in atherosclerosis. The results indicate that LZIP regulates the expressions of the chemokines and chemokine receptors that are involved in atherosclerosis development, and that LZIP is involved in foam cell formation in the early stages of atherogenesis. The modulation effect of CCR2 expression is due to the DNA binding ability of LZIP to the C/EBP element in CCR2 promoter. LZIP is highly elevated in

serum from atherosclerosis patients, suggesting that LZIP is a putative biomarker for atherosclerosis. In conclusion, the function of LZIP in development of atherosclerosis and in regulation of the chemokines and chemokine receptors that are involved in atherosclerosis was characterized. Further characterization of the role of LZIP in atherogenesis will contribute to an understanding of the development of atherosclerosis and the regulatory mechanisms of the chemokines and chemokine receptors that are involved in atherosclerosis.

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CONCLUSIONS

In this study, differential protein expression in atherosclerosis was analyzed using proteomic, and the regulatory role of human LZIP in expression of chemokines and their receptors was determined. Also, the function of human LZIP in development of atherosclerosis was investigated.

1. Thirty-nine differently expressed proteins were detected in atherosclerotic tissues.

   Protein expression patterns of normal and atherosclerotic tissues were examined using 2-DE (pH 4-7, 24 cm long), and proteins that were differentially expressed in each sample were analyzed.

2. Twenty-seven proteins were identified using MALDI-TOF mass spectrometric analysis and the MS-FIT database.

   Of the 39 proteins that were highly expressed in atherosclerosis,

27 proteins were identified from the MS-FIT database. Twelve protein spots were unidentified. These identified proteins are known to be involved in a number of biological processes in atherosclerosis progressions.

3. Confirmation of differential protein expression was performed by Western blot analysis.

   The expression levels of DcR1, ANX-5, and 14-3-3γ were increased approximately 2-fold in atherosclerotic samples.

4. LZIP increases mRNA expressions of MCP-1 and CCR2 in THP-1 cells.

   RNase protection assay in THP-1 cells transfected with the mock vector or LZIP was performed. MCP-1 and CCR2 mRNA expressions were increased in cells transfected with LZIP compared to cells transfected with the mock vector.

5. Surface expressions of CCR1 and CCR2 are increased by LZIP.

Strong CCR1 and CCR2 surface expressions were detected by flow cytometry in LZIP transfected THP-1 cells compared to cells transfected with the mock vector. These data indicate that LZIP up-regulates protein expressions of both CCR1 and CCR2.

6. LZIP enhances THP-1 cell migration in response to MCP-1 and Lkn-1.

THP-1 cell migration in response to MCP-1 was increased 2-fold based on the chemotactic index in cells transfected with LZIP and the chemotactic activity of Lkn-1 was also increased 5-fold in LZIP transfected cells.

7. LZIP is involved in formation of foam cells.

LZIP mRNA expression was increased 6-fold in foam cells

treated with PMA and oxLDL compared to control THP-1 cells and cells transfected with siRNA-LZIP (siLZIP) failed to form foam cells.

8. LZIP increases mRNA expressions of MCP-1 and CCR2a in foam cells.

Foam cells transfected with LZIP highly expressed the mRNA of MCP-1, CCR2a compared to foam cells transfected with the mock vector and mRNA expressions of these were repressed in foam cells transfected with siLZIP.

9. LZIP binds to the C/EBP element in CCR2 promoter.

CCR2 promoter contains the CCAAT enhancer binding protein (C/EBP) element and LZIP binds to the C/EBP element and induces CCR2 mRNA and protein expression.

10. The LZIP level is highly elevated in serum from coronary atherosclerosis patients.

    LZIP was highly expressed in serum from atherosclerosis patients both in the vein and aorta compared to normal serum. These data indicate that LZIP probably plays an important role in atherosclerosis development and may be used as a biomarker for atherosclerosis.

In summary, this study is the first attempt to analyze the proteins that are differentially expressed in atherosclerotic tissues, and the function of LZIP in development of atherosclerosis and in regulation of the chemokines and chemokine receptors that are involved in atherosclerosis was characterized. These results will contribute to an understanding of the development of atherosclerosis and the regulatory mechanisms of the chemokines and chemokine receptors that are involved in atherosclerosis.

국문 요약

프로테오믹스를 이용한 동맥경화 특이 발현 단백질의 분석 및 LZIP의 동맥경화 발병과정에서의 역할에 관한 연구

동맥경화에서 특이적으로 발현하는 단백질을 발굴하기 위하여 동맥경화 환자의 병변 조직과 정상 조직을 채취한 후, 프로테오믹스를 이용하여 특이단백질을 분석하였다. 2 DE 기법을 이용하여 분석한 결과, 발현 차이가 나는 39개의 단백질을 발굴하여 MALDI-TOF을 이용하여 peptide mapping을 하였고, MS-FIT database를 이용하여 단백질을 분석하였다. 39개의 단백질 중 27개의 단백질을 동정 하였으며, 이 단백질들은 동맥경화 발병 과정에서 중요한 역할을 하는 단백질들이었다. 특히, 세포 내 칼슘 매개 진행과정, 혈관 연근육 세포의 유주 현상, MMP 물질의 활성 및 염증관여 사이토카인 전구체들의 조절 등에 관여하는 단백질들이 발견되었다.

케모카인 MCP-1과 그 수용체 CCR2는 동맥경화의 발병 단계에서 단핵구를 혈관벽으로 유주시키는 중요한 역할을 한다. LZIP은 백혈구의 유주 활성을 조절하는 새로운 전사인자이다. LZIP의 동맥경화 발병과정에서의 역할을 조사하기 위하여, LZIP에 의하여 발현되는 케모카인과 수용체들을 RNase protection 법을 이용하여 검색하였다. 단핵구에서 LZIP은 동맥경화에서 중요한 MCP-1, Lkn-1 등의 케모카인과 CCR1, CCR2 등의 수용체 발현을 mRNA와 단백질 수준에서 증가시켰다. LZIP은 MCP-1에 의한 화학주성을 증가시켰고, 동맥경화의 진행과정 중 형성되는 거품세포에서 과 발현되어 있었다. siRNA LZIP은 거품세포의 형성을 억제하여 LZIP이 거품세포 형성에 관여함을 관찰하였다. 또한, LZIP은 CCR2 촉진염기서열의 C/EBP 요소에 결합하여 CCR2 발현을 조절함을 규명하였다. 동맥경화 환자의 혈청에서 LZIP이 과 발현되어있음을 관찰하여 LZIP이 동맥경화의 표지자로 역할을 할 수 있음을 제시하였다.

본 연구에서 LZIP은 거품세포의 형성뿐만 아니라 동맥경화의 발병 과정에서 중요한 역할을 하는 단백질임을 알 수 있었고, 동맥경

화의 새로운 생체 표지자로 사용 될 수 있음을 알 수 있었다.

핵심어 : 동맥경화, 2 DE, LZIP, MCP-1, CCR2, 거품세포, CCR2 촉진 염기 서열, 환자 혈청

감사의 글

힘들었던 시간들을 뒤로하고 박사과정을 마치게 되었습니다. 이제서야 인생의 첫걸음을 내 딛을 수 있을 것 같습니다.

학위 하는 동안 지도해 주신 양 용석 교수님, 김 종배 교수님, 박 용석 교수님, 이 혜영 교수님, 오 옥두 교수님, 김 태우 교수님께 감사의 말씀을 드립니다. 연구소에서 연구자로서의 길을 가르쳐 주신 나 도선 교수님 그리고 아낌 없이 모든 것을 지원해 주시고 지도해 주신 고 제상 교수님께 감사의 말씀을 드립니다. 실험실에서 동고동락하며 많은 도움을 주신 장 성욱 박사님, 미국에 가서도 걱정해 주시고 도움을 주신 김 윤석 박사님, 끈임 없는 관심을 주신 김 인식 박사님에게도 감사의 말씀을 드립니다. 학교에 갈 때 마다 많은 도움을 주신 임 병혁 선생님과 지금 열심히 실험하고 있을 규상, 연임, 인수, 은주, 수정 그리고 여러 후배들에게도 감사의 말씀을 드립니다. 힘들었을 때 마다 같이 소주한잔을 기울여준 인귀 형, 친구 준표, 현석이 에게도 감사의 말씀을 드립니다.

마지막으로 오늘이 있기까지 전폭적인 지원과 사랑을 주신 아버님, 어머님, 동생 옥진이 에게 감사의 말씀을 드리며, 장모님과 처제 정은이 에게도 감사의 말씀을 드립니다. 그리고 옆에서 기쁜 일과 슬픈 일 모두 함께한 사랑하는 아내에게 고맙다는 말을 전하며 감사의 글을 마치겠습니다.