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Proteomic analysis of differential protein expression in atherosclerosis

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Abstract
Although recent studies have shown that several pro-inflammatory proteins can be used as biomarkers for atherosclerosis, the mechanism of atherogenesis is unclear and little information is available regarding proteins 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 analysed using two-dimensional electrophoresis-based proteomics. Thirty-nine proteins were detected that were differentially expressed in the atherosclerotic aorta compared with the normal aorta. 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 the results include the identification and characterization of signalling pathways involved in atherogenesis, and further exploration of the role of selected identified proteins in atherosclerosis.

Keywords: *Atherosclerosis, cardiovascular disease, inflammation, two-dimensional electrophoresis*

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Introduction

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

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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 and Fuster 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 tumour 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 (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 (Fassbender et al. 1999, Reape and Groot 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. In addition, 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 analysed using two-dimensional electrophoresis (2-DE)-based proteomics.

Materials and methods

Materials

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.

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

Frozen tissues were homogenized at 3000 rpm for 30 s using Tissue-Tearor™ (BioSpec Products, Inc., Bartlesville, OK, USA) 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 were removed by centrifugation at 15 000*g* for 30 min at 4°C. Before isoelectric focusing (IEF), 700 µg 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 kVh. 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 a vertical 10% SDS-polyacrylamide gels with a run of 2.5 W per gel for 30 min followed by 17 W per gel for 4–5 h at 10°C. For analytical purpose, the gels were silver stained as described (Gevaert and Vandekerchhove 2000).

Image acquisition and data analysis

Spot signals were measured by densitometric scanning using VersaDoc™ Imaging Systems (Bio-Rad). 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), 10 µl 100 ng μl^{-1} trypsin solution were 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) were 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^{-1} 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 CHCA matrix was added. The sample-matrix mixture was dried at room temperature and analysed in reflector mode. The time of flight was measured using following parameter: 20 kV accelerating voltage, 66% grid voltage, 0% guide wire voltage, 200 ns 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 fragments 1–17 (*m/z* 2094.3471), and 18–39 (*m/z* 2466.6449). Spectra were the sum of 200 laser shots, and those peaks with a signal-to-noise ratio >4:1 were selected for database searching.

Protein database search

The mono-isotopic masses for each peptide were entered into the program MS-FIT (available at www.prospector.ucsf.edu) for searches against the Swiss-Prot and NCBI databases. For MS-FIT searches, masses derived from trypsin, CHCA and keratin were excluded. Typically, the initial searching parameter were (1) search species: *Homo sapiens*; (2) pI range: 4–7; (3) mass tolerance: ±100 ppm; (4) a minimum of four peptide 'hits' required for a match; (5) cysteine as carboxylamidomethyl cysteine; and (6) methionine in an oxidized form.

Western blot analysis

Normal and atherosclerotic tissue samples were homogenized in phosphate-buffered saline at 3000 rpm for 30 s using Tissue-Tearor™ (BioSpec Products) 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 electrophoretically. 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 horseradish 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 Biotechnology, Piscataway, NJ, USA). The same blot was stripped and reprobed with anti-β-actin antibody for use as an internal control.

Results

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. We first

examined protein expression patterns of normal and atherosclerotic tissues using 2-DE (pH 4–7, 24 cm long), and proteins that were differentially expressed in each sample were analysed. To avoid experimental variation, 2-DE separation for each sample was repeated more than four times. Figure 1 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 visualized by silver staining. 2-D gel image scanning and densitometric scanning of spot signals were performed using the PDQuest™ software package. The expression patterns of more than 900 protein spots were analysed from normal and atherosclerotic tissues. Results from 2-DE image analyses showed that the protein profile was significantly different between normal and atherosclerotic tissues. However, protein profiles from the seven normal tissues were similar, indicating that individual protein expression variation was minimal (data not shown). 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 (data not shown).

Since protein profiles from the seven atherosclerotic tissues were not identical, we focused on the identification of proteins that showed increased expression levels in all

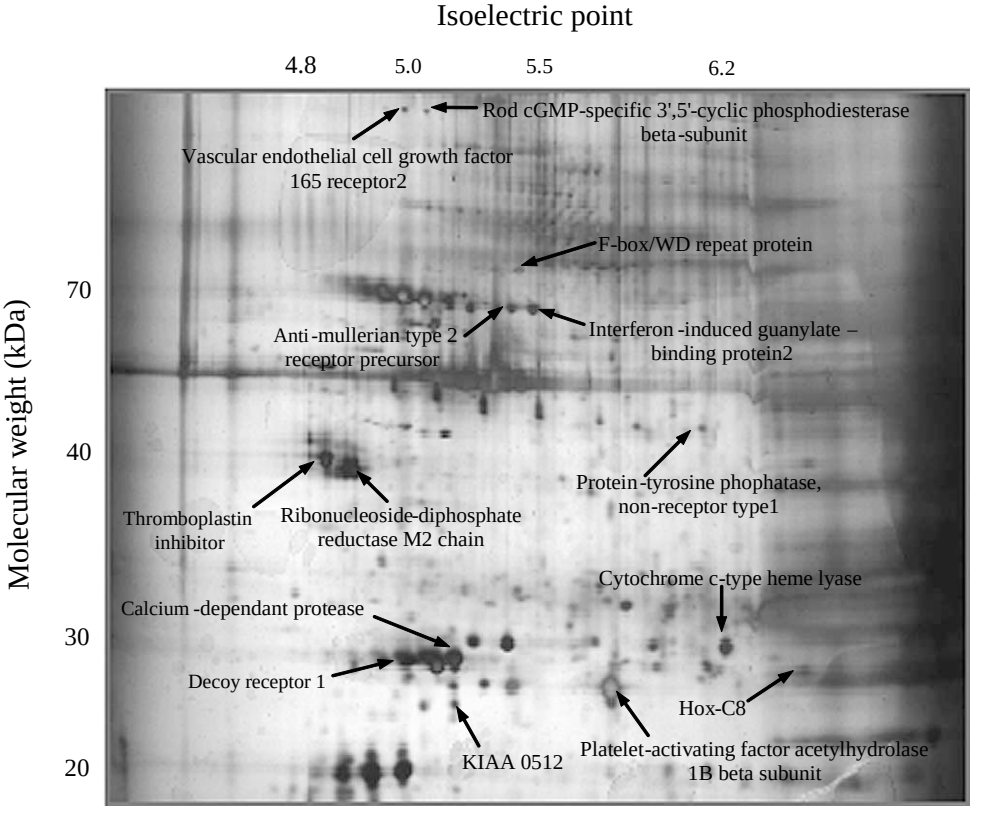

Figure 1. Typical 2-DE picture of atherosclerosis tissue. The gel was separated on a 25.5 × 20.5 plate and stained with silver stain, as described in the text. The horizontal axis is the IEF dimension, which stretches from pH 4 to 7, and the vertical represents a 10% SDS-PAGE gel. Total sample proteins (700 μg) were loaded. Proteins that were highly expressed in all seven atherosclerotic tissues were indicated.

seven atherosclerotic samples, but not or less expressed in normal aorta. Figure 2 shows the results of PDQuest™ image analysis. Images of the identified protein spots are presented with a histogram of the expression patterns. All protein spots from 2-D gels of each tissue sample were excised and trypsinized before MALDI-TOF mass spectrometric 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. Tables 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 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 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 were of focus, the protein expression levels of selective 3 proteins were examined. Results from Western blotting showed that the

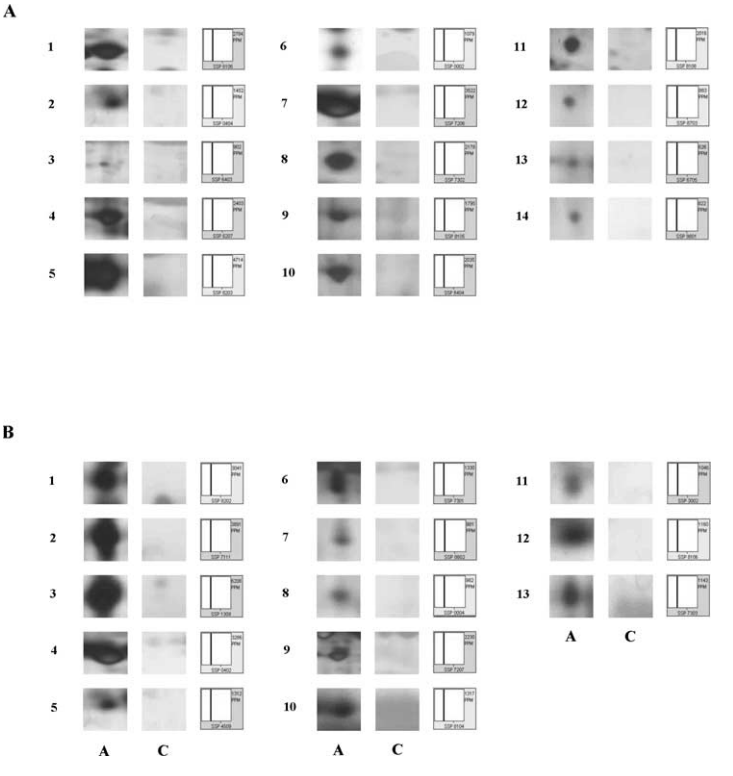

Figure 2. Expression kinetics, determined by PDQuest™ software: (A) Proteins highly expressed in all seven atherosclerotic tissues; (B) proteins highly expressed in one or two atherosclerotic tissues. A, atherosclerotic tissue; C, control aorta tissue.

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

Spot ID	Protein name	MW	PI	Swiss-Prot Access Number	MOWSE score
1	Calcium-dependant protease	28 316	5.0	P04632	8.77E+02
2	Homeobox protein Hox-C8	27 755	6.6	P31273	8.24E+00
3	Protein-tyrosine phosphatase, non-receptor type 1	49 967	5.9	P18031	8.44E+01
4	Annexin A5 (thromboplastin inhibitor)	35 937	4.9	P08758	9.75E+01
5	Ribonucleoside-diphodphate reductase M2 chain	44 878	5.3	P31350	5.42E+01
6	Human hypothetical protein KIAA 0512	32 234	5.3	Q14165	3.67E+01
7	Tumour necrosis factor receptor superfamily member 10C precursor (decoy receptor 1)	27 395	4.8	O14798	9.48E+00
8	Platelet-activating factor acetylhydrolase 1B beta subunit	25 569	5.6	Q29459	7.93E+00
9	Anti-mullerian type 2 receptor precursor	62 750	5.5	Q16671	8.52E+01
10	Interferon-induced guanylate-binding protein 2 (guanine nucleotide-binding protein 2)	67 184	5.5	P32456	6.80E+00
11	Cytochrome c-type heme lyase	30 602	6.2	P53701	9.21E+00
12	Neuropilin-2 precursor (vascular endothelial cell growth factor 165 receptor 2)	104 832	5.0	O60462	8.9E+01
13	Rod Cgmp-specific 3′,5′-cyclic phosphodiesterase beta-subunit	98 408	5.1	P35913	5.84E+00
14	F-box/WD repeat protein	67 297	5.3	Q8N3Y1	7.02E+00

protein expression levels of DcR1, ANX-5 and 14-3-3γ were increased in atherosclerotic samples compared with normal samples (Figure 3A). These results were also confirmed using densitometry. The expression levels of DcR1, ANX-5 and 14-3-3γ were increased approximately twofold in atherosclerotic samples (Figure 3B).

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

Spot ID	Protein name	MW	PI	Swiss-Prot Access Number	MOWSE score
1	Myosin regulatory light chain2 (cardiac muscle isoform)	19 827	4.8	P24844	7.28E+01
2	Myosin regulatory light chain2 (cardiac muscle isoform)	19 827	4.8	P24844	6.48E+01
3	Myosin regulatory light chain2 (cardiac muscle isoform)	19 827	4.8	P24844	8.56E+00
4	14-3-3 protein gamma	28 303	4.8	P35214	9.07E+01
5	Calcineurin B subunit isoform 1 (protein phophatase 2B regulatory subunit 1)	19 300	4.6	P06705	4.27E+00
6	Methionine aminopeptidase 2	52 892	5.6	P50579	6.85E+00
7	SH3 domain-binding protein 5	47 030	5.1	O60239	4.93E+02
8	Guanine nucleotide-binding protein G (Y), alpha subunit	42 124	5.5	P29992	7.14E+00
9	ATP synthase beta chain, mitochondrial precursor	56 560	5.3	P06576	6.07E+00
10	Interferon regulatory factor 6	53 130	5.2	O14896	8.90E+00
11	Calcium-binding protein CaBP 5	19 826	4.5	Q9NP86	3.60E+02
12	Tropomyosin alpha 3 chain	32 819	4.7	P06753	7.27E+00
13	Serine/threonine protein phophatase 2A, 48kDa regulatory subunit B	47 661	4.6	Q9Y5P8	7.37E+01

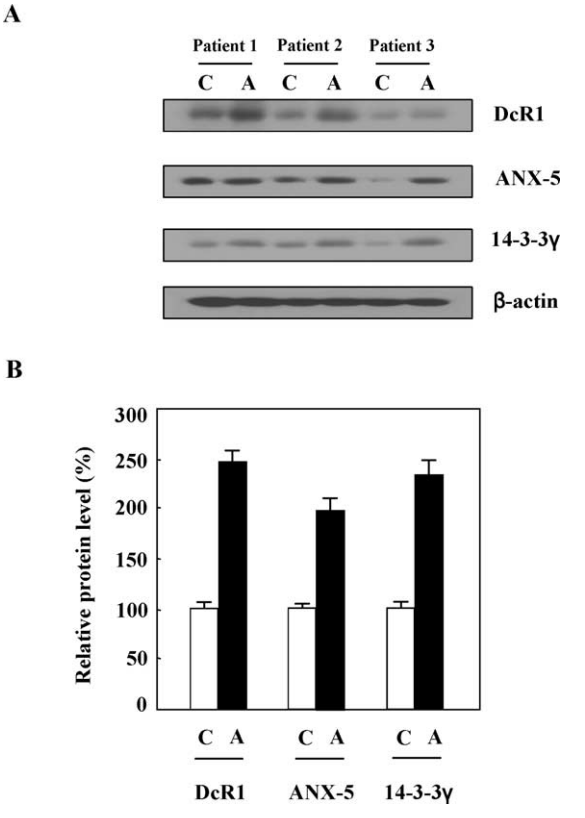

Figure 3. (A) Normal and atherosclerotic tissues from three patients were homogenized. A total of 40 μg total sample proteins were loaded on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The protein expression levels of DcR1, ANX-5 and 14-3-3γ were determined by Western blotting. 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 the mean ± standard deviation (the protein level of control aorta is set to 100%). Data represent three independent experiments.

Discussion

2-DE-based proteomics were 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 with normal aorta tissues (data not shown). However, we focused on proteins that showed increased expression levels in atherosclerosis in order to identify proteins responsible for atherogenesis or biomarkers for atherosclerosis. A typical 2-DE picture of atherosclerosis tissues shows the streaking on the gel. The reason for the streaking is probably lipid contamination in atherosclerotic tissues rather than protein modification.

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 (Andersson et al. 1996), oxidative stress (Andersson et al. 1998) and ischaemia reperfusion injury (Yoshida et al. 1995). In post-ischaemic myocardium, the proteolytic activity of this protein is related to degradation of the sarcomeric proteins troponin 1 and desmin (Gao et al. 1996, Papp et al. 2000), 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 migration of VSMCs and MMP activation, which are important for atherosclerotic progression (Uzui et al. 2000). Protein-tyrosine phophatase also plays a role in type 2 diabetes and obesity as a negative regulator of insulin (Ramachandran and Kennedy 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 signalling (Ashkenazi & Dixit 1999). The soluble decoy receptors are involved in the antagonizing receptor-mediated function in atherosclerosis (Jalkanen et al. 2003). Platelet-activating factor acetylhydrolase 1Bβ subunit is involved in pro-inflammatory responses and has correlation with asthma, stroke, myocardial infarction and non-familial cardiomypathy (Tjoelker and Stafforini 2000). This protein is also concerned with 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, Andersen 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 and Burgoyne 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 and Carbone 1999). Methionine aminopeptidase 2 (MetAP2) 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 we found putative biomarkers for atherosclerosis using 2-DE based proteomics, other proteomic techniques such as LC-MS/MS, Q-TOF will be helpful to determine the differential protein expression profiles in atherosclerosis. We are now analysing whether these proteins are detected in serum from atherosclerosis patients. We performed Western blot analysis to confirm the findings. However, a quantitative analysis method such as ELISA can be used to determine the differential protein expression. The possibility that these proteins can be used as diagnostic biomarkers for atherosclerosis is under investigation.

The present study isolated and identified differentially expressed proteins in atherosclerotic tissues using proteomics. Although the role of each protein involved in atherosclerosis has not been characterized, the 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 were analysed instead of plasma samples, well-known circulating biomarkers for inflammation, such as CRP, IL-6 and TNF-α, were not detected. In addition, 30% of the proteins analysed 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 our knowledge this is the first attempt at an analysis of proteins that are differentially expressed in atherosclerotic tissues. One can now begin to understand the molecular mechanisms of atherosclerosis, which are important pathological processes. Potential applications of these data include the identification and characterization of signalling pathways involved in atherogenesis, and further exploration of the role of selected identified proteins in clinical samples.

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