

KUOPION YLIOPISTON JULKAISUJA G. - A. I. VIRTANEN –INSTITUUTTI 18 .
KUOPIO UNIVERSITY PUBLICATIONS G.
A. I. VIRTANEN INSTITUTE FOR MOLECULAR SCIENCES 18 .



A. I. VIRTANEN
I N S T I T U T E

TIINA TUOMISTO

**Gene Expression in Atherosclerosis
and Skeletal Muscle Ischemia
A DNA Array Study**

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio
for public examination in Mediteknia Auditorium, University of Kuopio,
on Saturday 12th June 2004, at 12 noon

Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Kuopio

Distributor: Kuopio University Library
P.O. Box 1627
FIN-70211 Kuopio
FINLAND
Tel. +358 17 163 430
Fax +358 17 163 410
<http://www.uku.fi/kirjasto/julkaisutoiminta/julkmyyn.html>

Series editors: Professor Karl Åkerman, M.D., Ph.D.
Department of Neurobiology
A.I. Virtanen Institute for Molecular Sciences

Research Director Jarmo Wahlfors, Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences

Author's address: Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio
P.O. Box 1627
FIN-70211 Kuopio
FINLAND
E-mail: Tiina.Tuomisto@uku.fi

Supervisors: Professor Seppo Ylä-Herttuala, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Kuopio

Mikko Turunen, Ph.D.
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Kuopio

Reviewers: Professor Riitta Lahesmaa, M.D., Ph.D.
Turku Centre for Biotechnology
University of Turku and Åbo Akademi University

Markku Pentikäinen, M.D., Ph.D.
Wihuri Institute
University of Helsinki

Opponent: Professor Markku Savolainen, M.D., Ph.D.
Department of Internal Medicine
University of Oulu

ISBN 951-781-977-3
ISBN 951-27-0082-4 (PDF)
ISSN 1458-7335

Kopijyvä
Kuopio 2004
Finland

Tuomisto, Tiina. Gene expression in atherosclerosis and skeletal muscle ischemia- a DNA array study. Kuopio University Publications G. – A.I. Virtanen Institute for Molecular Sciences 18. 2004. 76 p.

ISBN 951-781-977-3

ISBN 951-27-0082-4 (PDF)

ISSN 1458-7335

ABSTRACT

Atherosclerosis and its clinical manifestations- coronary artery disease, cerebral artery disease and peripheral artery diseases- are the most common cause of death in Western countries. To develop new treatment strategies, such as gene therapy, more information of the pathobiological mechanisms underlying atherosclerosis and ischemic diseases is needed. The aim of this study was to utilize recently developed large-scale RNA expression and protein expression methods, DNA arrays and antibody arrays, in the analysis of gene and protein expression patterns in atherosclerosis and skeletal muscle ischemia. Firstly, we investigated gene expression changes in whole mount human atherosclerotic lesions as compared to normal artery, and found upregulation of genes in lesions that were connected to smooth muscle cell proliferation, formation of connective tissue and lipid metabolism, which are all important hallmarks of atherogenesis. In addition, upregulation of 75 genes, including unknown EST-sequences were found, which were not previously connected to atherosclerosis. Next, we utilized laser microdissection method to dissect macrophage-rich shoulder areas from human atherosclerotic lesions, analyzed gene expression patterns of these areas, and confirmed the results also in a cell culture model of monocyte-derived macrophages. Several genes that function in cell attachment and inflammation were found to be highly expressed in shoulder-area macrophages. In addition, a key enzyme in cholesterol biosynthesis, HMG-CoA reductase was highly expressed in shoulder-area macrophages, which, altogether with the high expression of cell surface integrins, may explain some beneficial effects of statins on the treatment of atherosclerosis. Gene expression patterns during monocyte-macrophage cell differentiation was also analyzed, and gene cluster analysis revealed clear patterns of activation of different gene classes during differentiation. At early stages, expression of early-response genes, such as transcription factors increased, and the genes that have roles in proliferation, migration, inflammation and lipid metabolism changed later. Gene and protein expression patterns in lipid-loaded macrophages was studied using DNA array and antibody array methods. It was found that among well-known macrophage genes, also novel transcription factors and genes functioning in cell signalling were upregulated both at mRNA and at protein level. Comparison of DNA array and protein array studies suggests that the prediction of protein level changes from changes at mRNA level might be difficult for many genes, which emphasizes the fact that the result from gene expression studies should also be confirmed at protein level. Finally, we analyzed gene expression in human lower limb ischemia to find factors involved in ischemia-induced angiogenesis. We found different expression patterns of growth factors and factors involved in cell death in acute and chronic ischemia. In conclusion, DNA and antibody array methods provide useful and powerful strategies for the analysis of molecular events during the development of atherosclerosis and ischemic diseases. Data gathered from human atherosclerotic lesions, macrophage-rich shoulder areas, and *in vitro* macrophages suggests that growth factor activities, inflammatory mediators, cell adhesion and lipid metabolism play important roles in the pathogenesis of atherosclerosis. Expression analysis of human skeletal muscle ischemia revealed the activation of different signalling pathways in acute and chronic ischemia, and offered rationale for therapeutic strategies.

National Library of Medicine Classification: QU 58.5, QZ 52, WG 300, WG 550, WE 500

Medical subject headings: arteriosclerosis; peripheral vascular diseases; genomics; proteomics; muscle, skeletal; ischemia; macrophages; oligonucleotide array sequence analysis; gene expression; up-regulation; expressed sequence tags; growth substances; inflammation mediators; cell adhesion; lipids / metabolism; signal transduction

ACKNOWLEDGEMENTS

This study was carried out at the Department of Molecular Medicine, A.I.Virtanen Institute, University of Kuopio, during the years 1999-2004.

I wish to express my deepest gratitude to my supervisor, Professor Seppo Ylä-Herttua. His endless knowledge in vascular biology and molecular medicine and enthusiasm for science, and, even more importantly, his constant encouragement, never ending optimism, and warm-hearted support made these studies possible.

I am grateful to the second supervisor of my thesis, Dr. Mikko Turunen, for his scientific knowledge and support.

I wish to thank the official reviewers Professor Riitta Lahesmaa and Dr. Markku Pentikäinen for their constructive criticism and guidance in improving my thesis.

I owe sincere thanks to my colleagues and collaborators in all phases of this work. I thank Mikko Hiltunen for introducing me to the field of molecular medicine. I sincerely thank Tuomas Rissanen for patient guidance and rewarding collaboration during these years. I am grateful to Mervi Riekkinen, Helena Viita, and Anna Korkeela for fruitful collaboration and invaluable assistance in performing this studies. I thank Juha Rutanen for his expertise in vascular pathology, Konrad Kölbl for the help with laser microdissection, and Jan Bräsen, Kari Karkola, and Ismo Vajanto for providing the human samples. I warmly thank Jani Rätty for his patient assistance with computers.

I wish to thank the whole SYH group for these memorable years. I want to express my warmest thanks to my colleague, roommate, and fellow student Päivi Turunen for her continuous support and friendship during these years.

Without the excellent technical assistance from Mervi Nieminen, Anne Martikainen, Aila Erkinheimo, Seija Sahrio, Sari Järveläinen, Tommi Heikura, and Janne Kokkonen, or secretarial help from Marja Poikolainen and Helena Pernu, this work would have never been possible. I want to thank Docent Garry Wong for revising the language of my thesis.

I would warmly thank all my dear friends, especially Taru, for their friendship, endless encouragement, and warm support during the ups and downs of my life.

I dedicate my warmest thanks to my little sisters Anna and Leena, mother Kaarina, father Juhani, and grandmother Anni for all the support and love they have given me. I wish warmly thank Tuomas for this wonderful time we have shared.

Kuopio, May 2004



Tiina Tuomisto

This study has been financially supported by grants from Finnish Academy, Sigrid Juselius Foundation, Finnish Foundation for Cardiovascular Research, Finnish Medical Foundation, Finnish Cultural Foundation of Northern Savo, Aarne Koskelo Foundation, Aarne and Aili Turunen Foundation, Helena Vuorenmies Foundation, Kuopio Naturalists' Society, and Aune and Johan Edvard Bergström Foundation.

ABBREVIATIONS

Ab	antibody	NOS	nitric oxide synthase
ABC	ATP-binding cassette transporter	oxLDL	oxidized low density lipoprotein
Apo	apolipoprotein	PAD	peripheral arterial disease
CAD	coronary artery disease	PCR	polymerase chain reaction
cDNA	complementary deoxyribonucleic acid	PDGF	platelet-derived growth factor
CLI	critical limb ischemia	PECAM	platelet-endothelial cell adhesion molecule
COX	cyclooxygenase	PMA	phorbol 13-myristate 12-acetate
CSF	colony stimulating factor	PPAR	peroxisome proliferator-activated receptor
Cy	cytidine	RNA	ribonucleic acid
DD	differential display	ROS	reactive oxygen species
DIT	diffuse intimal thickening	RT-PCR	reverse transcriptase polymerase chain reaction
DNA	deoxyribonucleic acid	SAGE	serial analysis of gene expression
EC	endothelial cell	SMC	smooth muscle cell
ECM	extracellular matrix	SOD	superoxide dismutase
EST	expressed sequence tag	SOM	self-organizing map
FGF	fibroblast growth factor	TGF	transforming growth factor
HDL	high density lipoprotein	Th-cell	T-helper cell
HIF	hypoxia-inducible factor	TIMP	tissue inhibitor of matrix metalloproteinase
HMG-CoA reductase	3-hydroxy-3-methyl glutaryl-coenzymeA reductase	TNF	tumor necrosis factor
ICAM	intercellular adhesion molecule	VCAM	vascular cell adhesion molecule
IFN	interferon	VEGF	vascular endothelial growth factor
IGF	insulin-like growth factor	VLDL	very low-density lipoprotein
IL	interleukin	2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
JAK	Janus kinase		
LDL	low density lipoprotein		
LDL-R	LDL receptor		
LMD	laser microdissection		
MCP-1	monocyte chemotactic protein-1		
MHC	major histocompatibility complex		
MMP	matrix metalloproteinase		
mRNA	messenger ribonucleic acid		
NF- κ B	nuclear factor- κ B		
NO	nitric oxide		

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to by their Roman numerals:

- I Mikko O. Hiltunen*, Tiina T. Tuomisto*, Mari Niemi, Jan H. Bräsen, Tuomas T. Rissanen, Petri Törönen, Ismo Vajanto, Seppo Ylä-Herttuala. **Changes in gene expression in atherosclerotic plaques analyzed using DNA array.** *Atherosclerosis*; 165:23-32 (2002).
- II Tiina T. Tuomisto, Anna Korkeela, Juha Rutanen, Helena Viita, Jan H. Bräsen, Mervi S. Riekkinen, Tuomas T. Rissanen, Kari Karkola, Zsolt Kiraly, Konrad Kölbl, Seppo Ylä-Herttuala. **Gene expression in macrophage-rich inflammatory cell infiltrates in human atherosclerotic lesions as studied by laser microdissection and DNA array- Overexpression of HMG-CoA reductase, colony stimulating factor receptors, CD11A/CD18 integrins, and interleukin receptors.** *Arterioscler Thromb Vasc Biol*; 23:2235-2240 (2003).
- III Tiina T. Tuomisto, Mervi S. Riekkinen, Helena Viita, Seppo Ylä-Herttuala. **Analysis of gene and protein expression during monocyte-macrophage differentiation and cholesterol loading- cDNA and protein array study.** *Submitted for publication.*
- IV Tiina T. Tuomisto*, Tuomas T. Rissanen*, Ismo Vajanto, Anna Korkeela, Juha Rutanen, Seppo Ylä-Herttuala. **HIF-VEGF-VEGFR-2, TNF- α and IGF-pathways are upregulated in critical human skeletal muscle ischemia as studied with DNA array.** *Atherosclerosis*; 174:111-120 (2004).

* Authors with equal contribution. Also some unpublished data are presented.

CONTENTS

1	INTRODUCTION.....	13
2	REVIEW OF THE LITERATURE.....	13
2.1	ATHEROSCLEROSIS.....	13
2.1.1	Types of atherosclerotic lesions.....	14
2.1.2	Pathogenesis of atherosclerosis	14
2.1.3	Gene expression in atherosclerosis	17
2.1.4	Clinical manifestations of atherosclerosis	23
2.1.5	Treatment of atherosclerosis and its clinical manifestations.....	24
2.2	GENOMICS AND PROTEOMICS IN THE RESEARCH OF ATHEROSCLEROSIS	24
2.2.1	Gene expression in the cardiovascular system.....	24
2.2.2	DNA array formats and the analytical method	26
2.2.3	Data analysis and bioinformatics.....	29
2.2.4	Applications of DNA arrays in the research of atherosclerosis	31
2.2.5	Protein arrays.....	36
2.2.6	Genomic vs. proteomic studies.....	37
2.3	LASER MICRODISSECTION.....	37
2.3.1	The principle of laser microdissection	37
2.3.2	Applications of laser microdissection.....	39
2.3.3	Applications of laser microdissection in the research of atherosclerosis.....	39
3	AIMS OF THE STUDY.....	40
4	MATERIALS AND METHODS	40
4.1	HUMAN TISSUE SAMPLES	40
4.1.1	Human atherosclerotic lesions (I and II).....	40
4.1.2	Human skeletal muscle samples (IV)	40
4.2	CELL CULTURE STUDIES (II AND III)	41
4.3	LASER MICRODISSECTION (II)	41
4.4	GENE EXPRESSION PROFILING (I-IV).....	41
4.4.1	DNA array analyses (I-IV)	41
4.4.2	RT-PCR (I-IV).....	43
4.4.3	<i>In situ</i> hybridization (I).....	43
4.5	PROTEIN EXPRESSION PROFILING (I-IV)	43
4.5.1	Immunohistochemistry (I, II, IV)	43
4.5.2	Protein array analysis (III).....	43
5	RESULTS	44
5.1	GENE EXPRESSION IN HUMAN ATHEROSCLEROTIC LESIONS (I AND II).....	44
5.2	GENE EXPRESSION CHANGES DURING MONOCYTE-MACROPHAGE-DIFFERENTIATION (III).....	45
5.3	PROTEIN EXPRESSION IN OXLDL-STIMULATED MACROPHAGES (III).....	47
5.4	COMPARISON OF GENE AND PROTEIN EXPRESSION IN OXLDL-STIMULATED MACROPHAGES (III).....	48
5.5	GENE EXPRESSION IN HUMAN ISCHEMIC SKELETAL MUSCLE (IV).....	48
6	DISCUSSION	50
6.1	GENE EXPRESSION IN HUMAN ATHEROSCLEROTIC LESIONS	50
6.2	GENE EXPRESSION IN MACROPHAGE-RICH SHOULDER AREA OF ATHEROSCLEROTIC LESION.....	52
6.3	GENE EXPRESSION DURING MONOCYTE MACROPHAGE-DIFFERENTIATION	54
6.4	GENE AND PROTEIN EXPRESSION IN OXLDL-STIMULATED MACROPHAGES	54
6.5	GENE EXPRESSION IN ISCHEMIC SKELETAL MUSCLE	56
7	CONCLUSIONS AND FUTURE DIRECTIONS	57
8	REFERENCES.....	59

1 INTRODUCTION

Atherosclerosis and its clinical manifestations- coronary artery disease, cerebrovascular disease, peripheral artery disease and kidney disorders-are the most common cause of death in Western countries. A number of patients cannot be effectively treated with conventional strategies such as with drug treatment, bypass surgery, and angioplasty. To improve current treatment methods and develop better ones, it is extremely important to understand the complicated molecular mechanisms underlying the atherosclerotic process.

Conventionally, the analysis of gene and protein expression patterns has been restricted to “one-gene” and “one-protein” methods, such as to RT-PCR and Western blot, respectively. However, in the mid 1990’s, high-throughput expression analysis methods, DNA and protein arrays, have been developed to enable the analysis of thousands of genes in a single experiment. These methods all together with the completion of the sequence of human genome have introduced a new field of biomedical research, “functional genomics”. Functional genomics concentrates on the understanding of the “central dogma” of molecular biology: how is the complex intra- and intercellular network, comprising of genes, mRNA molecules and proteins, regulated; and even more importantly, which are the molecular mechanisms of diseases.

Atherogenesis is a complicated process, which includes endothelial cell dysfunction, smooth muscle cell (SMC)

proliferation and migration, recruitment of inflammatory cells, accumulation of lipids, formation of connective tissue, and thrombus formation. The understanding of the fundamental pathobiological mechanisms of atherosclerosis demands information of gene and protein expression patterns in human lesions, specific cells in lesions, animal models and cell culture models of different cell types that play a role in the atherosclerotic process. In this study, we analyzed gene expression in human atherosclerotic lesions, macrophage-rich shoulder areas and *in vitro* macrophages, as well as in ischemic skeletal muscle using DNA array method.

2 REVIEW OF THE LITERATURE

2.1 ATHEROSCLEROSIS

Human arteries are composed of three layers: the intima, the media, and the adventitia. The intima, the innermost layer of the artery, is composed of endothelium, a few smooth muscle cells (SMC) and macrophages, and extracellular matrix (ECM) components such as collagen, elastin and proteoglycans (Stary et al., 1992). Internal elastic lamina separates the intima from the media, which is mainly made up by SMCs (Schoen and Cotran, 1999). The adventitia is separated from the media by external elastic lamina and is mainly composed of fibroblasts and connective tissue in which vasa vasorum and nerve fibers are dispersed (Schoen and Cotran, 1999; Gutterman, 1999).

When atherosclerotic lesions develop, they are first formed in areas with adaptive intimal thickening (atherosclerosis-prone locations). Adaptive intimal thickening is a physiological adaptation to different blood flow and wall tensions in distinct regions of arteries (Stary et al., 1992; Resnick et al., 2000). Adaptive intimal thickening does not directly lead to the development of lesions, but atherogenic stimuli, such as elevated plasma concentrations of lipoproteins, is also needed (Stary et al., 1992).

The most important risk factors for atherosclerosis are well known: genetic background, high serum LDL cholesterol and low HDL cholesterol levels, elevated blood pressure, diabetes, smoking, low physical activity, and the male gender (Libby et al., 2002; Ross, 1999; Doevendans et al., 2001).

2.1.1 Types of atherosclerotic lesions

Atherosclerotic lesions have been classified to six grades numbered from I to VI (Stary et al., 1995; Stary et al., 1994). Type I lesions, or initial lesions, contain few lipid droplets and some macrophage foam cells (Stary et al., 1994). Type II lesions, also known as fatty streaks, consist mainly of macrophage foam cell layers. Other cell types present in fatty streaks are T-lymphocytes and mast cells (Stary et al., 1994). Type III lesions, or intermediate lesions, form a morphological and pathophysiological bridge between fatty streaks and atheromas. Lipid droplets are mostly intracellular in type I and II lesions, whereas in type III lesions lipid particles are extracellular and replace intercellular matrix and drive SMCs apart (Stary et al., 1994). In type IV

lesions dense accumulation of extracellular lipid forms a lipid core in the intima (Stary et al., 1995). Type V lesions are characterized by new fibrous connective tissue in the intima above the lipid core and may also contain calcium (Stary et al., 1995). Type VI lesions, or complicated lesions, have generally the underlying morphology of type IV and V lesions with the disruption of the lesion surface (VIa), hematoma (VIb) or hemorrhage or thrombotic deposits (VIc) (Stary et al., 1995).

2.1.2 Pathogenesis of atherosclerosis

Over the years there have been several hypotheses on the development of atherosclerosis. Nowadays the response-to-injury hypothesis (Ross, 1986), the lipid hypothesis (Steinberg, 1983; Ross, 1986), and the monoclonal hypothesis (Benditt and Benditt, 1973; Schwartz et al., 1995) are the most accepted theories for the pathogenesis of atherosclerosis. The lipid hypothesis emphasizes the importance of lipids, especially low density lipoprotein (LDL) in the development of atherosclerosis (Steinberg, 1983). The monoclonal hypothesis stresses the significance of the clonal expansion of SMCs in developing plaques (Schwartz et al., 1995).

The response-to-injury theory considers atherosclerosis a chronic inflammatory response of the arterial wall initiated by injury to the endothelium. Endothelial injury can be caused by hyperlipidemia, hypertension, smoking, hemodynamic factors, inflammatory mediators, homocysteine, toxins, viruses and immune reactions. It leads to endothelial dysfunction with increased permeability and the adhesion

of blood monocytes, T-lymphocytes, and platelets (Ross, 1993; Schoen and Cotran, 1999; Ross, 1999). The adhesion of the above mentioned cells is caused by the appearance of specific adhesive glycoproteins on endothelial cells, and by chemoattractants secreted by the endothelium, its adherent leukocytes, and SMCs (Ross, 1993). When the process continues, adhered monocytes differentiate into macrophages, engulf lipids, become foam cells, and form the fatty streak together with lymphocytes, (Ross, 1993; Schoen and Cotran, 1999). During the lesion development, SMCs migrate from media to intima by the courtesy of chemokines and other factors produced by platelets and macrophages. SMCs proliferate and synthesize extracellular matrix in the intima (Ross, 1993; Schoen and Cotran, 1999). Cycles of accumulation of mononuclear cells, SMC migration and proliferation and formation of fibrous tissue produce an advanced lesion. In advanced lesions, fibrous cap overlies a core of lipids and necrotic tissue (Figure 1) (Ross, 1999).

The role of endothelial cell dysfunction is central in the development of atherosclerosis. Endothelial cells have many functions. For example, they form a nonthrombogenic and nonadherent surface, and secrete growth-regulatory molecules (Ross, 1993; Schoen and Cotran, 1999). Changes in those properties are typical for endothelial cell dysfunction (Ross, 1993). OxLDL, or otherwise modified LDL, has been considered as a key component in endothelial injury (Ross, 1993; Ross, 1999). OxLDL induces the formation of cell surface glycoproteins to which monocytes and leukocytes adhere. OxLDL is chemotactic for other monocytes, it participates in the

activation of the differentiation of monocytes into macrophages, and further, the uptake of oxLDL by macrophages leads to foam cell formation (Ross, 1993; Ross, 1999). The inflammatory response itself and mediators of inflammation, such as interleukin-1 and macrophage colony stimulating factors (CSFs), augments the binding of LDL to endothelium. Additionally, modified LDL induces the production of inflammatory agents (Ross, 1999). Changes in shear stress also play a role in the induction of adhesion molecules (Ross, 1999). Additionally, the thrombotic and coagulant activities of the endothelium are changed during the formation of atherosclerotic lesion (Ross, 1993).

Macrophages are present in all stages of atherosclerotic lesions. They internalize and oxidize LDL. Activated macrophages express class II histocompatibility antigens, which allow them to present antigens to T-lymphocytes (Ross, 1999). Macrophages play also a role in the fibroproliferative process by secreting numerous growth factors, such as PDGF and IL-1 (Ross, 1993). As a response to macrophage CSFs, macrophages may multiply in the lesion. On the other hand, under certain circumstances, they undergo apoptosis induced by inflammatory cytokines and thus partly form the necrotic core of advanced lesions. Activated macrophages release proteolytic enzymes, which may cause the thinning of the fibrous cap of the lesion that exposes the lesion to ulceration (Ross, 1999). Furthermore, macrophages can cause endothelial injury by forming toxic substances (Ross, 1986; Schoen and Cotran, 1999). Besides macrophages, T-cells play an important role in the inflammatory process and

immune response in atherosclerosis. They elaborate inflammatory cytokines, such as interferon- γ (IFN- γ), which activate macrophages, endothelial cells, and SMCs (Ross, 1999; Libby et al., 2002). During lesion development, there is a switch from very proinflammatory, macrophage-activating, Th1-response to less inflammatory, protective Th2-response. Cytotoxic T-cells have also been found in atherosclerotic lesions (Zhou et al., 1998; Hansson et al., 2002). It has been proposed that mast cells also contribute to atherogenesis, especially by influencing the lipid metabolism in the arterial wall and activating proteolytic enzymes (Kovanen, 1996; Libby, 2002). Activated platelets secrete proatherogenic substances that stimulate SMC and leukocyte migration and ECM

production, and form the thrombus (Ross, 1999; Libby, 2002).

There are at least two different phenotypes of SMCs in the arteries. SMCs of contractile phenotype respond to agents inducing either vasoconstriction or vasodilatation. Synthetic SMCs can produce several growth-regulatory molecules and cytokines, such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), and synthesize extracellular matrix (Ross, 1993). Additionally, the SMCs in the intima and the media differ from each other (Schwartz et al., 1995). In addition to macrophages, SMCs can also take up lipoproteins and form foam cells (Stary et al., 1994).

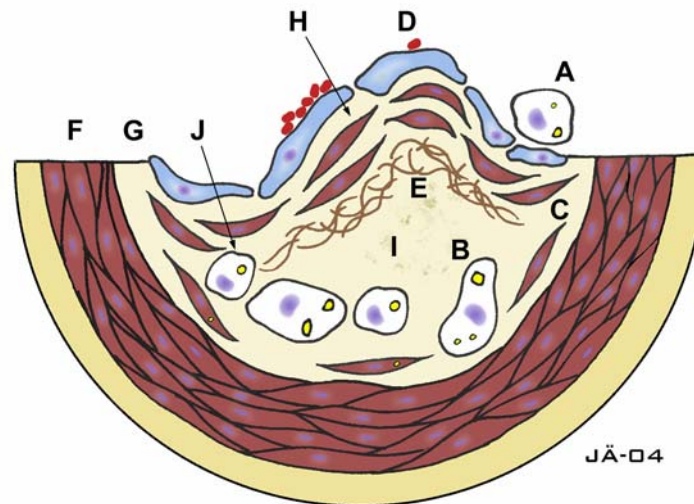


Figure 1. An atherosclerotic lesion. The vessel wall consists of three layers, an intima (G), media (F) and adventitia underlying the media. The development of an atherosclerotic lesion is accompanied by monocyte (A) adhesion to endothelium and differentiation to macrophages (B), foam cell formation, SMC (C) migration and proliferation, T-cell and platelet (D) adherence and ECM (E) production. An advanced lesion consists of fibrous cap (H), necrotic core (I) and macrophage-rich shoulder area (J).

2.1.3 Gene expression in atherosclerosis

2.1.3.1 Genes affecting the lipid metabolism in the arterial wall

In atherosclerosis, the lipid metabolism in intima is disturbed. Lipids, especially cholesterol originating from plasma LDL, are consequently accumulated extracellularly and intracellularly in macrophages and SMCs. Most of the blood cholesterol is carried in LDL particles. LDL particles are formed in the capillary system from VLDL particles in a process mediated by endothelial lipoprotein lipase. Most of the LDL receptors are situated in the liver, but there are also LDL receptors in extrahepatic tissues to provide cells with the cholesterol they need. Apo B-100 protein on the surface of LDL particles is the mediator of the adherence of LDL particles to their receptors. The uptake of LDL into cells is strictly controlled by plasma LDL levels via the synthesis and recycling of LDL receptors. In addition, intra- and extracellular levels of cholesterol and lipids in the extrahepatic system are controlled by the lymphatic removal (Brown and Goldstein, 1979; Brown et al., 1981; Pentikainen et al., 2000; Kovanen, 2000; Guyton and Klemp, 1996). The uptake of triglyceride-rich lipoproteins, such as chylomicron remnants and very-low density lipoproteins (VLDL), by liver lipoprotein receptors is mediated by apoE on the surface of these lipoproteins (Tall et al., 1999).

The fate of LDL particles that enter the arterial intima differs from that in other extrahepatic tissues. LDL-particles are

trapped by intimal proteoglycans, and therefore retained and accumulated in intima. They are also modified in many ways. (Kovanen, 2000; Pentikainen et al., 2000). When the plasma levels of LDL are elevated, more LDL is transported into intima. For LDL particles to become atherogenic, they have to be modified. Modifications include: proteolytic modifications, especially the degradation of apoB-100 by intimal cell proteases (Pentikainen et al., 2000; Kovanen, 1996); lipolytic modification by phospholipases (Murakami and Kudo, 2003; Pentikainen et al., 2000), and cholesterol esterases (Li and Hui, 1997); and most importantly, oxidative modification by lipoxygenases, peroxidases and oxygen-derived free radicals (Yla-Herttuala, 1994; Kuhn and Chan, 1997; Yla-Herttuala et al., 1989). Modification of LDL enhances the trapping of LDL particles by proteoglycans, causes endothelial damage, triggers various pro-inflammatory signals (e.g. the upregulation of monocyte adhesion molecules, the augmentation of the immunological response, increased chemoattraction), as well as pro-atherogenic signals (e.g. mitogenic signals for SMCs) (Steinberg et al., 1989; Guyton and Klemp, 1996; Pentikainen et al., 2000; Steinberg and Witztum, 1999). LDL particles are also engulfed by macrophages via scavenger receptors, and the macrophages become foam cells (Matsumoto et al., 1990; Nakata et al., 1999; de Winther et al., 2000). Besides LDL, also intermediate-density lipoprotein (IDL), VLDL, chylomicron remnants and lipoprotein (a) are accumulated into intima, and this accumulation might accelerate the formation of atheroma (Nordestgaard, 1996).

In addition to atherogenic lipoproteins, also plasma high density lipoprotein (HDL) particles are drifted to intima (Yla-Herttuala et al., 1987). They carry away cholesterol particles from intimal macrophages and other peripheral tissues in a process called “reverse cholesterol transport” (Tall et al., 1999; Rader, 2003). Several factors are needed in this process: cholesterol ester hydrolases in macrophages, which catalyzes the hydrolysis of cholesterol ester stores (Ghosh et al., 2003); ATP-binding cassette transporter ABCG, which functions in the intracellular cholesterol transport; interaction of apoA1- surface protein on HDL particles and ATP-binding cassette transporter ABCA1 on macrophages (Rader, 2003; Schmitz et al., 2001); lecithin-cholesterol acetyltransferase (LCAT), lipoprotein lipases, and cholesterol ester transfer protein (CETP) (Tall et al., 1999; Rader, 2003). Besides reverse cholesterol transport, HDL has also other antiatherogenic effects. They contain antioxidant enzymes, such as paraoxonase, which can break down oxidized lipids and protect LDL from oxidation (Libby et al., 2002). HDL also attenuates the inflammatory response (Rader, 2003).

In atherosclerosis, lipid metabolism in intima is disturbed mainly due to elevated LDL levels, inadequate levels of HDL particles and the modification of LDL particles in intima (Kovanen, 2000; Tall et al., 1999). Genetic factors, such as deficiencies or variances in LDL receptor genes, in lipoprotein lipases, in apolipoproteins apoAI, -B100, -C and -E and lipoprotein (a), have great impact on the genesis of dyslipidemias and atheromas (Tall et al., 1999; Doevendans et al., 2001).

2.1.3.2 Scavenger receptors

Scavenger receptors (ScRs) are membrane glycoproteins that are involved in the internalization of unmodified and modified lipoproteins, as well as glycosylation end products, apoptotic cells and bacteria (Yamada et al., 1998; Terpstra et al., 2000). Scavenger receptors can be divided to class A-F receptors. They are expressed in hepatic and/or extrahepatic tissue, such as in macrophages and endothelial cells (Terpstra et al., 2000; Yamada et al., 1998). Macrophage scavenger receptors classes A and B are both upregulated in atherosclerotic lesions (Yla-Herttuala et al., 1991; Nakata et al., 1999; Hiltunen et al., 1998). Class A receptors bind acetylated LDL and oxLDL, and play a role in cell adhesion and foam cell formation in atherosclerotic lesions (Brown and Goldstein, 1979; Kodama et al., 1990; de Winther et al., 2000). CD36 (class B ScR) also binds oxLDL, and has a major role in macrophage foam cell formation (Endemann et al., 1993; Huh et al., 1996), as well as human CD68 (class D ScR) (Ramprasad et al., 1996; Tsukamoto et al., 2002). Besides macrophages, endothelial cells and SMCs express scavenger receptors important for atherogenesis; both lectin like receptor for oxidized LDL (LOX-1, class E ScR) (Sawamura et al., 1997) and ScR expressed by endothelial cells I (SREC-I, class F ScR) (Ishii et al., 2002) internalize oxLDL. SMCs express class A ScRs (Mietus-Snyder et al., 2000), CD36 (Zingg et al., 2002) and LOX-1 (Kume and Kita, 2001).

2.1.3.3 Adhesion molecules

Cellular adhesion molecules mediate the interaction between the endothelium and blood cells via cell-cell and cell-matrix interactions. These adhesion molecules participate also in cell migration, signalling functions, and other vascular physiological responses (Price and Loscalzo, 1999).

Cellular adhesion molecules can be divided into four classes based on their structure and function. The first class of adhesion molecules is integrins, which consist of α and β subunits. They can be further divided into three subclasses: VLA integrins, leukocyte integrins (CD11/CD18), and cytoadhesins. They are distributed among a variety of cell types in the vasculature (Springer, 1994; Price and Loscalzo, 1999). They have also important signalling functions, such as functions in cell growth, differentiation, and survival via their effects on cytoskeletal organization and various signal transduction molecules (Frenette and Wagner, 1996; Price and Loscalzo, 1999). The second class is immunoglobulin gene family that functions as endothelial ligands for integrins expressed on leukocytes and platelets, and participates also in signal transduction and cell migration (Price and Loscalzo, 1999). Members of this class include for example vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecules (ICAMs), and platelet-endothelial cell molecule (PECAM) (Dustin et al., 1986; Newman et al., 1990; Cybulsky et al., 1991). The third class of cellular adhesion molecules is selectins, which bind to carbohydrate ligands on leukocytes and endothelial cells, and mediate the first step in leukocyte adhesion to endothelium (McEver et al.,

1995). Three types of selectins have been identified: E- and P-selectins, which are expressed mainly on endothelial cells; and L-selectins, which are expressed on leukocytes (Price and Loscalzo, 1999; McEver et al., 1995). The fourth class of adhesion molecules, cadherins, support cell-cell contacts to maintain tissue cohesion, but they also participate in dynamic morphogenetic events, and have adhesion-activated cell signalling activities (Yap and Kovacs, 2003).

Leukocyte adhesion is a crucial step in atherogenesis. Several different adhesion molecules function in different steps of leukocyte adhesion. Tethering and rolling are mediated by selectin-carbohydrate interaction. In firm adhesion, integrins on neutrophils, monocytes and lymphocytes (CD11/CD18) attach to ICAM-1 and VCAM-1 on endothelial cells. Transmigration requires homophilical PECAM-1-interactions between attached cells and endothelium (Price and Loscalzo, 1999; Krieglstein and Granger, 2001; McEver et al., 1995). Adhesion molecule interactions are also involved in platelet adhesion, such as platelet integrin $\alpha_{IIb}\beta_3$ – endothelial von Willebrand factor-interaction (Price and Loscalzo, 1999). The expression of adhesion molecules is regulated by various cytokines, for example, ICAM-1 and VCAM-1 are strongly upregulated by IL-1 and TNF- α (Price and Loscalzo, 1999; Meager, 1999). Additionally, common atherosclerotic risk factors, such as hypercholesterolemia, diabetes, smoking, hypertension and homocysteinemia affect adhesion molecule expression. As an example, the expression of endothelial cell adhesion molecules VCAM-1, ICAM-1 and P-

selectin is increased in atherosclerotic lesions (Price and Loscalzo, 1999).

2.1.3.4 Growth factors

Growth factors regulate cellular functions including proliferation, differentiation, migration, and survival/apoptosis (Waltenberger, 1997). Many growth factors have important roles in the development of atherosclerosis (Ross, 1993). The binding of the growth factor to its specific cell-surface molecule leads to the dimerization of the receptor and autophosphorylation, and initiates activation of signal transducing cascades, which leads to the modulation of cellular functions (Claesson-Welsh, 1996; Claesson-Welsh, 2003; Waltenberger, 1997). The majority of growth factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) stimulate proliferation and inhibit apoptosis by influencing cell cycle regulators. On the other hand, members of transforming growth factor- β (TGF- β) family are negative regulators of the cell cycle (Waltenberger, 1997; Nasmyth, 1996).

Platelet-derived growth factor (PDGF) is produced by platelets, SMCs, fibroblasts, macrophages, and endothelial cells. It is a very potent SMC mitogen (Heldin, 1992; Heldin and Westermark, 1999; Bonthron et al., 1988). Furthermore, it has a chemotactic effect on SMCs, endothelial cells, fibroblasts and inflammatory cells (Siegbahn et al., 1990; Risau et al., 1992), induces angiogenesis (Risau et al., 1992), and stimulates production of ECM (Heldin, 1992).

Fibroblast growth factors (FGFs) are secreted by various cell types including endothelial cells and SMCs (Sidawy et al., 1998). bFGF stimulates endothelial cell and SMC proliferation (Lindner et al., 1990; Lindner et al., 1991), is a potent angiogenic factor (Folkman and Shing, 1992), and prevents apoptosis of SMCs (Fox and Shanley, 1996).

Transforming growth factor β (TGF- β) can act as a proliferative or antiproliferative stimulus depending on the target cell activation. The main effect on endothelial cells and SMCs is antiproliferative, whereas on fibroblasts the effect is proliferative (Sporn et al., 1987; Derynck et al., 1987; Pollman et al., 1999; McCaffrey, 2000). TGF- β stimulates ECM formation (Waltenberger et al., 1993a; Chen et al., 1987), it modulates the inflammatory and immunological responses (Waltenberger et al., 1993b; Tsunawaki et al., 1988), and it has also a role in the modification of actions of other growth factors, such as the actions of FGF and PDGF (Baird and Durkin, 1986; Mii et al., 1993). The cells in atherosclerotic lesions are resistant to the antiproliferative and apoptotic effects of TGF- β due to the reduction of TGF- β 1 receptor (McCaffrey, 2000).

Insulin-like growth factors (IGFs) induce proliferation and migration of endothelial cells and SMCs (Bayes-Genis et al., 2000; Grant et al., 1994). IGF-1 is also an angiogenic factor (Nicosia et al., 1994). Additionally, IGFs promote macrophage chemotaxis, LDL uptake and the release of proinflammatory cytokines from macrophages (Hochberg et al., 1992; Renier et al., 1996; Bayes-Genis et al., 2000).

Vascular endothelial growth factor A (VEGF) is a very potent endothelial cell

mitogen and angiogenic factor (Ferrara et al., 2003; Thomas, 1996). It and other members of the VEGF family (VEGF-A, -B, -C, -D and PlGF) have also a variety of other functions in vasculature: they promote monocyte chemotaxis, ECM modelling, vasodilatation, vascular permeability and lymphatic vessel growth. The members have slightly different effects due to divergent binding capacities to VEGF-receptors. The expression of VEGFs and their receptors is regulated by hypoxia and by several cytokines and growth factors (Gerber et al., 1997; Frank et al., 1995; Ferrara and Davis-Smyth, 1997; Ferrara et al., 2003; Thomas, 1996; Rissanen, 2003).

2.1.3.5 Cytokines and chemokines

Cytokines modulate the inflammatory and immunological responses in atherosclerosis (Ross, 1993). The expression of cytokines is induced by several proatherogenic stimuli, such as by oxLDL, by infection, and by cytokines produced by inflammatory cells (Krishnaswamy et al., 1999). Interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) are probably the most potent proinflammatory cytokines (Laukkanen and Yla-Herttuala, 2002; Dinarello, 1997; Sack, 2002). Stimulation of endothelial cells by IL-1 causes multiple responses, but most notably, induces the expression of adhesion molecules, which promote monocyte recruitment and infiltration into the arterial wall (Bevilacqua et al., 1985; Krishnaswamy et al., 1999). TNF- α has several proatherogenic properties. It enhances the recruitment of inflammatory cells and activates matrix metalloproteinase production (Sack, 2002; Pober and Cotran, 1990; Galis et

al., 1994a). IL-1 and TNF- α upregulate the expression of many genes, for example interleukins 1-8, CSFs, and adhesion molecules. Many of the effects of IL-1 and TNF- α are mediated through these molecules (Laukkanen and Yla-Herttuala, 2002; Krishnaswamy et al., 1999). Interferon- γ (IFN- γ) is produced by activated Th1-cells in lesions. It increases VCAM-1-expression on endothelial cells, augments class II MHC expression in macrophages, and modulates scavenger receptor expression and cholesterol efflux in macrophages (Li et al., 1995; Wang et al., 2002; Li et al., 1993; Hansson et al., 1989; Libby et al., 2002).

Interleukin-10 has several anti-inflammatory properties, such as the inhibition of cytokine production in macrophages and lymphocytes, and the inhibition of monocyte adhesion (Tedgui and Mallat, 2001; Krakauer, 1995; Mallat et al., 1999). IL-4 and IL-13 are also anti-inflammatory cytokines produced by Th2-cells that suppress the production of cytokines by macrophages and monocytes (Tedgui and Mallat, 2001). They are also capable of promoting angiogenesis (Fukushi et al., 2000).

Chemokines (CCs, such as monocyte chemoattractant protein-1, and CXC-chemokines, such as IL-8) are activators and chemoattractants to leukocytes and monocytes (Gerszten et al., 2000). They also cause angiogenesis and SMC migration and proliferation (Burke-Gaffney et al., 2002). They are abundantly expressed in lesional cells, and their expression is induced by a number of proatherogenic stimuli including growth factors, cytokines, oxLDL and endothelial injury (Gerszten et al., 2000; Burke-Gaffney et al., 2002; Nelken et al., 1991; Reape et al., 1999).

2.1.3.6 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) degrade extracellular matrix components, which is essential for matrix remodelling, infiltration of inflammatory cells, apoptosis, plaque rupture and angiogenesis (George, 1998). MMPs can be divided into three classes: collagenases (substrates mainly different collagens and proteoglycans), gelatinases (substrates collagens and gelatine), and stromelysins (various substrates, such as collagens, laminin, fibronectin, elastin and proteoglycans) (George, 1998). There are elevated levels of many MMPs in atherosclerotic lesions, especially in macrophage-rich areas (Galis et al., 1994b). MMP expression is regulated by several cytokines and growth factors (George, 1998). Stimulatory factors include for example IL1, TNF- α and PDGF (Fabunmi et al., 1996; Galis et al., 1994a), and inhibitory factors include for example IFN- γ (Xie et al., 1994). MMP activity is also regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). The complex regulatory balance of MMP activity by cytokines and TIMPs is crucial for the plaque evolution (George, 1998). TIMPs have also growth inhibitory and pro-apoptotic activities (Baker et al., 1998).

2.1.3.7 Nitric oxide

Endothelial production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) plays a central role in the maintenance of vascular health (Cannon, III, 1998; Preli et al., 2002). NO functions in the regulation of

vascular tone, it decreases platelet activation and adhesion, it inhibits growth factor activities, and has several anti-inflammatory properties such as the inhibition of the expression of cytokines and leukocyte adhesion molecules (ICAM-1, VCAM-1) (Bath et al., 1991; Radomski and Moncada, 1993; Cannon, III, 1998). The production of NO by eNOS is regulated for example by shear stress (Joannides et al., 1995).

2.1.3.8 Oxidative stress

Increased production of reactive oxygen species (ROS) in endothelium leads to increased lipid oxidation and endothelial dysfunction, and, subsequently, to the loss of vasodilatation, increased platelet aggregation, SMC growth and inflammatory response. ROS include hydroxyl and nitric oxide radicals, which are produced by NADH/NAPDH oxidases, xanthine oxidase, and eNOS. Several known atherogenic stimuli, such as hypertension, diabetes and cigarette smoking increase the production of ROS in endothelium (Cai and Harrison, 2000; Harrison et al., 2003). Vascular oxidative stress is regulated by antioxidative mechanisms, which include superoxide dismutases (SOD) and catalases (Muzykantov, 2001).

2.1.3.9 Transcription factors

Peroxisome proliferator-activated receptors (PPAR- α , - γ and - δ) are nuclear receptor-type transcription factors (Walczak and Tontonoz, 2002; Barbier et al., 2002; Chinetti et al., 2000). PPAR- γ is highly expressed in atherosclerotic lesions, especially in

macrophage-rich areas (Marx et al., 1998; Tontonoz et al., 1998). PPAR- α and - γ modulate inflammatory responses, for example by inhibiting the expression of cytokines (e.g. MCP-1) and adhesion molecules (e.g. VCAM-1). They modulate also lipid metabolism, such as cholesterol efflux and foam cell formation, for example by inducing ABCA1 and CD36 expression. Additionally, they play a role in thrombus formation, as well as in plaque stability (e.g. by inhibiting the activity of MMPs). The net effect of PPARs in vessel wall is antiatherogenic (Tontonoz et al., 1998; Chinetti et al., 2000; Barbier et al., 2002; Marx et al., 1998).

Nuclear factor- κ B (NF- κ B) is a transcription factor associated with oxidative stress and inflammation (Meyer et al., 1991; Collins and Cybulsky, 2001). Active NF- κ B can be detected in atherosclerotic lesions (Brand et al., 1996). NF- κ B activates the expression of many important proatherogenic genes, which include adhesion molecules VCAM-1 and ICAM-1, chemokines and cytokines, as well as genes that regulate cell proliferation and cell survival (Collins and Cybulsky, 2001; Brand et al., 1996). The activity of NF- κ B is controlled by the redox status of the cells, by cytokines (Li and Karin, 1999), by oxLDL (Brand et al., 1997), as well as by shear stress and advanced glycosylation end products (Collins and Cybulsky, 2001).

In addition, several other transcription factors mediate anti- and proatherogenic stimuli. These include early growth response-1 (Egr-1), which modulates the expression of PDGF-B (Khachigian et al., 1996) and FGF (Biesiada et al., 1996). Activator protein-1 (AP-1) complex regulates cell proliferation and survival, and is activated by several

cytokines, such as by TNF- α (Shaulian and Karin, 2002). Additionally, Janus kinase- signal transducers of activators of transcription (JAK-STAT)-pathways mediate signals from various cytokines, such as from IFN- γ and CSFs (Ihle and Kerr, 1995).

2.1.4 Clinical manifestations of atherosclerosis

2.1.4.1 Coronary artery disease

Chronic coronary artery disease (CAD) is most commonly due to the obstruction of coronary arteries by an atheromatous plaque. CAD predisposes patients to myocardial infarction, unstable angina pectoris, and sudden cardiac death (Gersh et al., 2001). CAD is the most common cause of death in Western countries. In Finland, about 25 % of all deaths are caused by CAD. Its socioeconomical impact is very significant. In Finland in during 1997, ~170000 patients received compensation from the Social Insurance Institution for the treatment of CAD (Reunanen, 2000).

2.1.4.2 Other clinical manifestations

Peripheral arterial disease (PAD) is caused by atherosclerosis when it obstructs blood supply to lower or upper extremities. The most prominent symptom of PAD is intermittent claudication. The patients suffering from PAD have increased risk for adverse cardiovascular events, as well as the risk for limb loss (Creager and Libby, 2001). In addition, atherosclerosis predisposes to cerebrovascular diseases, such as to stroke and transient ischemic attacks

(TIA) (Wasserman, 2002), and to renal disorders, such as to thromboembolism and renal artery stenosis (Boudoulas and Leier, 2001).

2.1.5 Treatment of atherosclerosis and its clinical manifestations

Noninvasive treatment of CAD and other clinical manifestations of atherosclerosis include: drugs affecting lipid metabolism, such as statins; antihypertensive drugs, such as beta-blockers, diuretics and angiotensin-converting enzyme (ACE)-inhibitors; antiplatelet drugs, such as acetylsalicylic acid; and vasodilators, such as nitrates in conjunction with risk factor modification (O'Toole and Grech, 2003). Percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG) are the primary interventional therapies of stable CAD (O'Toole and Grech, 2003). Primary interventional therapies for PAD include percutaneous transluminal angioplasty (PTA) and open surgery with vein grafts (Dormandy and Rutherford, 2000). However, the outcome of the conventional therapy is not always satisfactory, for example 20-30 % of patients with chronic critical limb ischemia as a consequence of PAD cannot be treated with conventional strategies and require amputation (Dormandy and Rutherford, 2000). Recently, gene therapy strategies to induce therapeutic angiogenesis, prevent restenosis, and affect lipid metabolism have been introduced (Yla-Herttuala and Martin, 2000; Rissanen et al., 2001; Rutanen et al., 2001).

2.2 GENOMICS AND PROTEOMICS IN THE RESEARCH OF ATHEROSCLEROSIS

2.2.1 Gene expression in the cardiovascular system

The protein-coding segments of the genome, or genes, are transcribed into mRNA molecules in a phenomenon called "gene expression". mRNA molecules are then translated to proteins, which are considered as the main functional molecules of a living cell. Transcription and translation processes include various modifications, such as alternative splicing of transcripts and different posttranslational modifications of proteins, which include glycosylation and phosphorylation of proteins (Zubay et al., 1995). Consequently, the relationship between the abundance of mRNA and corresponding proteins is not straightforward, as discussed in section 2.2.6.

Functional genomics and proteomics are terms introduced in the 1990s to describe the methods to understand the "central dogma" of molecular biology, which is: how the genes are transcribed and translated, and how these processes are regulated in the entire cellular network. Conventionally, gene expression at the mRNA level has been studied using "one-gene methods" such as RT-PCR, Northern Blot and *in situ* hybridization, and also in large-scale using serial analysis of gene expression (SAGE), differential display (DD), and large-scale expressed sequence tag (EST) sequencing (Ausubel et al., 2003; Stanton, 2001). However, in the mid 1990s, more powerful methods, called DNA arrays were developed to

revolutionize gene expression studies (Lockhart et al., 1996; Schena et al., 1995). Correspondingly, efficient large scale protein expression analysis methods, such as protein arrays, 2D-PAGE and mass spectrometry, have been developed beside “one-protein methods”, which include Western Blot and immunohistochemistry (Zhu and Snyder, 2003; Ausubel et al., 2003).

The number of genes encoded by the human genome has been estimated to be ~32000-38000 (Venter et al., 2001). Using EST sequencing and array analysis it was estimated that ~20900-27100 genes are expressed in the cardiovascular system (Dempsey et al., 2001). The gene expression changes during atherosclerotic events have been studied extensively by DD and SAGE, but these studies have been restricted to cellular models, only.

SMCs have been stimulated with several atherogenic triggers, such as by lipopolysaccharide (LPS) and IFN- γ (Wang et al., 1996) and by products from activated macrophages (De Vries et al., 2000; Beauchamp et al., 2003). Wang et al (1996) were able to find elevated expression levels of interferon-inducible protein-10 (IP10) in activated SMCs. IP10 is involved in SMC migration, proliferation and inflammatory response (Wang et al., 1996). As a response to cytokines and growth factors secreted from macrophages, there are alterations in the expression of ICAM-1, GM-CSF, IL-8, and in the NF- κ B signalling (De Vries et al., 2000). Products of macrophages also change the expression of various cytokines, growth factors, and ECM proteins (Beauchamp et al., 2003).

In addition to SMCs, vascular endothelial cells have been stimulated by atherosclerotic stimuli, such as by TNF-

α (Horrevoets et al., 1999), by native LDL (Rodriguez et al., 2002), and by conditioned macrophage medium (de Waard et al., 1999). It was found that TNF- α induced changes in leukocyte trafficking, cell cycle control, apoptosis, and oxidative stress response (Horrevoets et al., 1999). Conditioned macrophage medium affected mainly cell adhesion proteins (de Waard et al., 1999). LDL treatment was shown to downregulate enzymes such as lysyl oxidase involved in maintaining ECM structure. This effect could be related to an increase in the endothelial permeability following LDL exposure (Rodriguez et al., 2002). In addition, steady laminar shear stress was shown to induce expression of atheroprotective genes, such as cyclooxygenase-2 (COX-2), Mn-SOD and eNOS (Topper et al., 1996).

The effects of oxLDL stimulation in macrophages was studied by DD. Induction of adipophilin, which might have a role in foam cell formation, was detected (Wang et al., 1999). Hashimoto et al (1999) studied gene expression changes during monocyte-macrophage differentiation as induced by CSFs. They found that lipid-metabolism related genes, such as apoE, were induced (Hashimoto et al., 1999).

The use of large-scale expression proteomics in atherosclerosis research has been published only once so far: 2D-PAGE was used to detect differences between SMCs from newborn and aged rats (Cremona et al., 1995). Because cellular retinol-binding protein was upregulated in aged SMCs, they suggested that retinoids might play a role in SMC differentiation and vascular ageing.

These studies have given valuable insight into the proatherogenic and

antiatherogenic processes in the different cell types of the atherosclerotic lesions, and emphasized the importance of inflammatory processes, growth factor activities, ECM modeling, lipid metabolism, and cell adhesion in the atherosclerotic process.

2.2.2 DNA array formats and the analytical method

2.2.2.1 The principle of DNA arrays

DNA arrays can be used to monitor global gene expression patterns in many different organisms (Lockhart and Winzler, 2000). Additionally, DNA array can be used in large-scale detection of single nucleotide polymorphisms (SNP arrays) and mutations (Roses, 2000). DNA array-based gene expression monitoring has been applied to cell culture studies (Cho et al., 1998; Iyer et al., 1999), studies of diseases such as cancer (DeRisi et al., 1996; Golub et al., 1999), or cardiovascular diseases (Tuomisto and Yla-Herttuala, 2003). Moreover, DNA array-based expression screening can be used in drug development (Marian and Gollob, 2003).

DNA array technology is derived from the Southern blot technique (Southern, 1975), which is based on the observation that single-stranded DNA attached to nitrocellulose membrane can hybridize to its complementary RNA (Gillespie and Spiegelman, 1965). DNA array systems share the following components: the array, which contains immobilized nucleic acid sequences or “probes”; one or more labeled samples or “targets” that are hybridized to the arrays; and a detection system that quantifies the hybridization signals (Figure 2).

Two types of arrays that have been utilized in gene expression profiling include cDNA arrays and oligonucleotide arrays. For cDNA arrays, probes are prepared by amplifying cDNA clones by PCR, which are then printed onto nylon membranes (macroarrays) (Zhao et al., 1995) or to microscopic glass slides (microarrays) (Schena et al., 1995). Probes can be either known genes or novel genes, termed EST-sequences (Borsani et al., 1998). The targets are generated from the RNA of interest and labeled during cDNA synthesis by radioactive labels (usually ³³P) for nylon macroarrays, or by fluorescent labels (usually Cy3 and Cy5) for glass microarrays. It is also possible to amplify RNA linearly in a T7-polymerase mediated *in vitro* transcription reaction, if the source of RNA is limited, such as in samples from laser microdissection and tissue biopsies (Luo et al., 1999; Feldman et al., 2002). The advantage of using fluorescent dyes is the possibility to hybridize two or more differentially labeled targets simultaneously, which allows the comparison of control and experimental samples in one assay. Arrays based on radioactive labeling require parallel hybridizations.

Oligonucleotide arrays or “chips” are high-density arrays composed of synthetic oligonucleotide probes (Lockhart et al., 1996). Oligonucleotide arrays are synthesized *in situ* by photolithography (Fodor et al., 1991). In the Affymetrix system, which is the most commonly used chip system, there are 10-25 oligonucleotide pairs, perfect match (PM) and mismatch (MM), as probes for each gene. Biotin-labeled fragmented target cRNA is hybridized to the array, and stained with phycoerythrin-conjugated streptavidin

(www.affymetrix.com). Similar to methods based on radioactive detection, the Affymetrix system requires parallel hybridizations. In addition to *in situ* synthesis, oligonucleotide arrays can be manufactured by printing prefabricated oligonucleotides to glass slides (Rogers et al., 1999).

As a detection system, a phosphorimager is used for radioactive labels and a confocal microscope scanner for fluorescent labels. Images are analyzed using software that quantifies the signals. The signal intensity is proportional to the amount of mRNA in each sample (Duggan et al., 1999). In addition to differences in probes, matrix material and labelling, systems differ with respect to methods of arraying, chemistry, linkers, hybridization, and detection (Gerhold et al., 1999).

The advantages of cDNA arrays as compared to oligonucleotide arrays are the lower costs of the array itself. Nylon arrays are reusable and analysis equipment is inexpensive. It is also possible to customize the arrays if clone sets are available. Because sequence data is not necessary for probe selection, it is also possible to also examine unknown genes. However, the handling of cDNA clone sets is time-consuming and prone to contamination. The sensitivity and specificity of different array methods are highly dependent on the probe design. High sensitivity and specificity can be obtained when multiple probes are used for each gene, as in Affymetrix system. This enables the detection of closely related genes and splice variants reliably. However, the array design for oligonucleotide arrays is very challenging and time-demanding (Schinke et al., 2003). The expression

measurements made by the two different array types have been compared, and it was found that the correlation between the measurements from different array types is highly dependent on the array design (Jenssen et al., 2002; Li et al., 2002b). Differences between the results gained from different array types may partly arise from cross-hybridization, which is a problem especially in cDNA arrays, and from different mRNA splice-variants. The genome data, which is used for oligonucleotide probe design is still incomplete, and makes optimal probe design difficult. There are also some differences in the detection sensitivities of Cy3 and Cy5-labels. Some differences may be due to inter-array variations as well as in the differences in the different data analysis methods for cDNA and oligonucleotide arrays.

2.2.2.2 Sensitivity of DNA arrays

At the moment, one DNA array can contain up to almost 50 000 different transcripts (www.affymetrix.com). Typically, the amount of total RNA required for target preparation is about 5-200 µg (2-5 µg of mRNA) depending on the array method used (Duggan et al., 1999; Gerhold et al., 1999). The sensitivity of both the cDNA-based and the oligonucleotide-based assays is less than 1 copy in 100000 in a mRNA population. Because the amount of mRNA in a cell is about ~0.5-1.0 pg (or 10000-100000 mRNA molecules), very-low-copy transcripts can be detected (Gerhold et al., 1999; Todd and Margolin, 2002).

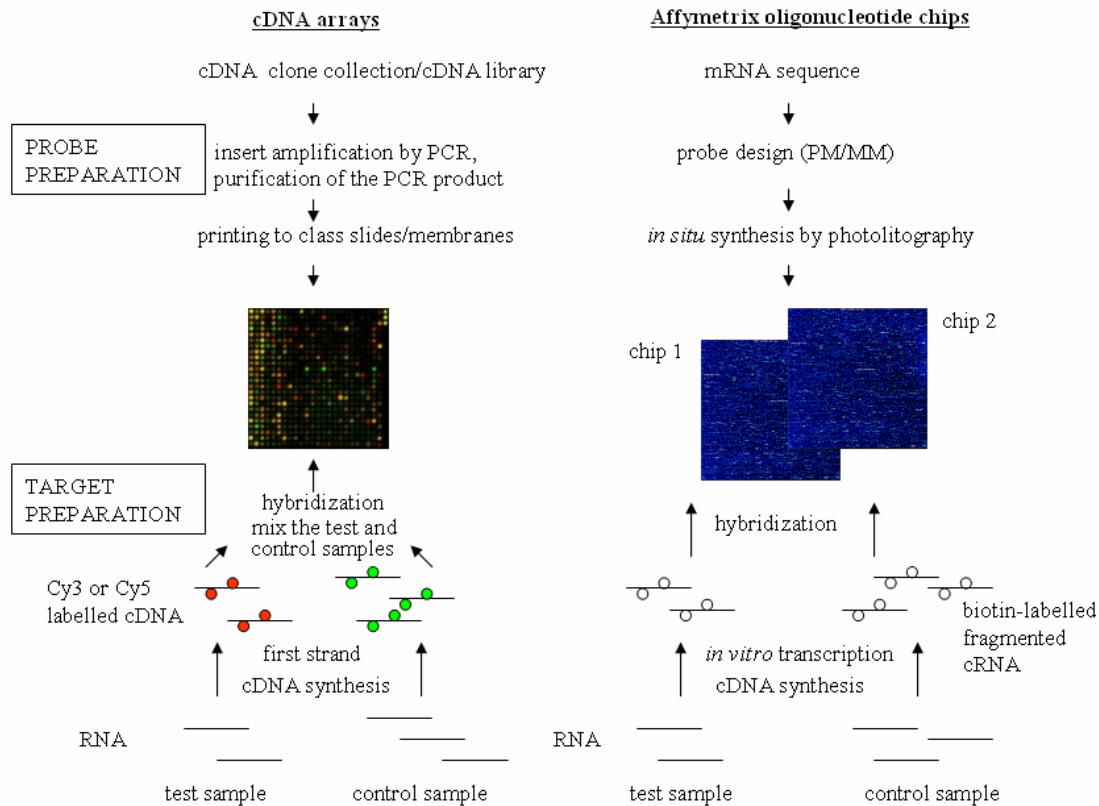


Figure 2. Schematic overview of the probe and target preparation for cDNA array vs. Affymetrix oligonucleotide chip. Probes for cDNA arrays are manufactured by PCR amplification of cDNA clones. Probes for Affymetrix oligonucleotide chips are synthesized *in situ* by photolithography. Targets for cDNA arrays are prepared by labelling RNA from test and control experiment with Cy3 or Cy5, which are hybridized to the same array. Targets for Affymetrix oligonucleotide chips are labelled with biotin during *in vitro* transcription, and hybridized to separate chips. Adapted from Wilson (Wilson et al., 2002).

2.2.2.3 Limitations in DNA array analyses

DNA microarray systems have limitations, some of which reflect the relatively early stages of development of this technology and the genome project which it is based on. Some of the errors may arise from the arrays itself, such as from the selection of proper target

(crosshybridization of nearly similar sequences, incomplete genome sequence data available for probe design) or from the printing errors during the manufacture of the arrays. Tissue heterogeneity, inter-individual differences, and the treatment of experimental animals (stress, circadian rhythm) might also contribute to experimental variation. The quality of RNA is extremely important. Challenges in tissue preservation and post-mortem

changes in human samples must be met (Johnston et al., 1997). Several artifacts can be derived from different sources during the various processing steps, including the sample preparation, labeling (different efficiency of dye incorporation, problems in the linearity of RNA amplification), hybridization (for example dust) and scanning. To overcome these problems, proper experimental design is extremely important, including the choice of optimal controls, adequate replicates and the confirmation of the results with independent methods (Kerr and Churchill, 2001; Butte, 2002; Abab et al., 2003).

2.2.3 Data analysis and bioinformatics

2.2.3.1 Preprocessing and normalization of the data

A schematic overview of the different steps in the analysis of DNA array data is presented in figure 3. After hybridization of probes to the arrays, the signals are detected with appropriate scanners. Special software is used to quantify the intensity of the signals and the background. The data should be filtered to get rid of too low and high intensities (for example saturated spots). Normalization of the data is needed to adjust the data for effects resulting from systematic non-biological differences between arrays. These differences can be caused by the differences in the efficiencies of probe labelling (Brazma and Vilo, 2000; Dopazo et al., 2001; Forster et al., 2003). Before choosing the

normalization method, the linearity of the data should be tested using a MA plot (Tseng et al., 2001). If the data is linear, the possible approaches that can be used in normalization are the use of housekeeping genes (Heller et al., 1997), artificial control probes (Forster et al., 2003), or more preferred, the mean or median value of the intensities of all measured genes (Alizadeh et al., 2000; Dopazo et al., 2001). For non-linear data, more sophisticated methods for data normalization are available, for example LOWESS and SNOMAD techniques can be used (Forster et al., 2003; Holloway et al., 2002; Colantuoni et al., 2002).

2.2.3.2 Finding differentially expressed genes

After normalization, expression ratios are calculated, and usually log transformed to make the distribution more symmetric (Dopazo et al., 2001). Typically, ratios of 1.5-2 are considered as a change in gene expression (Brazma and Vilo, 2000). The reproducibility of repeated samples is evaluated and the statistical significance is tested using classical statistical techniques, which include standard t-test, paired t-test, and nonparametric tests (Claverie, 1999; Dopazo et al., 2001; Forster et al., 2003; Tuimala, 2003). Unfortunately, the analysis is often based only on arbitrary assigned fold differences (Dopazo et al., 2001). If more than two groups are compared, an analysis of variance (ANOVA) is the most commonly used test (Kerr et al., 2000).

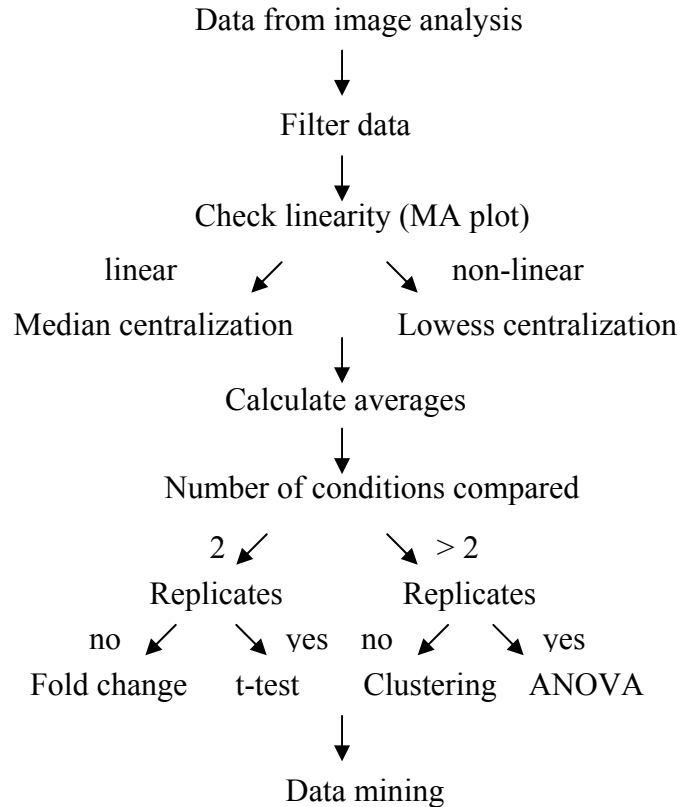


Figure 3. Schematic overview of the steps in array data analysis. Adapted from Laine and Tuimala (Laine and Tuimala, 2003).

2.2.3.3 Clustering methods

If the array analysis is performed in different timepoints, or in different stages of diseases, it is possible to use clustering techniques to identify genes with similar expression patterns. Clustering methods can be grouped as supervised and unsupervised. Supervised methods assign some predefined classes to a data set, whereas in unsupervised methods, no prior assumptions are applied. The hierarchical (Eisen et al., 1998) and K-mean clustering algorithms (Tavazoie et al., 1999), as well as self-organizing maps (SOMs) (Tamayo et al., 1999; Toronen et al., 1999) have been used as clustering methods and are examples of unsupervised clustering

methods (Vihinen and Tuimala, 2003). Supervised clustering methods, such as decision trees, can also be used (Brazma and Vilo, 2000).

2.2.3.4 Data mining

There are attempts to build up an international repository of gene expression data that is also linked to all relevant molecular biology databases. The MIAME set of protocols is the leading proposal for data submission and databases. MIAME provides rules in reporting the array data regarding experimental design, array design, treatment of samples, hybridization conditions, measurements such as images and quantification, and normalization (Stoeckert, Jr. et al., 2002;

Brazma et al., 2001). Many journals currently require that array data is reported according to MIAME rules.

Public databases that are useful in the analysis of data from genomic and proteomic analyses include GenBank, which contains sequences of all genes and UniGene, which contains clusters of the EST-sequences, BLAST that can be used in comparing the gene sequences, and OMIM, which contains the information of the function of the genes and their mutations. All are available at National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) (Table 1). There are also several microarray databases available, such as ArrayExpress of the European Bioinformatics Institute, Gene Expression Omnibus (GEO) of NCBI, Stanford Microarray database and the RNA abundance Database of the University of Pennsylvania Center for Bioinformatics (Table 1) (Stoeckert, Jr. et al., 2002; Argraves et al., 2003; Galperin, 2004)

2.2.4 Applications of DNA arrays in the research of atherosclerosis

2.2.4.1 Studies using cell culture models

Studies published so far regarding the gene expression profiles in atherogenesis have been mainly focused on cultured cells or animal models. The effect of several proatherogenic stimuli, such as oxLDL (Shiffman et al., 2000; Andersson et al., 2001; Virgili et al.,

2003; Sukhanov et al., 2003), VLDL and oxVLDL (Norata et al., 2003), homocysteine (Li et al., 2002a) and copper (Svensson et al., 2003) have been studied in cell culture models. Macrophages were differentiated to foam cells by oxLDL stimulation (Shiffman et al., 2000). There were changes in the ECM interactions and in the inflammatory response. As a response to internal lipid loads, there were also changes in the production of proteins involved in sterol synthesis, storage and efflux. Additionally, it was suggested that there is no enhancement in macrophage proliferation due to oxLDL exposure (Shiffman et al., 2000). Another study of macrophage oxLDL-stimulation drew similar conclusions regarding the proliferation of macrophages and the inflammatory response (Andersson et al., 2001). OxLDL treatment of endothelial cells lead to upregulation of transcription factors and adhesion molecules. There were changes in the inflammatory response and lipid metabolism, as well (Virgili et al., 2003). SMCs react to oxLDL stimulation quite similarly as other cell types studied (Sukhanov et al., 2003). Cell-cell interaction molecules, such as integrins, and transcription factors were induced. On the other hand, factors that function in lipid metabolism and in humoral response were attenuated after oxLDL stimulation (Sukhanov et al., 2003). Effect of VLDL and oxVLDL on endothelial cells was studied by Norata et al (2003). It was found that cell adhesion and inflammatory responses were induced by both stimuli.

Table 1. Some important molecular biology databases.

Database name	Description	URL
Nucleotide sequence databases		
GenBank	Collection of all publically available nucleotide and protein sequences	http://www.ncbi.nlm.nih.gov
EMBL Nucleotide Sequence Database	Collection of all publically available nucleotide and protein sequences	http://www.ebi.ac.uk/embl.html
TIGR Gene Indices	Organism-specific databases of genes and ESTs	http://www.tigr.org/tdb/tgi.shtml
UniGene	Clusters of ESTs and mRNA sequences	http://www.ncbi.nlm.nih.gov/UniGene
Protein sequence databases		
NCBI Protein database	All protein sequences	http://www.ncbi.nlm.nih.gov/entrez
SwissProt	Protein sequence database	http://www.expasy.org/sprot
TrEMBL	Translations of EMBL nucleotide database	http://www.expasy.org/sprot
Signalling pathways		
ENZYME	Enzyme nomenclature and properties	http://www.expasy.org/enzyme
KEGG	Metabolic and regulatory pathways	http://www.genome.ad.jp/kegg
GeneNet	Database on gene network components	http://wwwmgs.bionet.nsc.ru/mgs/gnw/genenet
Human genome maps		
Ensembl	Annotated information on eukaryotic genomes	http://www.ensembl.org
GeneCards	Human genes, maps, proteins and diseases	http://bioinfo.weizmann.ac.il/cards/
GeneLoc	Gene location database	http://genecards.weizmann.ac.il/geneloc/
Human genes and diseases		
OMIM	Catalog of human genetic and genomic disorders	http://www.ncbi.nlm.nih.gov/Omim/
HGMD	Human mutation database	http://www.hgmd.org/
Microarray and gene expression databases		
ArrayExpress	Collection of microarray data	http://www.ebi.ac.uk/arrayexpress
HugeIndex	Expression levels of human genes in normal tissues	http://hugeindex.org
Stanford Microarray database	Microarray data	http://genome-www.stanford.edu/microarray
Proteomic databases		
SWISS-2DPAGE	Annotated 2D gel electrophoresis database	http://www.expasy.org/ch2d

However, native and oxidized forms of VLDL activated different intracellular pathways (ERK 1/2, and p38 MAPK pathways, respectively). Native VLDL was shown to be pro-proliferative, whereas oxVLDL was cytotoxic to the cells (Norata et al., 2003).

The molecular link between hyperhomocysteinemia and atherosclerosis has been unclear. Endothelial cells were treated with homocysteine and it was found that HMG-CoA reductase was induced (Li et al., 2002a). It was suggested that via HMG-CoA reductase homocysteine causes accumulation of cholesterol into endothelial cells. This provides a rationale for statin therapy in hyperhomocysteinemic patients without hyperlipidemia. Also, the link between copper and atherosclerosis has been unclear. Svensson et al (2003) have shown that copper induces in macrophages the expression of several cholesterol-metabolism-related genes, including HMG-CoA reductase and LDL receptors (Svensson et al., 2003).

To mimic the inflammatory process in atherogenesis, different cell lines have been stimulated with inflammatory cytokines (Haley et al., 2000) or lipopolysaccharide (Mikita et al., 2001). Haley et al (2000) treated aortic SMCs with TNF- α and found increased expression of the chemokine eotaxin. The expression of its receptor, CCR3, was localized primarily in macrophage-rich areas of lesions. These findings suggest that TNF- α produced by activated SMCs may recruit and activate macrophages through the CCR3 receptor (Haley et al., 2000). The effect of oxLDL-treatment in the alteration of inflammatory response stimulated by lipopolysaccharide in macrophages was

studied by Mikita et al (2001). Their results indicate that oxLDL significantly alters the inflammatory response, in a process mediated by NF- κ B signaling and activation of nuclear receptors retinoid X receptor and PPAR γ . Also, there were changes in cytokine and chemokine expression, which may have effects on the recruitment and behaviour of monocytes and macrophages in atherosclerotic lesions (Mikita et al., 2001).

In addition to chemical atherogenic stimuli, the effects of mechanical stress to endothelial cells have been studied (McCormick et al., 2001; Ohura et al., 2003). The results from these studies suggest that shear stress causes alterations mainly in ECM modulation, oxidative stress and in the inflammatory properties. Laminar shear stress is protective, whereas turbulent shear stress is proatherogenic for endothelial cells (Ohura et al., 2003). Also, the differences between the different phenotypes of SMCs, quiescent and proliferative, have been studied (Blindt et al., 2002). Gene expression differences were found especially in genes functioning in ECM modulation, such as in TIMPs and MMPs, in cell adhesion molecules, and in proteins functioning in cell motility (Blindt et al., 2002). Inhibition of SMC proliferation by butyrate was examined by Ranganna et al (2003). It was found that butyrate treatment affects cell growth, differentiation, and stress response (Ranganna et al., 2003). This study shows that DNA array method can be applied to the clarification of the molecular mechanism of drug treatments. In conclusion, the above mentioned studies have given valuable information on the effects of different

atherogenic stimuli on different cell types present in atherosclerotic lesions. It has been found that the atherogenic effects are mainly mediated by the alterations in ECM modulation, inflammation, intercellular communication, and lipid metabolism.

2.2.4.2 Studies in animal models

An initial array study in animals compared differences between the phenotypes of macaque aorta and vena cava media (Adams et al., 2000). It was found that regulator of G-protein signalling (RGS5) was highly upregulated in aortic tissue. It was suggested that RGS5 might be involved in the adaptation of arteries to pressure changes (Adams et al., 2000). Since then, various animal models have been used in gene expression analysis of atherosclerotic events. Gene expression during atherogenesis was studied using ApoE deficient mice with western diet, as compared to normal mice fed with normal chow (Wuttge et al., 2001). Several well-known proatherogenic factors, such as ICAM-1, VCAM-1, CD-antigens 14, 18, and 3, and PDGF were upregulated in ApoE-mice. Several new candidate genes for atherosclerosis, such as nerve growth factor and hepatocyte growth factor were upregulated as well (Wuttge et al., 2001). The effects of known atherosclerotic stimuli, such as maternal hypercholesterolemia (Napoli et al., 2002), and hyperhomocysteinemia (HHcy) (Ungvari et al., 2003) have been also studied. In a study of maternal hypercholesterolemia, LDL-R knockout mice were fed with high-cholesterol diet and the lesions of offsprings at age of 3 months were analyzed using Affymetrix chips (Napoli et al., 2002). Surprisingly, it was found that instead of macrophage-

related genes, genes related to matrix components and intracellular proteins showed increased expression levels. Signal transduction-specific array was used to study the effects of HHcy in rats, and the induction of TNF- α and iNOS was found (Ungvari et al., 2003). This data confirms that the influence of homocysteine on atherogenesis is mediated via oxidative stress.

The clinical manifestations of atherosclerosis, such as aneurysms (Nakahashi et al., 2002), have also been studied in animals. It was found that genes functioning in oxidative stress, such as hemeoxygenase, and genes functioning in matrix remodelling, such as MMP-2 and MMP-9, were upregulated in experimental rat aneurysm (Nakahashi et al., 2002). Neointima formation, which hinders angioplasty and the use of vein grafts, has been studied in primates after aortic and vein grafting (Geary et al., 2002). There were marked changes only in matrix proteins and proteins functioning in matrix synthesis in neointima. The inhibitory effects of ribozyme targeting PDGF-A chain mRNA on neointima formation in rat carotid artery after angioplasty has been also studied (Kotani et al., 2003). Since there were suppression in intracellular signalling systems, kinases and cell cycle-related peptides, the ribozyme treatment was considered specific and efficient (Kotani et al., 2003).

2.2.4.3 Studies using human samples

In addition to animal and cell culture models of atherosclerosis, a few studies using human samples have also been published. The expression patterns of fibrous cap versus adjacent media in

samples from endarterectomy (n=13) were compared, and strong induction of early growth response gene, Egr-1, and Egr-1-inducible genes, such as SOD, ICAM-1, PDGF, and TNF, was detected (McCaffrey et al., 2000). It was also observed in LDL-R knockout mice that Egr-1 expression increases progressively during atherogenesis. Plaque rupture leads to serious clinical problems, such as vascular occlusion. The gene expression changes predisposing to plaque rupture were studied comparing whole mount stable and ruptured lesions from different individuals (n=3), and strong induction of a perilipin was found (Faber et al., 2001). Perilipin expression was localized in foam cells and in cells surrounding cholesterol crystals, and it was suggested that perilipin expression may indicate reduced lipolysis in plaques. Apoptosis in plaque can predispose the plaque to rupture. Two studies utilizing endarterectomy samples and apoptosis-specific arrays have been published. Martinet et al (2002) used mammary artery as a control, and found elevated expression levels in DAP kinase, caspase-1, and apoptosis regulator BAX. The expression of DAP kinase was localized in foam cells of SMC origin (Martinet et al., 2002). In another study, proximal normal zone and zone immediately adjacent to stenotic lesion were compared (n=5), and the induction of TNF-pathway member TRAIL-receptor, and the downregulation of TNF-R1, TNF-R2 and IGFBP-3 was detected (Woodside et al., 2003).

Composition of the atherosclerotic plaque is a crucial factor in determining other clinical events, such as the stability of angina and the susceptibility to aneurysms. In a study using coronary atherosclerotic plaques, it was found that

lymphocyte adhesion molecule MadCAM and tissue factor expression were highly induced in samples with unstable angina (Randi et al., 2003). Abdominal aorta aneurysms (AAA) are associated with atherosclerosis, and the underlying pathobiologies share similarities. AAA tissue was compared to normal aorta (n=4), and expression changes were found in genes affecting inflammation, such as in IL-8, in extracellular matrix degradation (MMP-9), and in SMC depletion (downregulation of myosin light chain kinase) (Tung et al., 2001). In another study, AAAs were compared to samples with arterial occlusive disease and normal arteries (n=7, 5, 5, respectively) using cell interaction molecule-specific arrays, and induction of MMP-9, ICAM, and TNF- β -receptor was detected (Armstrong et al., 2002).

Neointima formation is a severe problem complicating the use of angioplasty in the treatment of atherosclerosis. Upregulation of FK506-binding protein 12, which is a target for restenosis-inhibitory drug Rapamycin, as well as the upregulation of known neointima formation-related genes, such as COX-2 and thrombospondin-1, was detected when human stent-induced neointima samples (n=10) were compared to normal arteries (n=12) (Zohlhofer et al., 2001).

All of these studies have some limitations, such as the small number of samples analyzed, problems in choosing an optimal control sample (variations between different individuals or different origins of arterial samples), limited number of genes in the arrays (usually less than 1000 genes, meaning less than 5 % of genes in genome), and especially, the inadequate confirmation of the results with independent methods.

2.2.5 Protein arrays

2.2.5.1 Protein array formats and the analytical method

As the DNA microarray technology has become a valuable tool in large-scale RNA expression analysis, similar large-scale miniaturized protein arrays have also been developed in the past few years. Different array formats, such as peptide arrays (Houseman et al., 2002), antigen-antibody arrays (Lueking et al., 1999; Joos et al., 2000; Haab et al., 2001), and protein arrays (Arenkov et al., 2000; MacBeath and Schreiber, 2000; Zhu et al., 2000) have been applied to the studies of antibody-antigen and protein-protein, as well as enzyme-substrate, protein-drug, and receptor-ligand interactions (Zhu and Snyder, 2003).

Usually nitrocellulose, poly-L-lysine, or avidin-coated microscopic slides have been used as surface material (Zhu and Snyder, 2003; Walter et al., 2002). Proteins are attached to the surface material by absorption, covalent cross-linking, or affinity attachment (Zhu and Snyder, 2003). Fluorescence, radioactive labelling or ELISA are the most commonly used detection methods (Zhu et al., 2000; Houseman et al., 2002; Walter et al., 2002; Zhu and Snyder, 2003). The principles of data analysis and bioinformatics are similar to that of DNA arrays as discussed in chapter 2.2.3.

The amount of proteins arrayed varies from hundreds of proteins on commercial microarrays to several thousands on large membrane-based arrays (de Wildt et al., 2000). Sensitivity of protein detection using antibody array has been reported to be adequate for clinical research using patient serum

samples, which means the ng/ml- µg/ml range (Haab et al., 2001; Schweitzer and Kingsmore, 2002). However, the vast range of protein concentrations in cells (by up to a factor of 10^{10}) poses many challenges to protein array technology (Liotta et al., 2003). The most important limitations of the use of the protein arrays are the problems in specificity, especially when only one antibody is used for the detection of each antigen. Additionally, there are difficulties in the production of proteins in large scale, in the selection of specific antigen/antibody pairs, and in the manufacturing of the arrays so that the functionality of the proteins is preserved (Zhu and Snyder, 2003; Schweitzer and Kingsmore, 2002). The limitations of specificity can be overcome by the use of sandwich assays (Zhu and Snyder, 2003), and several different large-scale protein expression systems are under development (Walter et al., 2002). In addition to technical problems with arrays itself, the heterogeneous cell content of tissue samples limits the analysis of protein expression.

2.2.5.2 Applications of protein arrays

Protein arrays can be used both in analytical and functional purposes. Analytical microarrays, for example antibody-antigen microarrays, can be used in diagnostics, such as an allergy test (Hiller et al., 2002; Wiltshire et al., 2000), or in autoimmune diagnostics (Joos et al., 2000; Robinson et al., 2002). They can also be used in the profiling of protein expression in different diseases, such as in cancer (Sreekumar et al., 2001). Functional purposes of protein arrays include the use of protein arrays in the research of protein-protein,

protein-ligand, and kinase-substrate interactions (MacBeath and Schreiber, 2000), as well as in the studies of posttranslational modifications (Liotta et al., 2003).

2.2.6 Genomic vs. proteomic studies

One of the major limitations in interpreting the data from genomic and proteomic analyses is the non-linear correlation between mRNA and protein expression. Not all genes are transcribed. There might be several transcript variants to those genes transcribed. Furthermore, all the transcripts are not translated to active proteins. In addition, proteins are modified posttranslationally in many ways. For example, proteins can be phosphorylated or glycosylated. There are also different subcellular locations for proteins, different turnover rates for proteins and mRNA transcripts, and functional interactions within different proteins. Also, the regulation of gene expression can function only at the mRNA level or at both mRNA and protein level, or solely at the protein level.

There is only a limited number of studies in which genomic and proteomic analyses have been compared. The correlation between the mRNA and protein levels has been found to be poor, as the correlation coefficients have been between 0.356 and 0.58, depending the methodology used. Predominantly, SAGE, DD, and DNA array have been used for mRNA quantification and 2D-electrophoresis for protein quantification (Anderson and Seilhamer, 1997; Anderson and Anderson, 1998; Gygi et al., 1999; Lian et al., 2001; Lian et al., 2002; Fessler et al., 2002). These discrepancies between the mRNA and

protein abundancies have been suggested to be derived from various posttranscriptional and posttranslational processes, and also at least in part, from technical limitations of the current methods (Lian et al., 2002). Because of this, it is important to confirm the results from RNA expression analyses also at the protein level before any conclusions are made. So far, studies comparing the results from DNA and protein arrays have not been reported.

2.3 LASER MICRODISSECTION

2.3.1 The principle of laser microdissection

A major problem with the analysis of gene and protein expression in tissue samples is the heterogeneity of the tissue. To overcome this problem, several methods for tissue microdissection have been reported, such as the irradiation of manually ink-stained sections to destroy unwanted material (Shibata et al., 1992), or manual microdissection (Emmert-Buck et al., 1994). However, these methods are time-consuming and labor-intensive. Emmert-Buck et al (1996) developed a new method, laser capture microdissection, which enables fast and precise dissection of single cells from tissue sections (Emmert-Buck et al., 1996). In this method, cells from tissue sections are catapulted to thin transparent film with the aid of focused pulse from an infrared laser (Emmert-Buck et al., 1996). The disadvantage of this method is a direct contact of film with the tissue sample, which predisposes the analysis for contamination. A few years later, Kolble (2000) reported the next generation laser

microdissection system, in which the tissue section is placed on a specific plastic foil, and microdissection is performed without direct contact to cells (Figure 4) (Kolble, 2000).

Laser microdissection methods can be applied to mRNA expression, mutation, and protein expression studies (Emmert-Buck et al., 1996; Fend and Raffeld, 2000). Generally, cryosections are used in mRNA and protein expression studies,

and paraffin-embedded sections in DNA analysis. Tissues can be fixed with conventional fixatives, such as acetone, ethanol or formaldehyde, the first two ones giving better results. Besides hematoxylin-eosin staining, fast immunostaining protocols can be used, which helps the dissection of specific cell populations (Fend et al., 1999; Fend and Raffeld, 2000; Fend et al., 2000).

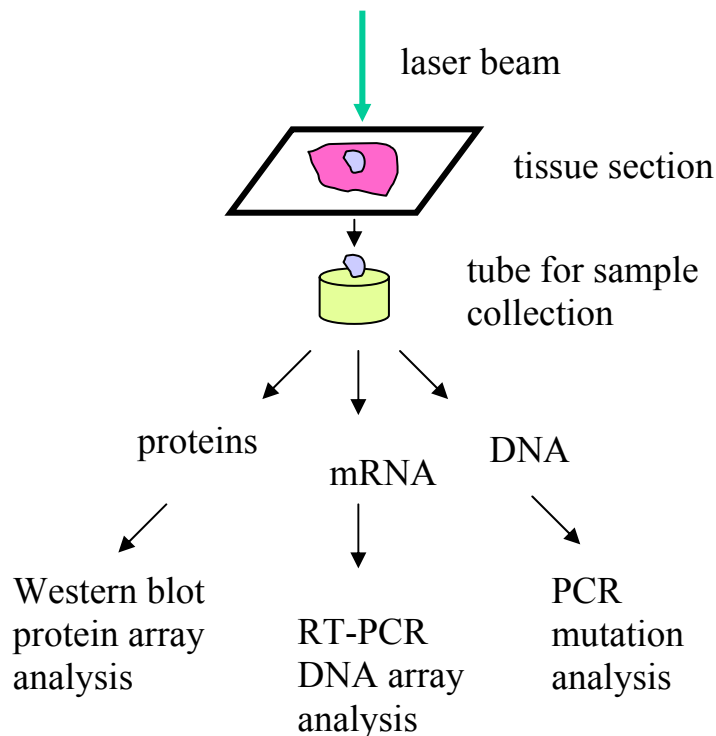


Figure 4. The principle and applications of laser microdissection technology. Using a laser beam, small cell populations can be dissected from tissue sections. Dissected cells can be then used for conventional mRNA, DNA and protein analyses (Tuomisto and Yla-Herttuala, 2003).

2.3.2 Applications of laser microdissection

Large-scale gene expression analysis methods, DNA arrays (Luo et al., 1999; Ohyama et al., 2000), and differential display (Okulicz et al., 2003) have been successfully combined with laser microdissection. However, DNA array analyses of microdissected samples have been mainly focused on cancer research (Leethanakul et al., 2000a; Luzzi et al., 2001; Hasegawa et al., 2002; Kemmner et al., 2003; Ma et al., 2003). The help of laser microdissection has also been used in the generation of cDNA libraries (Leethanakul et al., 2000b). Moreover, several different techniques of mutation research have been utilized in the studies of microdissected samples (Dietmaier et al., 1999; Dillon et al., 2001; Aldridge et al., 2003; Oesterreich et al., 2001; DiFrancesco et al., 2000). Additionally, the combinations of laser microdissection with variable protein analysis methods, such as with MALDI MS (Xu et al., 2002), with 2D gel electrophoresis (Banks et al., 1999; Emmert-Buck et al., 2000), and recently, with protein array (Batorfi et al., 2003) have been reported.

2.3.3 Applications of laser microdissection in the research of atherosclerosis

The analysis of single cell types in atherosclerotic lesions is hampered by the cellular heterogeneity of the arterial tissue. Arterial tissue contains macrophages, lymphocytes, SMCs, and

endothelial cells, as well as adventitial fibroblasts. Trogan et al (2002) stimulated apoE deficient mice with lipopolysaccharide and dissected macrophage foam cells from the lesions. In subsequent DNA array analysis, they found the upregulation of VCAM-1, ICAM-1 and MCP-1 (Trojan et al., 2002). Martinet et al (2003) dissected caspase-2 positive macrophages from human lesions and detected anti-apoptotic isoform of caspase-2 by Western blot (Martinet et al., 2003). With the help of microdissection Engelse et al (2002) confirmed the *in vivo* effects of activin-A adenovirus in the inhibition of neointima formation in murine arteries (Engelse et al., 2002).

3 AIMS OF THE STUDY

The aim of this thesis was to study gene expression patterns in human atherosclerosis and ischemic muscle using DNA array methods. This goal was divided into specific aims as follows:

1. Profile gene expression patterns in human atherosclerotic lesions (I and III).
2. Explore gene expression patterns in macrophage-rich inflammatory cell infiltrates in human atherosclerotic lesions with the aid of laser microdissection (II).
3. Analyze gene expression patterns *in vitro* during monocyte-macrophage differentiation and oxLDL-stimulation (II and III).
4. Analyze protein expression in oxLDL-stimulated macrophages (III).
5. Compare the results from DNA array and protein array analyses (III).
6. Profile gene expression in ischemic human skeletal muscle (IV).

4 MATERIALS AND METHODS

4.1 HUMAN TISSUE SAMPLES

4.1.1 Human atherosclerotic lesions (I and II)

Fresh arterial samples (study I: n=12, study II: n=13) from 10 individuals were obtained from organ donors, amputation operations, and fast autopsies. Some patients received antihypertensive treatment but none were on statin therapy. Macroscopically normal and

atherosclerotic areas were dissected and samples were divided into two parts. One part was immersion-fixed in 4 % paraformaldehyde / 15 % sucrose (pH 7.4) and embedded in paraffin (Yla-Herttuala et al., 1991). The other part was frozen in liquid nitrogen and stored for RNA analysis, or it was frozen in isopentane and embedded in OCT compound for LMD analysis (II). According to histology, the samples were classified to normal, fatty streaks (AHA classes I and II), and atherosclerotic lesions (AHA classes IV-VI) (Stary et al., 1995). Studies of human tissues were approved by the Ethical Committee of Kuopio University Hospital.

4.1.2 Human skeletal muscle samples (IV)

Ischemic human skeletal muscle samples were collected from lower limb amputation operations. The donors (n=8) suffered either from chronic critical limb ischemia (CLI) caused by peripheral arterial disease (PAD) with rest-pain and/or tissue loss lasting > 2 weeks, or acute-on-chronic CLI of 24-48h in duration due to an embolism or a major thrombus of native artery or prosthesis. Acute-on-chronic CLI means the worsening of chronic limb ischemia into CLI in less than 14 days. Upon amputation, all patients had ankle-brachial index (ABI) of 0-0.3 indicating critical ischemia. In the acute-on-chronic CLI cases, the underlying chronic ischemia was milder than in the chronic CLI cases on the basis of the walking distance and ABI measurements 4 weeks before amputation (Table I, in IV). Samples were collected from two regions of each amputee. One set of

samples, serving as controls, was collected from the amputation stump. The other set of samples was chosen from a region that was ischemic but still viable on the basis of macroscopic and microscopic investigation (nuclei were still present in myocytes). Samples were not collected from or near infected parts of chronically ischemic limbs. Samples for immunohistochemistry and RNA expression analyses were prepared similarly as arterial samples (see 4.1.1). Studies of human tissues were approved by the Ethical Committee of Kuopio University Hospital.

4.2 CELL CULTURE STUDIES (II AND III)

Human monocytic THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 medium according to ATCC's instructions. Unstimulated cells were collected for control, and rest of the cells were stimulated by 0.1 μ M PMA (phorbol 13-myristate 12-acetate) (Sigma) to induce differentiation into macrophages (Tsuchiya et al., 1982), and collected at 3h, 12h, 24h, and 72h (II, III). To study the effects of oxLDL stimulation, THP-1 cells were activated with PMA for 24 h, and further incubated with 100 μ g/ml of copper-oxidized LDL (Yla-Herttuala et al., 1989) for 72 h to induce foam cell formation (Laukkanen et al., 2000) (III). PMA-stimulated THP-1 cells, which were collected after 24-hour PMA-stimulation, were used as a control. In each analysis, two independent cell culture experiments were performed.

4.3 LASER MICRODISSECTION (II)

For LMD, 7 μ m thick OCT sections were used. Sections were placed on Poly-L-Lysine (Sigma) coated framed membrane slides (Leica Nilomark Oy). Sections were fixed in 70% ethanol and counterstained with haematoxylin/eosin and dehydrated in graded ethanol and xylene. Macrophage-rich inflammatory cell infiltrates from lesion shoulder areas were dissected using Leica DM LMD system (Leica Nilomark Oy). Macroscopically normal DIT was used as a control.

4.4 GENE EXPRESSION PROFILING (I-IV)

4.4.1 DNA array analyses (I-IV)

For gene expression profiling with nylon DNA arrays (I and IV), mRNA was isolated using Micro-Fast Track 2.0 kit (Invitrogen). For microarray analysis (II and III), total RNA was isolated from cultured cells using Trizol reagent (Gibco BRL) and from LMD samples using Strataprep total RNA microprep kit (Stratagene). RNA was quantified spectrophotometrically (A_{260}/A_{280}) (tissue samples and cell extracts) or with the RiboGreen RNA quantification kit (Molecular Probes) (LMD samples). The quality of RNA was checked with agarose gel electrophoresis. RNA from LMD samples was amplified using T7-based amplification using three cycles (Luo et al., 1999).

The Gene Discovery Array 1.3 nylon filters (Incyte Genomics) containing 18376 double-spotted human cDNA clones from the I.M.A.G.E collection were used to profile gene expression

patterns in homogenized arterial samples (I). LifeGrid 1.1 filter (Incyte Genomics) containing 8400 double-spotted human cDNA clones from the Incyte Genomics' library were used to profile gene expression in skeletal muscle samples (IV). Sanger centre Hver1.2.1 microarrays containing approximately 10000 clones from the I.M.A.G.E collection representing approximately 6000 different genes were used in studies II and III. Probes were labelled with ^{33}P -dCTP (I and IV) or Cy3-dCTP and Cy5-dCTP (II and III) during cDNA synthesis. Hybridization (O/N at 42°C for nylon arrays and at 47°C for microarrays) and washing (SSC+SDS) were performed according to manufacturers' instructions. All analyses with microarrays were repeated three times, including the use of reversed fluorophores (II and III).

Signal detection was performed with phosphoimager (Storm 860, Molecular Dynamics) (I and IV) or ScanArray 5000 (GSI Lumonics) (II and III). Quantification of the signals was performed with ArrayVision software (Imaging Research) (I and IV) or with QuantArray software (GSI Lumonics) (II and III), and the background was subtracted from the signal intensities. Images were also visually inspected to ensure that the signals were not due to artefacts or high background. In study I, signals were normalized and intensity scores were calculated using the following formula:

$$score = \frac{\text{int.GDA}1 + n}{m \times \text{int.GDA}2 + n}$$

where the int. GDA 1 and int. GDA 2 are intensities on filters 1 and 2, m is the average of all intensities on filter 1

divided by the average of intensities on filter 2 and n is $0.2 \times m$. The rationale for using the formula was to avoid false results caused by very low signal intensities which with the current formulation only produce values ≈ 1 . Gene expression was considered significantly changed, if the score was ≥ 1.5 . In studies II-IV, data analysis was performed with GeneSpring software (Silicon Genetics). The linearity of the signal intensities was checked using GeneSpring software using MA plot, where M represents the log ratio of the test and control samples and the A is the total log intensity of each spot. Because the data was linear (i.e plot displayed 45° rotation), the data was normalized to the median of the signals according to the protocol of the Finnish Center for Scientific Computing (Laine and Tuimala, 2003). Signals were normalized using GeneSpring software by dividing each gene by the median of its measurements in all samples and intensity ratios were calculated. Gene expression was considered significantly up- or downregulated when the intensity ratio test/control was ≥ 2.0 or ≤ 0.5 , respectively. Statistical significances of the differences were calculated according to Claverie (Claverie, 1999) (I-IV). To reach the 5 % significance level $|\text{Int}_{\text{test}} - \text{Int}_{\text{control}}|$ must be $\geq 2.8\sigma_{\text{control}}$ or $\geq 4.3\sigma_{\text{control}}$, when analyzed in triplicates or duplicates, respectively, where Int_{test} and $\text{Int}_{\text{control}}$ are the averages of normalized signal intensities in test and control samples in repeated experiments and σ is the distribution of signal intensities in the control sample. Cluster analysis (III) was performed using GeneSpring by generating SOMs (Tamayo et al., 1999) using default options of 6 rows, 5 columns and 90 000 iterations.

4.4.2 RT-PCR (I-IV)

After DNase I treatment, cDNA synthesis was performed using 3 µg of total RNA, random hexamers (Promega), and M-MuLV reverse transcriptase (MBI Fermentas). RT-PCRs were then performed with specific primers for JAK-1, VEGFR-2, PECAM, unknown gene and β-actin (control) (I), for HMG-CoA reductase, IRF-5, VEGF, NOS, EC-SOD, and β-actin (control) (II), for CD36, COX-2, Cdk-1, TFII-I, NEMO-like kinase, Elf-5, TRADD, and GAPDH (control) (III), VEGF, IGF-1, IGF-2, and GAPDH (control) (IV). Controls without reverse transcriptase were included in every analysis (Vajanto et al., 2002).

4.4.3 *In situ* hybridization (I)

In situ hybridization was performed using ³³P-UTP labeled riboprobes as described (Yla-Herttuala et al., 1990). The following probes were used: JAK-1 (nucleotides 2181-2749; GenBank NM002227); VEGF receptor-2 (nucleotides 1756-2262; GenBank AF063658); the unknown gene (nucleotides 95-644; GenBank AL049448). For each *in situ* hybridization, corresponding sense probes were used as controls (Yla-Herttuala et al., 1990).

4.5 PROTEIN EXPRESSION PROFILING (I-IV)

4.5.1 Immunohistochemistry (I, II, IV)

Paraffin sections were used for immunostainings. Immunohistochemistry was performed with the

avidin-biotin-horseradish peroxidase system (Vector Laboratories) using the 3'-5'-diaminobenzidine (DAB, Zymed). The antibodies used are presented in table 2. Controls included incubations where primary antibodies were omitted. The tissue sections were also incubated with class- and species-matched irrelevant immunoglobulins (Yla-Herttuala et al., 1990). Photographs of histological sections were taken using Olympus AX70 microscope (Olympus Optical) with analySIS imaging software (Soft Imaging System).

4.5.2 Protein array analysis (III)

Macrophages collected before and after oxLDL stimulation were analyzed by Ab Microarrays™ (Clontech) according to manufacturers' instructions (www.clontech.com). Briefly, proteins were extracted, labelled covalently with Cy3 and Cy5 and hybridized to arrays containing antibodies for 384 proteins. Analysis was repeated with reversed fluorophores. Images were analyzed and quantified as for cDNA arrays. Data analysis was performed as described in the manufacturer's protocol. Ratios were considered significant if they were >1.5 or <0.66.

Table 2. Antibodies used in immunohistochemistry.

Antibody	Target	Code/ Clone	IgG concentration ($\mu\text{g/ml}$)	Producer	Article
CD31/ PECAM1	Endothelium	JC70A	3.5-7.0	DAKO	I, II, IV
CD68	Macrophages	KP1	1.9-3.9	DAKO	I, II, IV
HHF-35	SMC	HHF-35	3.0	DAKO, Enzo Diagnostics	I, II
CD3	T-cells	PS1	2.0	DAKO	I, II
VEGFR- 2	VEGFR-2	VEGFR2scX/ sc6251	2.0, 0.4	RDI; Santa Cruz Biotechnology	I, IV
HIF-1 α	HIF-1 α	Ab-4, H1alpha67	2.0	Neomarkers	IV
VEGF	VEGF	sc-7269	0.4	Santa Cruz Biotechnology	IV
IGF-1	IGF-1	I8773	5.0	Sigma	IV

All antibodies except IGF-1 were monoclonal antibodies. IGF-1 antibody was polyclonal.

5 RESULTS

5.1 GENE EXPRESSION IN HUMAN ATHEROSCLEROTIC LESIONS (I AND II)

We analyzed gene expression in human normal arteries and atherosclerotic lesions with an array containing 18376 cDNA fragments (I). To validate the array analysis we used a group of 17 genes that have been previously linked to atherogenesis. These genes contained five genes that have a role in SMC proliferation and formation of connective tissue (γ -actin, 2.8-fold increase; cathepsin S, 1.7-fold; TIMP, 1.5-fold; fibronectin, 2.5-fold; and alpha-2-macroglobulin, 2.2-fold) and genes involved in lipid metabolism (cytochrome p450, 2.5-fold; CD68, 2.6-

fold; Fc-receptor, 1.8-fold; apolipoprotein E, 2.5-fold; and fibronectin, 2.5-fold). Eight genes previously connected to atherosclerosis showed decreased expression levels, for example heparin sulphate proteoglycan (syndecan 2) (2.6-fold decrease) and phospholipase D (2.0-fold). In addition to genes previously connected to atherosclerosis, we identified 75 genes that changed expression levels. Forty-four of these genes were upregulated and 31 genes downregulated. These genes contained also many unknown EST-sequences. For further analyses, we selected four genes, which belong to the functional group of cell signalling and communication and are known to play important roles in tissue pathology. The genes also represented a wide range of score values (1.6-95.5-fold). The selected genes were JAK-1, VEGF receptor-2, PECAM-1 and an unknown gene. The expression of them was

confirmed with RT-PCR and *in situ* hybridization and/or immunohistochemistry. According to *in situ* hybridization and/or immunohistochemistry, JAK-1 expression was localized in intimal macrophages; VEGFR2 in endothelium and SMCs, PECAM-1 in endothelium and the unknown gene in endothelium and macrophages. The selected unknown gene was chosen since it was highly upregulated in lesions (95.5-fold increase), and it had no significant homology to any known gene. In more detailed studies, it became evident that it has a strong macrophage-specific expression. The cloning and the characterization of this gene is underway in our laboratory.

The macrophage-rich shoulder area from human atherosclerotic lesions and diffuse intimal thickening (DIT) for control were dissected using a laser microdissection-device (II). DNA array analysis was performed after T7 polymerase-based amplification of RNA using microarrays that contained about 6000 genes. According to histological analyses, the shoulder area consisted mainly of macrophages, whereas the amounts of T-cells, macrophage-derived foam cells and SMCs, were about 1-2 %, 5%, and 10%, respectively. DIT samples consisted almost exclusively of SMCs. To validate the array analysis, we studied further genes typical for macrophages. Array analysis detected 33 genes that had well-known macrophage-functions, such as CSF receptors, and interleukin receptors and integrins. The expression of endothelial cell or T-cell specific genes was not significantly changed. We confirmed the results from the array analysis of dissected *in vivo* macrophages with an array analysis of *in vitro* macrophages. Monocyte-

macrophage differentiation, as induced by PMA-stimulation in THP-1 cells, were used as a cell model. When comparing the results from *in vitro* and *in vivo* macrophages, 62 genes were found that had changed expression levels in both analyses. These genes included several genes that have a role in signal transduction, for example, protein kinases and adenylate cyclases. Also, several KIAA proteins that have unknown functions had changed expression levels. The upregulation of HMG-CoA reductase, interferon binding protein 5 (IRF-5), eNOS and ecSOD, and the downregulation of VEGF, was confirmed by RT-PCR in PMA-stimulated THP-1 cells as compared to control THP-1 cells.

The expression changes of these selected genes were also studied after oxLDL stimulation of THP-1-derived macrophages by RT-PCR. The expression of HMG-CoA reductase and IRF-5 was downregulated, and the expression of eNOS, EC-SOD, and VEGF remained unchanged, when oxLDL-stimulated macrophages were compared to control macrophages (PMA-stimulated THP-1 cells). However, quantitative real-time RT-PCR is needed before firm conclusions can be made from the expression patterns of these genes.

5.2 GENE EXPRESSION CHANGES DURING MONOCYTE-MACROPHAGE-DIFFERENTIATION (III)

PMA-stimulation of THP-1 cells was used as a model of monocyte-macrophage differentiation that takes place during atherogenesis (III). Cells

were collected at 3 h, 12 h, 24 h, and 72 h timepoints. At 72 h, 213 genes were upregulated and 332 genes were downregulated, which means changes in approximately 10 % of genes present on the arrays. At 72 h, many macrophage-related genes were strongly upregulated. These genes included interleukin-1 beta (37.8-fold increase), macrophage inflammatory protein 3 (37.4-fold), MMP-9 (35.8-fold), interleukin-2 receptor (10.4-fold), and monocyte differentiation antigen CD14 (9.9-fold). Additionally, many genes that were upregulated to a lesser extent were connected to inflammation. These included macrophage inflammatory protein 4 (4.8-fold), M-CSF receptor (3.2-fold) and osteopontin (3.2-fold). It is worth noting that several cathepsins were upregulated, including cathepsins G, L, and H (9.2, 3.6 and 3.3-fold, respectively).

Genes were divided into clusters according to their expression patterns during monocyte-macrophage differentiation. Cluster analysis was performed using SOMs. Four well-defined clusters were found (Figure 5). Cluster 1 contained genes whose expression increased steadily. Among these genes were inflammatory genes such as BCL-6 (example gene in figure 5), integrin beta-5, CSF receptors, and many genes functioning in cell adhesion, signal transduction, cell proliferation and oxidative stress. In cluster 2, there were several early-response genes including somatostatin transcription factor 1 (STF 1), transcription factor JUN-B, EGF-response factor ERF-1, and translation initiation factor EIF-5. Cluster 2 contained also many mediators of intracellular signalling, such as GTP-binding proteins, protein kinases and phosphatases, nuclear hormone receptors

NOR-1 (example gene in figure 5), and NURR1. Later in differentiation (cluster 3), several genes functioning in cell adhesion, such as cathepsin B and cytohesin; genes functioning in cell movement, such as coronin-like protein p57 and actin-related proteins, and genes functioning in inflammation, such as interleukin-7 receptor (example gene in figure 5), galectin and interferon regulatory factor 7 were upregulated. Many genes that have a role in lipid metabolism had increased expression at 12, 24, and 72 h, such as fatty acid binding proteins (clusters 2 and 3), apolipoproteins A and E, scavenger receptor B, 1-AGP acyltransferase, oxysterol-binding proteins, and bile acid receptor (cluster 1). Cluster 4 included genes that were attenuated during monocyte-macrophage-differentiation. These genes included several genes functioning in initiation of translation and transcription, such as translation initiation factors 4E and ELF-2B, and c-myc binding protein (example gene in figure 5) and cell cycle progression proteins. To summarize these results, clear patterns of time-dependency of activation of different gene classes were found during monocyte-macrophage activation. In first phase, genes functioning in signal transduction, transcription and translation were activated. This was followed by activation of processes typical for macrophages (i.e. inflammation, proliferation and lipid metabolism). On the other hand, some transcription and translation factors and cell-cycle activators were downregulated during the differentiation process. Interestingly, many genes that have been characterized as neuronal genes, such as neuronal cadherin-2, neurexin 1-beta, neuronal-specific septin 3 as neuroendocrine

protein 7B2, were strongly upregulated during monocyte-macrophage differentiation. The expression changes in genes that has been known to be highly expressed in macrophages, such as CD36 and cyclooxygenase-2 (COX-2), and genes not previously connected to macrophages, such as transcription factors cyclin-dependent kinase 1 (Cdk1), general transcription factor TFII-I and Elf-5, and cell signalling-genes NEMO-like kinase and TRADD were confirmed also by RT-PCR.

5.3 PROTEIN EXPRESSION IN OXLDL-STIMULATED MACROPHAGES (III)

Protein expression patterns of oxLDL-stimulated macrophages were compared to control macrophages (PMA-stimulated monocytes) using antibody arrays that contained 384 proteins. Surprisingly, there were no large changes in the protein expression following the oxLDL stimulation. Most of the proteins presented in the antibody array were connected to cellular signalling and communication. Upregulated proteins included for example CD36, which is a scavenger receptor responsible for oxLDL uptake, inflammatory gene COX-2, and cell attachment-gene leukocyte antigen related protein phosphatase (LAR).

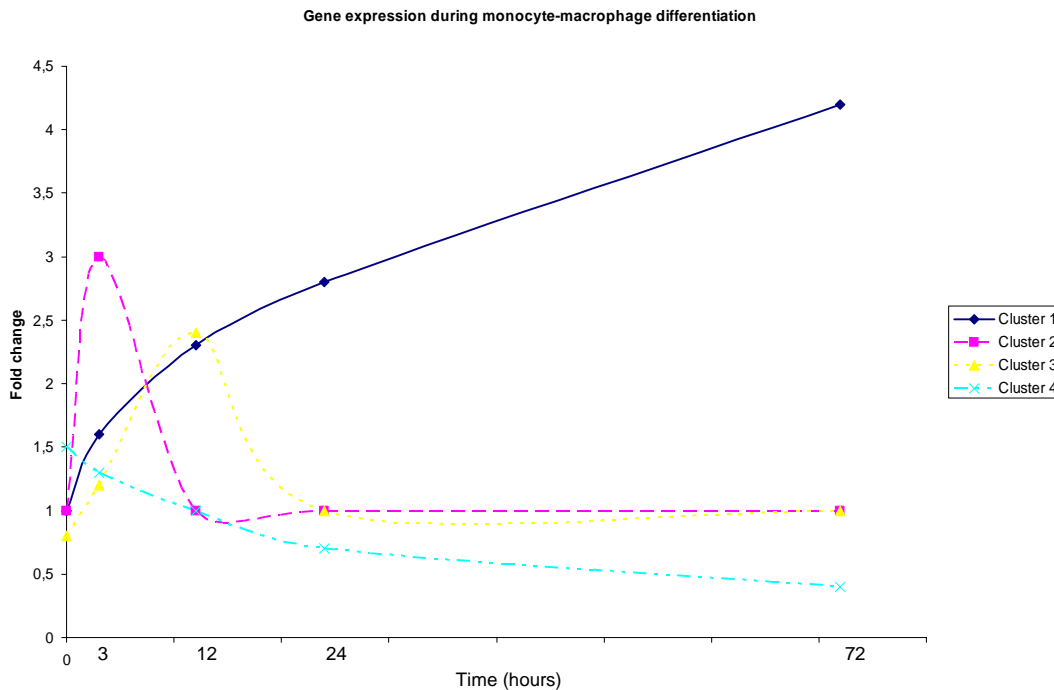


Figure 5. Gene expression changes during monocyte-macrophage-differentiation. Exemplar genes from each cluster are shown: BCL-6 from cluster 1 (continuously increasing expression), NOR-1 from cluster 2 (early-responding genes), interleukin-7 receptor from cluster 3 (late-responding genes), and c-myc binding protein from cluster 4 (continuously decreasing expression).

These proteins have all previously been connected to macrophage function. We also found changes in proteins that have not been previously connected to macrophages and have a role in cell division and transcription. Such proteins included signal-induced proliferation-associated gene, cell division cycle 27, CLIP-115, transcription factor TFII-I, and proliferation antigen Ki-67 (1.63-fold increase). On the other hand, factors promoting cell death, for example caspase-1 (1.68-fold), caspase-6 (1.65-fold), caspase-8 (1.62-fold), and TRADD (1.59-fold) were upregulated, suggesting that the balance between death-preventing and death-promoting events in cholesterol-loaded macrophages is strictly regulated. Although there were significant changes at the mRNA level in some oxidative stress-related genes (heme oxygenase 1, 3.4-fold), and heat shock proteins (Hsp100, 3.4-fold; Hsp10, 2.8-fold), which reflect the cell response to increased oxidative stress and toxic effects of oxLDL, respectively, and in genes functioning in lipid metabolism (ApoE, 2.8-fold) the changes at protein level were very minute with ratios ranging from 1.05 to 1.2.

5.4 COMPARISON OF GENE AND PROTEIN EXPRESSION IN OXLDL-STIMULATED MACROPHAGES (III)

After oxLDL-stimulation, 115 genes were upregulated and 189 downregulated, which means changes in about 5 % of the genes present on the array. Many genes connected to lipid metabolism, such as scavenger receptors, inflammatory genes, such as several interleukins, and genes that function in

extracellular matrix production, such as fibronectins, were strongly induced. Fifty-two % (198) of the targets in protein arrays were present also in cDNA-arrays. The expression ratios gained by cDNA arrays were compared to the ratios gained by the protein array analysis. The changes in the twenty most highly upregulated proteins that were also present on the DNA arrays appeared to be mostly parallel at mRNA and protein levels (Table 3, III). Although it became evident that the scale of ratios gained from protein array analysis (0.7-1.9-fold) was significantly narrower than the scale of ratios from cDNA array analysis (0.05-37.8-fold). This suggests that it might be difficult to predict changes at the protein level by the changes at the mRNA level. The expression ratios gained from protein arrays were plotted against the ratios gained from cDNA arrays for those 198 genes that were present on both protein and cDNA arrays (Figure 6). The correlation coefficient was 0.32.

5.5 GENE EXPRESSION IN HUMAN ISCHEMIC SKELETAL MUSCLE (IV)

In acute-on-chronic ischemia 291 genes were significantly upregulated and 174 genes were downregulated as compared to the control sample of the same limb (a change in 5.5 % of all genes present on the array). The most prominently upregulated gene was VEGF (7.2-fold increase), with induction of its hypoxia-inducible regulator gene HIF-1 α (3.1-fold) and its receptor VEGFR-2 (2.2-fold).

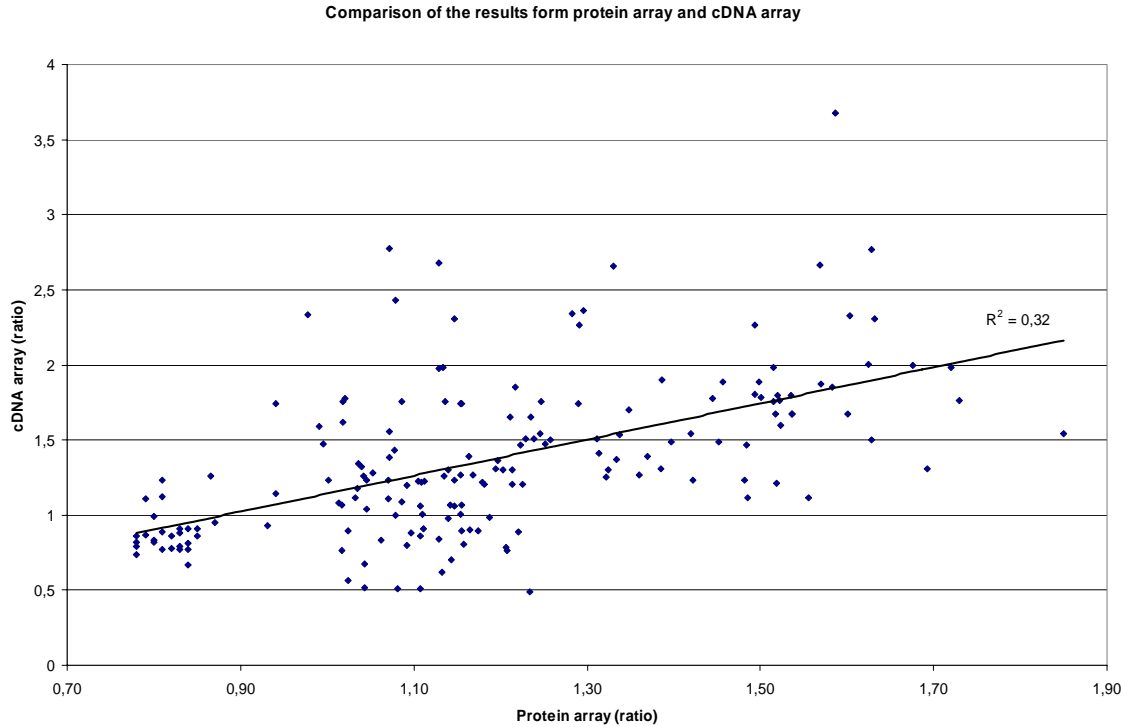


Figure 6. Comparison of results from protein arrays and cDNA arrays. The ratios gained from protein arrays are plotted against the ratios gained from cDNA arrays for those 198 genes that were present both in antibody arrays and cDNA arrays.

In addition, another hypoxia-regulated transcription factor HIF-2 α (3.5-fold), and several other factors involved in angiogenesis, such as Ephrins A1, B3 and B6, Tie-2, plasminogen-activator inhibitor-1 (PAI-1), c-met and TIMP-3 were significantly upregulated.

The localization of the expression patterns of HIF-1 α , VEGF and VEGFR-2 were studied using immunohistochemistry. In acute ischemia, VEGF was diffusely expressed in ischemic muscle with HIF-1 α colocalizing in the nuclei of the same cells. VEGFR-2 was detected in these acutely ischemic myocytes as well. VEGF upregulation was confirmed also at the mRNA level by RT-PCR. In addition to HIF-1 α -VEGF-VEGFR-2 pathway, TNF- α cascade was

remarkably upregulated. The cascade included TNF- α (3.7-fold), TNF- α convertase (15.3-fold) and TNF- α receptor-1 (2.9-fold) as well as the intracellular signalling mediators FADD (6.8-fold) and TRAF2 (1.6-fold).

In chronic CLI changes in gene expression patterns were much less striking than in acute-on-chronic CLI as only 74 genes were significantly upregulated and 34 downregulated as compared to the control samples collected from the same limb (change in 1.3 % of all genes). Most importantly, potent anabolic and survival factors IGF-1 (9.8-fold) and IGF-2 (2.4-fold) were strongly upregulated in chronic ischemia. These findings were confirmed by RT-PCR and immunohistochemistry (IGF-1). The

expression of IGF-1 in chronic ischemia was localized in atrophic and regenerating myocytes, whereas in agreement with the cDNA array data, no detectable IGF-1 immunostaining could be observed in acute ischemia. Interestingly, the same atrophic myocytes expressed VEGF, VEGFR-2, and HIF-1 α although at lower levels than in acute-on-chronic ischemia. In addition to the currently known genes, many unknown ESTs were highly upregulated under ischemia.

6 DISCUSSION

6.1 GENE EXPRESSION IN HUMAN ATHEROSCLEROTIC LESIONS

In recent years, DNA array technology has offered a powerful research tool in molecular pathobiology of various diseases (Lockhart and Winzeler, 2000). We utilized the DNA array method in the research of molecular mechanisms of atherosclerosis. Gene expression changes between whole mount human atherosclerotic lesions and normal arteries was studied using an array of 18376 genes. Because the isolation of truly normal arteries and small fatty streaks presents a significant problem, we only reported changes in advanced lesions. The results were first validated using a group of genes that are known to be affected in atherosclerosis, such as genes connected to SMC proliferation, formation of connective tissue and lipid metabolism, which are hallmarks in the development of atherosclerotic lesions. In addition to well-known atherosclerosis-related genes, 75 genes, including unknown EST-sequences,

were observed to have changed expression levels.

The expression changes in four genes functioning in cell signalling and communication, and representing a wide range of score values, namely PECAM-1, VEGFR-2, JAK-1, and an unknown EST-sequence, were confirmed and localized with independent methods. PECAM-1 is involved in the monocyte adhesion to endothelium. VEGFR-2 mediates the VEGF-mediated endothelial cell proliferation. JAK-1 mediates signalling from several cytokines and growth factors, such as from IFN- γ and PDGF (see 2.1.3 for further details). In addition to these signalling molecules, there was upregulation in other important signalling molecules, such as in tumour adhesion molecule MUC18 and in MAP-kinase activator. MAP-kinase activator has a role in mediating signals from several cytokines and growth factors, such as from IFN- γ , and functions also as a downstream target of JAK. Also genes functioning in gene expression and cell division (proto-oncogenes CRK-II, c-fos, pim-1) were upregulated. This data suggests that as a response to cytokines and growth factors, secreted by cells present in lesions, many cell signalling pathways are activated, and this activation leads to changes in gene expression, cell division and cell adhesion. Additionally, the upregulation of inflammatory and immunological genes, such as MHC I complex, Ig lambda chain, and complement component 7, stresses the importance of inflammatory and immunological response in the development of atherosclerosis.

In addition to expression changes in well-characterized genes, there were expression changes in unknown EST-

sequences. To clarify the role of these EST sequences in atherosclerosis, the full sequence of the clones should be determined. This can be done by screening and sequencing cDNA and/or genomic libraries, or using PCR-based methods, such as RACE (rapid amplification of cDNA ends). Nowadays, with the growing number of sequence information in the bioinformatical databases, the cloning could be done also *in silico* (Kato, 2002).

This study demonstrated that the DNA array method was able to detect multiple changes in gene expression, and can be used to study the molecular mechanisms underlying atherosclerosis. However, DNA array analyses of the pathogenesis of atherosclerosis have many limitations. First, atherosclerotic lesions consist of many cell types, such as SMCs, endothelial cells and macrophages, and it is evident that different cellular compositions of lesions have major impact on the results. Therefore, it is extremely important to properly characterize the lesions, if whole mount lesions are used. However, the use of laser microdissection overcomes this problem. Secondly, as a general problem in all analyses utilizing human tissue, the availability of tissue samples, especially normal control samples, is limited. Also, the differences between individuals, such as age, gender, and health status of different individuals, have an impact on the results. Ideally, the diseased and control samples should be obtained from the same individuals. It can also be debated whether the atherosclerotic process is similar in different arterial locations, and what is the effect of changes in shear stress on gene expression profiles. An adequate number of samples, pooling of samples

before array analysis, and statistical methods can help to diminish variations between individual samples. Thirdly, the array analysis itself is prone to several problems as discussed in detail in 2.2.2.3. Especially, the use of filters with radioactive detection requires parallel hybridizations and is susceptible to inter-array variations. Multiple hybridizations and statistical analyses of the results are used to reduce these variations. Unfortunately, high costs of array analyses limits the number of replicates that can be made. It is also extremely important to confirm the array results with independent methods at both at mRNA level and at protein level, because DNA arrays might produce false signals for example due to sequence homologies, and it is not straightforward to predict the changes at protein level from the changes at mRNA level, as discussed in detail in 2.2.6.

In addition to this study, several studies utilizing DNA array methods in the research of atherosclerosis have been published. Using mouse models of atherosclerosis, the development of atherosclerotic lesions have been studied. Wuttge et al (2001) were able to find expression changes in genes that are connected to macrophages, T-cells or are growth factors and participants in lipid metabolism (Wuttge et al., 2001). Based on gene cluster analysis, they proposed that gene expression during atherogenesis is not a linear process with maximal expression at advanced lesion stage, and that the pathogenesis of atherosclerosis should be evaluated in detail also at early time points. Napoli et al (2002) studied the atherosclerotic lesions of offsprings of hypercholesterolemic mice, and found changes, surprisingly, mainly in matrix components and enzymes, not in

macrophage-related genes (Napoli et al., 2002). Although animal models offer a possibility for well-defined study protocols and matching controls, the results cannot be directly applied to human beings (Hofker et al., 1998). Therefore, the atherosclerotic process has also been studied in human samples. McCaffrey et al (2000) found that Egr-1 is strongly upregulated in atherosclerotic plaques, and its upregulation may partly explain the activation of several growth factors and cytokines in lesions (McCaffrey, 2000). Gene expression changes predisposing the plaque to rupture have been studied by several groups. Induction of lipid-metabolism gene perilipin (Faber et al., 2001), induction of several genes regulating apoptosis, such as DAP-kinase (Martinet et al., 2002; Woodside et al., 2003), and induction of genes functioning in cell adhesion, such as MadCAM and downregulation of genes maintaining vascular homeostasis, such as chemokines MCP-1 and MCP-2 (Randi et al., 2003) were found. Generally, all these studies have some limitations, such as the variability in samples, controls, and array methods, especially the small number of genes on the arrays (usually less than 1000 genes) used. The results have been often inadequately confirmed with independent methods, although some findings, (e.g. the upregulation of Egr-1) were confirmed properly. However, according to these studies, it is evident that inflammatory responses and ECM modulation play important roles in the atherosclerotic process.

6.2 GENE EXPRESSION IN MACROPHAGE-RICH SHOULDER AREA OF ATHEROSCLEROTIC LESION

To overcome the effects of tissue heterogeneity on gene expression patterns in atherosclerotic lesions, we used laser microdissection to isolate macrophage-rich shoulder areas of human atherosclerotic lesions and compared it to DIT. Because the amount of mRNA that could be extracted from LMD samples was very low, T7-polymerase amplification was needed prior to DNA array analysis. Array analysis was performed with fluorescence-based microarrays that contained about 6000 genes. Although, the current method does not enable the isolation of single cells, and dissected shoulder areas contained small amounts of other cells, we concluded that we were able to successfully isolate macrophage-rich areas since: (i) several genes typical for macrophages were identified; (ii) expression changes in T-cell and endothelial cell-specific genes were not significant; and (iii) similar results were found *in vitro* in monocyte-macrophage differentiation. Upregulation of 72 genes in addition to macrophage-related 33 genes was identified. It is worth noting that the expression changes for LMD macrophages were consistently bigger than the changes in cultured macrophages, which suggests that in the lesions the macrophages are under the influence of complex system of interleukins and growth factors secreted by other cells, which cannot be mimicked in cell culture models. As a most interesting finding, a key enzyme in cholesterol biosynthesis, HMG-CoA

reductase, was highly upregulated both in *in vitro* and *in vivo* macrophages. The reason for the high expression may be the proliferation and/or differentiation of the macrophages in lesions. Also cell surface integrins CD11a/CD18, which bind to cell adhesion molecules VCAM-1 and ICAM-1 on endothelial cells, and therefore contribute to cell movements in atherosclerotic lesions (Rosseau et al., 2000), were upregulated in LMD macrophages. HMG-CoA partly regulates the activity of integrins CD11a/CD18. Additionally, in cell culture studies statins have been shown to downregulate the expression of these integrins (Yoshida et al., 2001). Thus, we suggest that statin therapy may have favourable effects on the treatment of atherosclerosis through the downregulation of HMG-CoA reductase and subsequent reduction in macrophage trafficking in lesions via the reduction of the activity of cell surface integrins CD11a/CD18. There are also a growing number of studies indicating that clinical benefits of statins are related to their non-lipid-lowering, pleiotropic effects on the vasculature. These include effects: on endothelial function, such as the inhibition of endothelin-1 expression; on inflammation, such as the inhibition of macrophage cytokine production; on immune response, such as the reduction of MHC-II expression; and on SMC proliferation and thrombosis (Altieri, 2001; Palinski and Napoli, 2002). Thus, HMG-CoA reductase has an important role in versatile cellular functions, not only in lipid metabolism. Interestingly, the induction of HMG-CoA reductase has been noticed in two recent array studies, due to hyperhomocysteinemia in endothelial cells (Li et al., 2002a), and following copper-treatment of

macrophages (Svensson et al., 2003). These studies together suggest that the treatment of atherosclerosis with statins would be beneficial in many cases, even without hyperlipidemia. Some of the pleiotropic effects of statins have been demonstrated to be truly cholesterol-independent, such as the activation of eNOS by phosphorylation (Harris et al., 2004). However, to unravel the mechanisms of statin actions and the multiple roles of HMG-CoA reductase in atherosclerosis, further studies are needed.

This study was the first to report the use of laser microdissection and cDNA array in the research of human atherosclerotic lesions. Trogan et al (2002) dissected foam cells from lesions of lipopolysaccharide-treated apoE-deficient mice and detected upregulation of adhesion molecules VCAM-1, ICAM-1 and MCP-1. The results suggested that atherosclerosis might actually be caused by the upregulation of inflammatory and cell adhesion molecules expressed by macrophage foam cells in atherosclerotic lesions (Trogan et al., 2002). These results are very well in agreement with ours, as the upregulation of molecules that function in cell movements and inflammation was found.

The advanced atherosclerotic lesions consist of distinct areas with different cellular contents, such as of fibrous cap and necrotic core in addition to shoulder area. It would be interesting to study the gene expression patterns in these areas utilizing LMD and cDNA arrays. Also, it has been debated if single cell types, such as macrophages, have different gene expression patterns and functions in different areas of lesions. For example, it could be imagined that macrophages in shoulder areas might have more inflammatory properties than

macrophages that surround lipid-rich core area, which on behalf might have more lipid-metabolism-related functions.

When the expression patterns of LMD macrophages were compared to the two cell culture models of macrophages used in this thesis, monocyte-macrophage differentiation (II, III) and oxLDL stimulation of macrophages (study III), it became evident that the LMD macrophages share more similarities with the PMA-stimulated monocytes than with the oxLDL-stimulated macrophages. In LMD macrophages, genes that function in macrophage differentiation, inflammation and immune response, such as CSF-receptors, integrins and interleukin receptors, were activated. On the contrary, there was only minor changes in lipid-metabolism-related genes, such as in scavenger receptors. This data suggests that monocyte-macrophage-differentiation might be a better model for lesion macrophages than the oxLDL-stimulation of macrophages.

6.3 GENE EXPRESSION DURING MONOCYTE MACROPHAGE-DIFFERENTIATION

We studied gene expression changes during monocyte-macrophage differentiation in PMA-stimulated monocytic THP-1-cells. PMA induces differentiation by activating protein kinase C isoenzymes and AP-1 transcription factors (see 2.1.3.9) (Vuong et al., 2002). Array analysis was validated by looking for genes that were previously connected to macrophages. Among the 10 most highly upregulated genes at 72 h, 5 genes were previously connected to inflammation or macrophages. Also, many other genes

having lower ratios were connected to macrophages and their inflammatory or lipid-related properties. However, it is worth noting that genes having low expression ratios with high expression intensities might often be biologically more relevant than genes having high ratios with low expression levels.

Gene expression patterns during monocyte-macrophage differentiation were studied by cluster analysis. It was found that at early timepoints, the expression of early-response genes, such as transcription factors, increased, and genes that have roles in proliferation, migration, inflammation, and lipid metabolism changed later. The expression patterns in macrophages vs. monocytes have been studied before using SAGE (Hashimoto et al., 1999). Many findings are similar to ours, for example the upregulation of cathepsins and genes involved in lipid metabolism. However, they were not able to study the time-dependency of the gene expression changes.

6.4 GENE AND PROTEIN EXPRESSION IN OXLDL-STIMULATED MACROPHAGES

Antibody array analysis of oxLDL-stimulated macrophages showed expression changes in many genes that have been previously connected to macrophages such as CD36, which is one of the major scavenger receptors for oxLDL, and in inflammatory gene COX-2. More importantly, array analyses also revealed changes in many new genes that were not previously connected to macrophages. Such genes included leukocyte antigen related protein phosphatase, which functions in cell

adhesion, and rabaptin-5, which is a part of the proliferative signalling from PDGF- β receptor. These genes might become new therapeutic targets for vascular diseases, as the monocyte and leukocyte adhesion, and cell proliferation are important in atherogenesis. Although oxLDL has toxic effects and increases the oxidative stress load in macrophages, there were no significant changes in heat shock proteins or enzymes that protect cells from oxidative stress. However, it should be pointed out that 384 antibodies for specific proteins present on the current array are not yet enough to get a comprehensive view about the overall changes in the proteome.

The results from cDNA arrays and protein arrays from oxLDL-stimulated macrophages were compared to each other. In general, the expression changes at protein level were smaller than the changes at mRNA level. The scale of ratios obtained by protein array analysis was also narrower varying from 0.7 to 1.9 compared to ratios obtained by cDNA arrays from 0.05 to 37.8. The correlation coefficient for ratios from protein array vs. ratios from cDNA array was 0.32, which is well in concordance with the correlation coefficients gained in other studies, in which the scale of correlation coefficients ranged from 0.356 to 0.58 (see 2.2.6). Thus, for several proteins, it may be difficult to predict changes at the protein level by changes at the mRNA level as discussed in detail in 2.2.6. This might be due to the fact that there are several transcript variants for active genes and all of them are not necessarily translated. Moreover, proteins are modified after translation. Thus, it should be emphasized that findings from gene expression profiling studies should also be confirmed at the

protein level, using Western blot, immunohistochemistry, protein arrays, and also quantitatively by ELISA.

In this study, it was found that genes previously connected to macrophages, such as CD36 and COX-2, and many novel transcription factors such as Cdk-1, Elf-5 and TFII-I, and genes functioning in cell signalling, such as NEMO-like kinase and TRADD were upregulated after oxLDL-treatment both at the mRNA and at the protein level. Gene expression profiles in oxLDL-treated macrophages have been studied previously (Andersson et al., 2001; Shiffman et al., 2000), and expression changes have been found mainly in genes that function in cell proliferation, lipid metabolism, and inflammatory response, as in our study. Besides oxidative modification, also lipolytic and enzymatic modification of LDL takes place in lesions and makes the LDL particle atherogenic (see 2.1.3.1). The effects of these differentially modified LDL particles might diverge, as suggested by Han et al (2003), who studied the effects of enzymatically modified LDL in THP-1 cells. They found that the cell response to enzymatically modified LDL differs from the cell response to oxLDL: inflammatory genes were not upregulated following treatment with enzymatically modified LDL, but were upregulated following oxLDL treatment; and further, enzymatically modified LDL highly activated matrix-degrading enzymes, such as cathepsins (Han et al., 2003). It would be also useful to study the differences in cell responses to *in vitro* and *in vivo* oxidized/modified LDL.

The advantages of cell culture experiments in the research of atherosclerosis are versatile, such as the

low costs of cell culture facilities, the possibility to control different variables and the possibility to utilize sophisticated clustering methods for data analysis. However, it is evident that the understanding of the complex gene regulatory network including the effects of different environmental factors in atherosclerotic lesions, would not be possible using cell culture models. It should be debated how much the cell culture models resemble the cells *in vivo*. For example, as a cell model for macrophages, usually PMA-stimulated THP-1 and U937 cells are used. During monocyte-macrophage-differentiation these cells become adhesive, express differentiation antigens and no longer proliferate. They are also functionally similar to tissue macrophages: they can phagocytose and present antigens, and they are activated by chemotactic agents (Verhoeckx et al., 2004). However, there are no studies where these monocyte-macrophage models are compared to *in vivo* macrophages using large-scale expression analysis methods and the validity of these models is not precisely verified. Also, the malignant origin of these cell lines hinders the interpretation of the results from gene expression studies, especially the results concerning cell proliferation. Unfortunately, standardized experiments using human peripheral blood monocytes (HPBMs) are difficult to perform because of the donor differences and the spontaneous differentiation of HPBMs to macrophages (Dr. Anna-Liisa Levenon, personal communication).

6.5 GENE EXPRESSION IN ISCHEMIC SKELETAL MUSCLE

We utilized a DNA array of 8400 genes to gain more information on ischemia-induced angiogenesis in human skeletal muscle, which is an approach that has not been reported before. Induction of the hypoxia-inducible transcription factors HIF-1 α and HIF-2 α and their downstream targets VEGF, VEGFR-2, and PAI-1 as well as the inflammatory and cell-death promoting TNF- α pathway with its downstream signalling machinery were found in acute-on-chronic CLI. Fewer cascades related to cell-death were activated in chronically ischemic muscle. Instead, potent cell survival and anabolic factors IGF-1 and IGF-2 were upregulated in atrophic and regenerating myocytes, as confirmed by immunohistochemistry.

Despite the difficulties in obtaining an ideal control for ischemic muscle and the low number of comparisons, the array results are in agreement with previously published data regarding the induction of HIF-1 α -VEGF-VEGFR2 and TNF- α -pathways in acute ischemia and induction of IGFs in chronic ischemia (Paoni et al., 2002; Li et al., 1996). Moreover, the percentage of genes differentially expressed in response to CLI (5.5% and 1.3% in acute and chronic, respectively) was quite similar to that reported from various animal models (1.0-9.4%) (Kim et al., 2002; Stanton et al., 2000).

It can be concluded that the gene expression patterns are quite different in acute-on-chronic and chronic CLI. The angiogenic HIF-1 α -VEGF-VEGFR-2 pathway seems to be the most important response to acute ischemia together with the activation of TNF- α cascade while in

chronic CLI, survival factors IGF-1 and IGF-2 are produced. These results offer potentially useful therapeutic perspectives. Gene transfer of VEGF, IGF-1 and IGF-2 to induce angiogenesis, survival and regeneration may be useful for the treatment of chronically ischemic skeletal muscle. On the other hand, in acute CLI exogenous VEGF may not be useful as these regions already contain high amounts of endogenous VEGF. Instead, the suppression of signalling pathways promoting cell-death and inflammation, such as the TNF- α pathway, might be beneficial under acute ischemia.

7 CONCLUSIONS AND FUTURE DIRECTIONS

It has been shown here that DNA array methods provide useful and powerful strategies for the analysis of molecular events during the development of atherosclerosis and ischemic diseases. Data gathered from human atherosclerotic lesions, macrophage-rich shoulder areas of atherosclerotic lesions and *in vitro* macrophages suggests that growth factor activities, cell signalling, inflammatory mediators, cell adhesion and lipid metabolism play important roles in the pathogenesis of atherosclerosis.

In more detail, it was shown that signalling cascades mediating IFN- γ signalling, several proto-oncogenes and genes functioning in inflammatory and immunological response, were highly expressed in atherosclerotic lesions. Also, strong macrophage-specific expression of an unknown gene was found. It was noticed that macrophages isolated from lesions using laser microdissection strongly expressed CSF

receptors, interleukin receptors, and integrins. Interestingly, HMG-CoA reductase was highly upregulated in lesion macrophages, and this upregulation together with the upregulation of CD11a/CD18 integrins suggests a new mechanism for the beneficial effects of statins in the treatment of atherosclerosis, which is the attenuation of monocytic cell trafficking to lesions. *In vivo* macrophages were also compared with different *in vitro* cell models. It can be proposed that monocyte-macrophage-differentiation is a better model for lesion macrophages than oxLDL stimulation of macrophages. To further validate the use of DNA arrays as research tool, we compared the results gained from DNA and protein arrays. It became evident that it might be difficult to predict the expression changes at protein level from the expression changes from mRNA level for each gene. This stresses the importance of careful confirmation of the results from DNA array studies both at mRNA and protein levels.

Expression analysis of human skeletal muscle ischemia revealed the activation of different signalling pathways in acute and chronic ischemia. In acute ischemia the angiogenic HIF- α -VEGF-VEGFR2 and proinflammatory TNF- α - pathways were activated, whereas in chronic ischemia survival factors IGF-1 and IGF-2 were activated. This data offers a rationale for therapeutic strategies.

It is evident that different high-throughput array methods, such as DNA array (Lockhart and Winzeler, 2000), protein array (Zhu and Snyder, 2003), and tissue array (Kallioniemi et al., 2001) will revolutionize biomedical research. These methods can be used for various purposes, such as for the analysis of molecular changes during

disease processes, classifying diseases, finding new therapeutic targets, and for the verification of new therapeutic strategies. However, several obstacles have to be overcome before one can fully take advantage of the capacity of these methods. The human genome encodes ~32000-38000 genes, and the number of different proteins may exceed ~500000. Therefore, huge technical improvements have to be made before the whole genome and, even more difficult, the whole proteome can be screened in a single experiment. However, the current analysis strategies could be greatly improved simply by proper experimental design, by adequate confirmation of the results both at mRNA and at protein levels, and by utilizing the data already available from array experiments in databases, and most importantly, by efforts to generate public array databases sharing general rules for performing the experiments and reporting the data.

In atherosclerotic research, cell models, animal models, and human tissue samples are all needed. The combination of laser microdissection and powerful arrays makes it possible to study gene expression patterns in different areas of atherosclerotic lesions, for example in macrophages in different locations. These elegant research tools, as integrated with cell cultures and animal models, will produce an accurate and reliable view of the molecular mechanisms of the atherosclerotic process. Moreover, combining the information about genetic variation and linkage studies, with gene and protein expression studies, would be essential for getting a more complete view of the disease process (Cheung and Spielman, 2002).

This thesis study produced enormous amounts of information about gene expression changes in atherosclerotic lesions. Several genes were found that have not been previously linked to the development of atherosclerosis, including completely unknown genes. However, further studies are needed to clarify the role of these genes in atherosclerotic process. This data can be used in the development of new therapeutic strategies for the treatment of atherosclerosis and ischemic disease.

8 REFERENCES

- Abab,S., Husain,M., and Liu,P. (2003) Principles of cDNA Microarrays as Applied in Heart Failure Research. In Proteomic and Genomic Analysis of Cardiovascular Disease, J.Van Eyk and M.J.Dunn, eds. (Darmstadt, Germany: Wiley-VCH Verlag GmbH & Co.), pp. 61-80.
- Adams,L.D., Geary,R.L., McManus,B., and Schwartz,S.M. (2000) A comparison of aorta and vena cava medial message expression by cDNA array analysis identifies a set of 68 consistently differentially expressed genes, all in aortic media. *Circ. Res.* 87, 623-631.
- Aldridge,B.A., Lim,S.D., Baumann,A.K., Hosseini,S., Buck,W., Almekinder,T.L., Sun,C.Q., and Petros,J.A. (2003) Automated sequencing of complete mitochondrial genomes from laser-capture microdissected samples. *Biotechniques* 35, 606-10, 612.
- Alizadeh,A.A., Eisen,M.B., Davis,R.E., Ma,C., Lossos,I.S., Rosenwald,A., Boldrick,J.C., Sabet,H., Tran,T., Yu,X., Powell,J.I., Yang,L., Marti,G.E., Moore,T., Hudson,J., Jr., Lu,L., Lewis,D.B., Tibshirani,R., Sherlock,G., Chan,W.C., Greiner,T.C., Weisenburger,D.D., Armitage,J.O., Warnke,R., Levy,R., Wilson,W., Grever,M.R., Byrd,J.C., Botstein,D., Brown,P.O., and Staudt,L.M. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403, 503-511.
- Altieri,D.C. (2001) Statins' benefits begin to sprout. *J. Clin. Invest* 108, 365-366.
- Anderson,L. and Seilhamer,J. (1997) A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18, 533-537.
- Anderson,N.L. and Anderson,N.G. (1998) Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis* 19, 1853-1861.
- Andersson,T., Borang,S., Larsson,M., Wirta,V., Wennborg,A., Lundeberg,J., and Odeberg,J. (2001) Novel candidate genes for atherosclerosis are identified by representational difference analysis-based transcript profiling of cholesterol-loaded macrophages. *Pathobiology* 69, 304-314.
- Arenkov,P., Kukhtin,A., Gemmell,A., Voloshchuk,S., Chupeeva,V., and Mirzabekov,A. (2000) Protein microchips: use for immunoassay and enzymatic reactions. *Anal. Biochem.* 278, 123-131.
- Argraves,G.L., Barth,J.L., and Argraves,W.S. (2003) The MUSC DNA Microarray Database. *Bioinformatics*. 19, 2473-2474.
- Armstrong,P.J., Johanning,J.M., Calton,W.C., Jr., Delatore,J.R., Franklin,D.P., Han,D.C., Carey,D.J., and Elmore,J.R. (2002) Differential gene expression in human abdominal aorta: aneurysmal versus occlusive disease. *J. Vasc. Surg.* 35, 346-355.
- Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A., and Struhl,K. (2003) In *Current Protocols in Molecular Biology*, F.M.Ausubel, R.Brent, R.E.Kingston, D.D.Moore, J.G.Seidman, J.A.Smith, and K.Struhl, eds. John Wiley & Sons, Inc.).
- Baird,A. and Durkin,T. (1986) Inhibition of endothelial cell proliferation by type beta-transforming growth factor: interactions with acidic and basic fibroblast growth factors. *Biochem. Biophys. Res. Commun.* 138, 476-482.
- Baker,A.H., Zaltsman,A.B., George,S.J., and Newby,A.C. (1998) Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. *TIMP-3 promotes apoptosis. J. Clin. Invest* 101, 1478-1487.
- Banks,R.E., Dunn,M.J., Forbes,M.A., Stanley,A., Pappin,D., Naven,T., Gough,M., Harnden,P., and Selby,P.J. (1999) The potential use of laser capture microdissection to selectively obtain distinct populations of cells for proteomic analysis--preliminary findings. *Electrophoresis* 20, 689-700.
- Barbier,O., Torra,I.P., Duguay,Y., Blanquart,C., Fruchart,J.C., Glineur,C., and Staels,B. (2002) Pleiotropic actions of peroxisome proliferator-activated

- receptors in lipid metabolism and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 22, 717-726.
- Bath,P.M., Hassall,D.G., Gladwin,A.M., Palmer,R.M., and Martin,J.F. (1991) Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler. Thromb.* 11, 254-260.
- Batorfi,J., Ye,B., Mok,S.C., Cseh,I., Berkowitz,R.S., and Fulop,V. (2003) Protein profiling of complete mole and normal placenta using ProteinChip analysis on laser capture microdissected cells. *Gynecol. Oncol.* 88, 424-428.
- Bayes-Genis,A., Conover,C.A., and Schwartz,R.S. (2000) The insulin-like growth factor axis: A review of atherosclerosis and restenosis. *Circ. Res.* 86, 125-130.
- Beauchamp,N.J., van Achterberg,T.A., Engelse,M.A., Pannekoek,H., and De Vries,C.J. (2003) Gene expression profiling of resting and activated vascular smooth muscle cells by serial analysis of gene expression and clustering analysis. *Genomics* 82, 288-299.
- Benditt,E.P. and Benditt,J.M. (1973) Evidence for a monoclonal origin of human atherosclerotic plaques. *Proc. Natl. Acad. Sci. U. S. A* 70, 1753-1756.
- Bevilacqua,M.P., Pober,J.S., Wheeler,M.E., Cotran,R.S., and Gimbrone,M.A., Jr. (1985) Interleukin-1 activation of vascular endothelium. Effects on procoagulant activity and leukocyte adhesion. *Am. J. Pathol.* 121, 394-403.
- Biesiada,E., Razandi,M., and Levin,E.R. (1996) Egr-1 activates basic fibroblast growth factor transcription. Mechanistic implications for astrocyte proliferation. *J. Biol. Chem.* 271, 18576-18581.
- Blindt,R., Vogt,F., Lamby,D., Zeiffer,U., Krott,N., Hilger-Eversheim,K., Hanrath,P., vom,D.J., and Bosserhoff,A.K. (2002) Characterization of differential gene expression in quiescent and invasive human arterial smooth muscle cells. *J. Vasc. Res.* 39, 340-352.
- Bonthron,D.T., Morton,C.C., Orkin,S.H., and Collins,T. (1988) Platelet-derived growth factor A chain: gene structure, chromosomal location, and basis for alternative mRNA splicing. *Proc. Natl. Acad. Sci. U. S. A* 85, 1492-1496.
- Borsani,G., Ballabio,A., and Banfi,S. (1998) A practical guide to orient yourself in the labyrinth of genome databases. *Hum. Mol. Genet.* 7, 1641-1648.
- Boudoulas,H. and Leier,C.V. (2001) Renal Disorders and Cardiovascular Disease. In *Heart Disease*, E.Braunwald, D.P.Zipes, and P.Libby, eds. W.B. Saunders Company), pp. 2280-2297.
- Brand,K., Eisele,T., Kreusel,U., Page,M., Page,S., Haas,M., Gerling,A., Kaltschmidt,C., Neumann,F.J., Mackman,N., Baeurele,P.A., Walli,A.K., and Neumeier,D. (1997) Dysregulation of monocyte nuclear factor-kappa B by oxidized low-density lipoprotein. *Arterioscler. Thromb. Vasc. Biol.* 17, 1901-1909.
- Brand,K., Page,S., Rogler,G., Bartsch,A., Brandl,R., Knuechel,R., Page,M., Kaltschmidt,C., Baeuerle,P.A., and Neumeier,D. (1996) Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J. Clin. Invest* 97, 1715-1722.
- Brazma,A., Hingamp,P., Quackenbush,J., Sherlock,G., Spellman,P., Stoeckert,C., Aach,J., Ansorge,W., Ball,C.A., Causton,H.C., Gaasterland,T., Glenisson,P., Holstege,F.C., Kim,I.F., Markowitz,V., Matese,J.C., Parkinson,H., Robinson,A., Sarkans,U., Schulze-Kremer,S., Stewart,J., Taylor,R., Vilo,J., and Vingron,M. (2001) Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat. Genet.* 29, 365-371.
- Brazma,A. and Vilo,J. (2000) Gene expression data analysis. *FEBS Lett.* 480, 17-24.
- Brown,M.S. and Goldstein,J.L. (1979) Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc. Natl. Acad. Sci. U. S. A* 76, 3330-3337.
- Brown,M.S., Kovanen,P.T., and Goldstein,J.L. (1981) Regulation of plasma cholesterol by lipoprotein receptors. *Science* 212, 628-635.
- Burke-Gaffney,A., Brooks,A.V., and Bogle,R.G. (2002) Regulation of chemokine expression in atherosclerosis. *Vascul. Pharmacol.* 38, 283-292.

- Butte,A. (2002) The use and analysis of microarray data. *Nat. Rev. Drug Discov.* 1, 951-960.
- Cai,H. and Harrison,D.G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ. Res.* 87, 840-844.
- Cannon,R.O., III (1998) Role of nitric oxide in cardiovascular disease: focus on the endothelium. *Clin. Chem.* 44, 1809-1819.
- Chen,J.K., Hoshi,H., and McKeehan,W.L. (1987) Transforming growth factor type beta specifically stimulates synthesis of proteoglycan in human adult arterial smooth muscle cells. *Proc. Natl. Acad. Sci. U. S. A* 84, 5287-5291.
- Cheung,V.G. and Spielman,R.S. (2002) The genetics of variation in gene expression. *Nat. Genet.* 32 *Suppl.*, 522-525.
- Chinetti,G., Fruchart,J.C., and Staels,B. (2000) Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. *Inflamm. Res.* 4911089900, 497-505.
- Cho,R.J., Campbell,M.J., Winzler,E.A., Steinmetz,L., Conway,A., Wodicka,L., Wolfsberg,T.G., Gabrielian,A.E., Landsman,D., Lockhart,D.J., and Davis,R.W. (1998) A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol. Cell* 2, 65-73.
- Claesson-Welsh,L. (1996) Mechanism of action of platelet-derived growth factor. *Int. J. Biochem. Cell Biol.* 28, 373-385.
- Claesson-Welsh,L. (2003) Signal transduction by vascular endothelial growth factor receptors. *Biochem. Soc. Trans.* 31, 20-24.
- Claverie,J.M. (1999) Computational methods for the identification of differential and coordinated gene expression. *Hum. Mol. Genet.* 8, 1821-1832.
- Colantuoni,C., Henry,G., Zeger,S., and Pevsner,J. (2002) SNOMAD (Standardization and Normalization of MicroArray Data): web-accessible gene expression data analysis. *Bioinformatics.* 18, 1540-1541.
- Collins,T. and Cybulsky,M.I. (2001) NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? *J. Clin. Invest* 107, 255-264.
- Creager,M.A. and Libby,P. (2001) Peripheral arterial disease. In *Heart Disease*, E.Braunwald, D.P.Zipes, and P.Libby, eds. W.B. Saunders Company), pp. 1457-1484.
- Cremona,O., Muda,M., Appel,R.D., Frutiger,S., Hughes,G.J., Hochstrasser,D.F., Geinoz,A., and Gabbiani,G. (1995) Differential protein expression in aortic smooth muscle cells cultured from newborn and aged rats. *Exp. Cell Res.* 217, 280-287.
- Cybulsky,M.I., Fries,J.W., Williams,A.J., Sultan,P., Eddy,R., Byers,M., Shows,T., Gimbrone,M.A., Jr., and Collins,T. (1991) Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM1 gene. *Proc. Natl. Acad. Sci. U. S. A* 88, 7859-7863.
- De Vries,C.J., van Achterberg,T.A., Horrevoets,A.J., ten Cate,J.W., and Pannekoek,H. (2000) Differential display identification of 40 genes with altered expression in activated human smooth muscle cells. Local expression in atherosclerotic lesions of smags, smooth muscle activation-specific genes. *J. Biol. Chem.* 275, 23939-23947.
- de Waard, van den Berg,B.M., Veken,J., Schultz-Heienbrok,R., Pannekoek,H., and van Zonneveld,A.J. (1999) Serial analysis of gene expression to assess the endothelial cell response to an atherogenic stimulus. *Gene* 226, 1-8.
- de Wildt,R.M., Mundy,C.R., Gorick,B.D., and Tomlinson,I.M. (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol.* 18, 989-994.
- de Winther,M.P., van Dijk,K.W., Havekes,L.M., and Hofker,M.H. (2000) Macrophage scavenger receptor class A: A multifunctional receptor in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 20, 290-297.
- Dempsey,A.A., Dzau,V.J., and Liew,C.C. (2001) Cardiovascular genomics: estimating the total number of genes expressed in the human cardiovascular system. *J. Mol. Cell Cardiol.* 33, 1879-1886.
- DeRisi,J., Penland,L., Brown,P.O., Bittner,M.L., Meltzer,P.S., Ray,M., Chen,Y., Su,Y.A., and Trent,J.M. (1996) Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat. Genet.* 14, 457-460.

- Derynck,R., Rhee,L., Chen,E.Y., and Van Tilburg,A. (1987) Intron-exon structure of the human transforming growth factor-beta precursor gene. *Nucleic Acids Res.* *15*, 3188-3189.
- Dietmaier,W., Hartmann,A., Wallinger,S., Heinmoller,E., Kerner,T., Endl,E., Jauch,K.W., Hofstadter,F., and Ruschoff,J. (1999) Multiple mutation analyses in single tumor cells with improved whole genome amplification. *Am. J. Pathol.* *154*, 83-95.
- DiFrancesco,L.M., Murthy,S.K., Luider,J., and Demetrick,D.J. (2000) Laser capture microdissection-guided fluorescence in situ hybridization and flow cytometric cell cycle analysis of purified nuclei from paraffin sections. *Mod. Pathol.* *13*, 705-711.
- Dillon,D., Zheng,K., and Costa,J. (2001) Rapid, efficient genotyping of clinical tumor samples by laser-capture microdissection/PCR/SSCP. *Exp. Mol. Pathol.* *70*, 195-200.
- Dinarello,C.A. (1997) Interleukin-1. *Cytokine Growth Factor Rev.* *8*, 253-265.
- Doevendans,P.A., Jukema,W., Spiering,W., Defesche,J.C., and Kastelein,J.J. (2001) Molecular genetics and gene expression in atherosclerosis. *Int. J. Cardiol.* *80*, 161-172.
- Dopazo,J., Zanders,E., Dragoni,I., Amphlett,G., and Falciani,F. (2001) Methods and approaches in the analysis of gene expression data. *J. Immunol. Methods* *250*, 93-112.
- Dormandy,J.A. and Rutherford,R.B. (2000) Management of peripheral arterial disease (PAD). TASC Working Group. TransAtlantic Inter-Society Consensus (TASC). *J. Vasc. Surg.* *31*, S1-S296.
- Duggan,D.J., Bittner,M., Chen,Y., Meltzer,P., and Trent,J.M. (1999) Expression profiling using cDNA microarrays. *Nat. Genet.* *21*, 10-14.
- Dustin,M.L., Rothlein,R., Bhan,A.K., Dinarello,C.A., and Springer,T.A. (1986) Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* *137*, 245-254.
- Eisen,M.B., Spellman,P.T., Brown,P.O., and Botstein,D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U. S. A* *95*, 14863-14868.
- Emmert-Buck,M.R., Bonner,R.F., Smith,P.D., Chuaqui,R.F., Zhuang,Z., Goldstein,S.R., Weiss,R.A., and Liotta,L.A. (1996) Laser capture microdissection. *Science* *274*, 998-1001.
- Emmert-Buck,M.R., Gillespie,J.W., Paweletz,C.P., Ornstein,D.K., Basrur,V., Appella,E., Wang,Q.H., Huang,J., Hu,N., Taylor,P., and Petricoin,E.F., III (2000) An approach to proteomic analysis of human tumors. *Mol. Carcinog.* *27*, 158-165.
- Emmert-Buck,M.R., Roth,M.J., Zhuang,Z., Campo,E., Rozhin,J., Sloane,B.F., Liotta,L.A., and Stetler-Stevenson,W.G. (1994) Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. *Am. J. Pathol.* *145*, 1285-1290.
- Endemann,G., Stanton,L.W., Madden,K.S., Bryant,C.M., White,R.T., and Protter,A.A. (1993) CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* *268*, 11811-11816.
- Engelse,M.A., Lardenoye,J.H., Neele,J.M., Grimbergen,J.M., De Vries,M.R., Lamfers,M.L., Pannekoek,H., Quax,P.H., and De Vries,C.J. (2002) Adenoviral activin a expression prevents intimal hyperplasia in human and murine blood vessels by maintaining the contractile smooth muscle cell phenotype. *Circ. Res.* *90*, 1128-1134.
- Faber,B.C., Cleutjens,K.B., Niessen,R.L., Aarts,P.L., Boon,W., Greenberg,A.S., Kitslaar,P.J., Tordoir,J.H., and Daemen,M.J. (2001) Identification of genes potentially involved in rupture of human atherosclerotic plaques. *Circ. Res.* *89*, 547-554.
- Fabunmi,R.P., Baker,A.H., Murray,E.J., Booth,R.F., and Newby,A.C. (1996) Divergent regulation by growth factors and cytokines of 95 kDa and 72 kDa gelatinases and tissue inhibitors or metalloproteinases-1, -2, and -3 in rabbit aortic smooth muscle cells. *Biochem. J.* *315 (Pt 1)*, 335-342.
- Feldman,A.L., Costouros,N.G., Wang,E., Qian,M., Marincola,F.M., Alexander,H.R., and Libutti,S.K. (2002)

- Advantages of mRNA amplification for microarray analysis. *Biotechniques* 33, 906-12, 914.
- Fend,F., Emmert-Buck,M.R., Chuaqui,R., Cole,K., Lee,J., Liotta,L.A., and Raffeld,M. (1999) Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am. J. Pathol.* 154, 61-66.
- Fend,F., Kremer,M., and Quintanilla-Martinez,L. (2000) Laser capture microdissection: methodical aspects and applications with emphasis on immuno-laser capture microdissection. *Pathobiology* 68, 209-214.
- Fend,F. and Raffeld,M. (2000) Laser capture microdissection in pathology. *J. Clin. Pathol.* 53, 666-672.
- Ferrara,N. and Davis-Smyth,T. (1997) The biology of vascular endothelial growth factor. *Endocr. Rev.* 18, 4-25.
- Ferrara,N., Gerber,H.P., and LeCouter,J. (2003) The biology of VEGF and its receptors. *Nat. Med.* 9, 669-676.
- Fessler,M.B., Malcolm,K.C., Duncan,M.W., and Worthen,G.S. (2002) A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase. *J. Biol. Chem.* 277, 31291-31302.
- Fodor,S.P., Read,J.L., Pirrung,M.C., Stryer,L., Lu,A.T., and Solas,D. (1991) Light-directed, spatially addressable parallel chemical synthesis. *Science* 251, 767-773.
- Folkman,J. and Shing,Y. (1992) Angiogenesis. *J. Biol. Chem.* 267, 10931-10934.
- Forster,T., Roy,D., and Ghazal,P. (2003) Experiments using microarray technology: limitations and standard operating procedures. *J. Endocrinol.* 178, 195-204.
- Fox,J.C. and Shanley,J.R. (1996) Antisense inhibition of basic fibroblast growth factor induces apoptosis in vascular smooth muscle cells. *J. Biol. Chem.* 271, 12578-12584.
- Frank,S., Hubner,G., Breier,G., Longaker,M.T., Greenhalgh,D.G., and Werner,S. (1995) Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. *J. Biol. Chem.* 270, 12607-12613.
- Frenette,P.S. and Wagner,D.D. (1996) Adhesion molecules--Part 1. *N. Engl. J. Med.* 334, 1526-1529.
- Fukushi,J., Ono,M., Morikawa,W., Iwamoto,Y., and Kuwano,M. (2000) The activity of soluble VCAM-1 in angiogenesis stimulated by IL-4 and IL-13. *J. Immunol.* 165, 2818-2823.
- Galis,Z.S., Muszynski,M., Sukhova,G.K., Simon-Morrissey,E., Unemori,E.N., Lark,M.W., Amento,E., and Libby,P. (1994a) Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ. Res.* 75, 181-189.
- Galis,Z.S., Sukhova,G.K., Lark,M.W., and Libby,P. (1994b) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J. Clin. Invest* 94, 2493-2503.
- Galperin,M.Y. (2004) The Molecular Biology Database Collection: 2004 update. *Nucleic Acids Res.* 32 Database issue, D3-22.
- Geary,R.L., Wong,J.M., Rossini,A., Schwartz,S.M., and Adams,L.D. (2002) Expression profiling identifies 147 genes contributing to a unique primate neointimal smooth muscle cell phenotype. *Arterioscler. Thromb. Vasc. Biol.* 22, 2010-2016.
- George,S.J. (1998) Tissue inhibitors of metalloproteinases and metalloproteinases in atherosclerosis. *Curr. Opin. Lipidol.* 9, 413-423.
- Gerber,H.P., Condorelli,F., Park,J., and Ferrara,N. (1997) Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J. Biol. Chem.* 272, 23659-23667.
- Gerhold,D., Rushmore,T., and Caskey,C.T. (1999) DNA chips: promising toys have become powerful tools. *Trends Biochem. Sci.* 24, 168-173.
- Gersh,B.J., Braunwald,E., and Bonow,R.O. (2001) Chronic coronary artery disease. In *Heart Disease*, E.Braunwald, D.P.Zipes, and P.Libby, eds. W.B. Saunders Company).
- Gerszten,R.E., Mach,F., Sauty,A., Rosenzweig,A., and Luster,A.D. (2000) Chemokines, leukocytes, and

- atherosclerosis. *J. Lab Clin. Med.* *136*, 87-92.
- Ghosh,S., St Clair,R.W., and Rudel,L.L. (2003) Mobilization of cytoplasmic CE droplets by overexpression of human macrophage cholesteryl ester hydrolase. *J. Lipid Res.* *44*, 1833-1840.
- Gillespie,D. and Spiegelman,S. (1965) A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* *12*, 829-842.
- Golub,T.R., Slonim,D.K., Tamayo,P., Huard,C., Gaasenbeek,M., Mesirov,J.P., Coller,H., Loh,M.L., Downing,J.R., Caligiuri,M.A., Bloomfield,C.D., and Lander,E.S. (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* *286*, 531-537.
- Grant,M.B., Wargovich,T.J., Ellis,E.A., Caballero,S., Mansour,M., and Pepine,C.J. (1994) Localization of insulin-like growth factor I and inhibition of coronary smooth muscle cell growth by somatostatin analogues in human coronary smooth muscle cells. A potential treatment for restenosis? *Circulation* *89*, 1511-1517.
- Gutterman,D.D. (1999) Adventitia-dependent influences on vascular function. *Am. J. Physiol* *277*, H1265-H1272.
- Guyton,J.R. and Klemp,K.F. (1996) Development of the lipid-rich core in human atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* *16*, 4-11.
- Gygi,S.P., Rochon,Y., Franza,B.R., and Aebersold,R. (1999) Correlation between protein and mRNA abundance in yeast. *Mol. Cell Biol.* *19*, 1720-1730.
- Haab,B.B., Dunham,M.J., and Brown,P.O. (2001) Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol.* *2*, RESEARCH0004.
- Haley,K.J., Lilly,C.M., Yang,J.H., Feng,Y., Kennedy,S.P., Turi,T.G., Thompson,J.F., Sukhova,G.H., Libby,P., and Lee,R.T. (2000) Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. *Circulation* *102*, 2185-2189.
- Han,S.R., Momeni,A., Strach,K., Suriyaphol,P., Fenske,D., Paprotka,K., Hashimoto,S.I., Torzewski,M., Bhakdi,S., and Husmann,M. (2003) Enzymatically modified LDL induces cathepsin H in human monocytes: potential relevance in early atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* *23*, 661-667.
- Hansson,G.K., Jonasson,L., Seifert,P.S., and Stemme,S. (1989) Immune mechanisms in atherosclerosis. *Arteriosclerosis* *9*, 567-578.
- Hansson,G.K., Libby,P., Schonbeck,U., and Yan,Z.Q. (2002) Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ. Res.* *91*, 281-291.
- Harris,M.B., Blackstone,M.A., Sood,S.G., Li,C., Goolsby,J.M., Venema,V.J., Kemp,B.E., and Venema,R.C. (2004) Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *Am. J. Physiol Heart Circ. Physiol.*
- Harrison,D., Griendling,K.K., Landmesser,U., Hornig,B., and Drexler,H. (2003) Role of oxidative stress in atherosclerosis. *Am. J. Cardiol.* *91*, 7A-11A.
- Hasegawa,S., Furukawa,Y., Li,M., Satoh,S., Kato,T., Watanabe,T., Katagiri,T., Tsunoda,T., Yamaoka,Y., and Nakamura,Y. (2002) Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. *Cancer Res.* *62*, 7012-7017.
- Hashimoto,S., Suzuki,T., Dong,H.Y., Yamazaki,N., and Matsushima,K. (1999) Serial analysis of gene expression in human monocytes and macrophages. *Blood* *94*, 837-844.
- Heldin,C.H. (1992) Structural and functional studies on platelet-derived growth factor. *EMBO J.* *11*, 4251-4259.
- Heldin,C.H. and Westermark,B. (1999) Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev.* *79*, 1283-1316.
- Heller,R.A., Schena,M., Chai,A., Shalon,D., Bedilion,T., Gilmore,J., Woolley,D.E., and Davis,R.W. (1997) Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. *Proc. Natl. Acad. Sci. U. S. A* *94*, 2150-2155.
- Hiller,R., Laffer,S., Harwanegg,C., Huber,M., Schmidt,W.M., Twardosz,A.,

- Barletta, B., Becker, W.M., Blaser, K., Breiteneder, H., Chapman, M., Cramer, R., Duchene, M., Ferreira, F., Fiebig, H., Hoffmann-Sommergruber, K., King, T.P., Kleber-Janke, T., Kurup, V.P., Lehrer, S.B., Lidholm, J., Muller, U., Pini, C., Reese, G., Scheiner, O., Scheynius, A., Shen, H.D., Spitzauer, S., Suck, R., Swoboda, I., Thomas, W., Tinghino, R., Hage-Hamsten, M., Virtanen, T., Kraft, D., Muller, M.W., and Valenta, R. (2002) Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J.* *16*, 414-416.
- Hiltunen, T.P., Luoma, J.S., Nikkari, T., and Yla-Herttuala, S. (1998) Expression of LDL receptor, VLDL receptor, LDL receptor-related protein, and scavenger receptor in rabbit atherosclerotic lesions: marked induction of scavenger receptor and VLDL receptor expression during lesion development. *Circulation* *97*, 1079-1086.
- Hochberg, Z., Hertz, P., Maor, G., Oiknine, J., and Aviram, M. (1992) Growth hormone and insulin-like growth factor-I increase macrophage uptake and degradation of low density lipoprotein. *Endocrinology* *131*, 430-435.
- Hofker, M.H., van Vlijmen, B.J., and Havekes, L.M. (1998) Transgenic mouse models to study the role of APOE in hyperlipidemia and atherosclerosis. *Atherosclerosis* *137*, 1-11.
- Holloway, A.J., van Laar, R.K., Tohill, R.W., and Bowtell, D.D. (2002) Options available--from start to finish--for obtaining data from DNA microarrays II. *Nat. Genet.* *32 Suppl*, 481-489.
- Horrevoets, A.J., Fontijn, R.D., van Zonneveld, A.J., De Vries, C.J., ten Cate, J.W., and Pannekoek, H. (1999) Vascular endothelial genes that are responsive to tumor necrosis factor- α in vitro are expressed in atherosclerotic lesions, including inhibitor of apoptosis protein-1, stannin, and two novel genes. *Blood* *93*, 3418-3431.
- Houseman, B.T., Huh, J.H., Kron, S.J., and Mrksich, M. (2002) Peptide chips for the quantitative evaluation of protein kinase activity. *Nat. Biotechnol.* *20*, 270-274.
- Huh, H.Y., Pearce, S.F., Yesner, L.M., Schindler, J.L., and Silverstein, R.L. (1996) Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation. *Blood* *87*, 2020-2028.
- Ihle, J.N. and Kerr, I.M. (1995) Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends Genet.* *11*, 69-74.
- Ishii, J., Adachi, H., Aoki, J., Koizumi, H., Tomita, S., Suzuki, T., Tsujimoto, M., Inoue, K., and Arai, H. (2002) SREC-II, a new member of the scavenger receptor type F family, trans-interacts with SREC-I through its extracellular domain. *J. Biol. Chem.* *277*, 39696-39702.
- Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C., Trent, J.M., Staudt, L.M., Hudson, J., Jr., Boguski, M.S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P.O. (1999) The transcriptional program in the response of human fibroblasts to serum. *Science* *283*, 83-87.
- Jenssen, T.K., Langaas, M., Kuo, W.P., Smith-Sorensen, B., Myklebost, O., and Hovig, E. (2002) Analysis of repeatability in spotted cDNA microarrays. *Nucleic Acids Res.* *30*, 3235-3244.
- Joannides, R., Haefeli, W.E., Linder, L., Richard, V., Bakkali, E.H., Thuillez, C., and Luscher, T.F. (1995) Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* *91*, 1314-1319.
- Johnston, N.L., Cervenak, J., Shore, A.D., Torrey, E.F., Yolken, R.H., and Cervenak, J. (1997) Multivariate analysis of RNA levels from postmortem human brains as measured by three different methods of RT-PCR. Stanley Neuropathology Consortium. *J. Neurosci. Methods* *77*, 83-92.
- Joos, T.O., Schrenk, M., Hopfl, P., Kroger, K., Chowdhury, U., Stoll, D., Schorner, D., Durr, M., Herick, K., Rupp, S., Sohn, K., and Hammerle, H. (2000) A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. *Electrophoresis* *21*, 2641-2650.
- Kallioniemi, O.P., Wagner, U., Kononen, J., and Sauter, G. (2001) Tissue microarray

- technology for high-throughput molecular profiling of cancer. *Hum. Mol. Genet.* *10*, 657-662.
- Katoh, M. (2002) Paradigm shift in gene-finding method: From bench-top approach to desk-top approach (review). *Int. J. Mol. Med.* *10*, 677-682.
- Kemmner, W., Roefzaad, C., Haensch, W., and Schlag, P.M. (2003) Glycosyltransferase expression in human colonic tissue examined by oligonucleotide arrays. *Biochim. Biophys. Acta* *1621*, 272-279.
- Kerr, M.K. and Churchill, G.A. (2001) Experimental design for gene expression microarrays. *Biostatistics.* *2*, 183-201.
- Kerr, M.K., Martin, M., and Churchill, G.A. (2000) Analysis of variance for gene expression microarray data. *J. Comput. Biol.* *7*, 819-837.
- Khachigian, L.M., Lindner, V., Williams, A.J., and Collins, T. (1996) Egr-1-induced endothelial gene expression: a common theme in vascular injury. *Science* *271*, 1427-1431.
- Kim, Y.D., Sohn, N.W., Kang, C., and Soh, Y. (2002) DNA array reveals altered gene expression in response to focal cerebral ischemia. *Brain Res. Bull.* *58*, 491-498.
- Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., and Krieger, M. (1990) Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature* *343*, 531-535.
- Kolble, K. (2000) The LEICA microdissection system: design and applications. *J. Mol. Med.* *78*, B24-B25.
- Kotani, M., Fukuda, N., Ando, H., Hu, W.Y., Kunimoto, S., Saito, S., and Kanmatsuse, K. (2003) Chimeric DNA-RNA hammerhead ribozyme targeting PDGF A-chain mRNA specifically inhibits neointima formation in rat carotid artery after balloon injury. *Cardiovasc. Res.* *57*, 265-276.
- Kovanen, P.T. (2000) Ateroskleroosin patologia ja molekulaariset syntytyvat. In *Kardiologia*, J.Heikkilä, H.Huikuri, K.Luomanmäki, M.S.Nieminen, and K.Peuhkurinen, eds. *Duodecim*, pp. 351-376.
- Kovanen, P.T. (1996) Mast cells in human fatty streaks and atheromas: implications for intimal lipid accumulation. *Curr. Opin. Lipidol.* *7*, 281-286.
- Krakauer, T. (1995) IL-10 inhibits the adhesion of leukocytic cells to IL-1-activated human endothelial cells. *Immunol. Lett.* *45*, 61-65.
- Kriegelstein, C.F. and Granger, D.N. (2001) Adhesion molecules and their role in vascular disease. *Am. J. Hypertens.* *14*, 44S-54S.
- Krishnaswamy, G., Kelley, J., Yerra, L., Smith, J.K., and Chi, D.S. (1999) Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. *J. Interferon Cytokine Res.* *19*, 91-104.
- Kuhn, H. and Chan, L. (1997) The role of 15-lipoxygenase in atherogenesis: pro- and antiatherogenic actions. *Curr. Opin. Lipidol.* *8*, 111-117.
- Kume, N. and Kita, T. (2001) Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in atherogenesis. *Trends Cardiovasc. Med.* *11*, 22-25.
- Laine, M.M. and Tuimala, J. (2003) Overview of data analysis. In *DNA Microarray Data Analysis*, J.Tuimala and M.M.Laine, eds. (Helsinki, Finland: CSC-Scientific Computing Ltd.), pp. 34-37.
- Laukkanen, J., Lehtolainen, P., Gough, P.J., Greaves, D.R., Gordon, S., and Yla-Herttuala, S. (2000) Adenovirus-mediated gene transfer of a secreted form of human macrophage scavenger receptor inhibits modified low-density lipoprotein degradation and foam-cell formation in macrophages. *Circulation* *101*, 1091-1096.
- Laukkanen, J. and Yla-Herttuala, S. (2002) Genes involved in atherosclerosis. *Exp. Nephrol.* *10*, 150-163.
- Leethanakul, C., Patel, V., Gillespie, J., Pallente, M., Ensley, J.F., Koontongkaew, S., Liotta, L.A., Emmert-Buck, M., and Gutkind, J.S. (2000a) Distinct pattern of expression of differentiation and growth-related genes in squamous cell carcinomas of the head and neck revealed by the use of laser capture microdissection and cDNA arrays. *Oncogene* *19*, 3220-3224.
- Leethanakul, C., Patel, V., Gillespie, J., Shillitoe, E., Kellman, R.M., Ensley, J.F., Limwongse, V., Emmert-Buck, M.R., Krizman, D.B., and Gutkind, J.S. (2000b) Gene expression profiles in squamous cell carcinomas of the oral

- cavity: use of laser capture microdissection for the construction and analysis of stage-specific cDNA libraries. *Oral Oncol.* 36, 474-483.
- Li, F. and Hui, D.Y. (1997) Modified low density lipoprotein enhances the secretion of bile salt-stimulated cholesterol esterase by human monocyte-macrophages. species-specific difference in macrophage cholesteryl ester hydrolase. *J. Biol. Chem.* 272, 28666-28671.
- Li, H., Cybulsky, M.I., Gimbrone, M.A., Jr., and Libby, P. (1993) Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. *Am. J. Pathol.* 143, 1551-1559.
- Li, H., Freeman, M.W., and Libby, P. (1995) Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and in vitro by cytokines. *J. Clin. Invest* 95, 122-133.
- Li, H., Lewis, A., Brodsky, S., Rieger, R., Iden, C., and Goligorsky, M.S. (2002a) Homocysteine induces 3-hydroxy-3-methylglutaryl coenzyme a reductase in vascular endothelial cells: a mechanism for development of atherosclerosis? *Circulation* 105, 1037-1043.
- Li, J., Brown, L.F., Hibberd, M.G., Grossman, J.D., Morgan, J.P., and Simons, M. (1996) VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. *Am. J. Physiol* 270, H1803-H1811.
- Li, J., Pankratz, M., and Johnson, J.A. (2002b) Differential gene expression patterns revealed by oligonucleotide versus long cDNA arrays. *Toxicol. Sci.* 69, 383-390.
- Li, N. and Karin, M. (1999) Is NF-kappaB the sensor of oxidative stress? *FASEB J.* 13, 1137-1143.
- Lian, Z., Kluger, Y., Greenbaum, D.S., Tuck, D., Gerstein, M., Berliner, N., Weissman, S.M., and Newburger, P.E. (2002) Genomic and proteomic analysis of the myeloid differentiation program: global analysis of gene expression during induced differentiation in the MPRO cell line. *Blood* 100, 3209-3220.
- Lian, Z., Wang, L., Yamaga, S., Bonds, W., Beazer-Barclay, Y., Kluger, Y., Gerstein, M., Newburger, P.E., Berliner, N., and Weissman, S.M. (2001) Genomic and proteomic analysis of the myeloid differentiation program. *Blood* 98, 513-524.
- Libby, P. (2002) Inflammation in atherosclerosis. *Nature* 420, 868-874.
- Libby, P., Ridker, P.M., and Maseri, A. (2002) Inflammation and atherosclerosis. *Circulation* 105, 1135-1143.
- Lindner, V., Lappi, D.A., Baird, A., Majack, R.A., and Reidy, M.A. (1991) Role of basic fibroblast growth factor in vascular lesion formation. *Circ. Res.* 68, 106-113.
- Lindner, V., Majack, R.A., and Reidy, M.A. (1990) Basic fibroblast growth factor stimulates endothelial regrowth and proliferation in denuded arteries. *J. Clin. Invest* 85, 2004-2008.
- Liotta, L.A., Espina, V., Mehta, A.I., Calvert, V., Rosenblatt, K., Geho, D., Munson, P.J., Young, L., Wulfkuhle, J., and Petricoin, E.F., III (2003) Protein microarrays: meeting analytical challenges for clinical applications. *Cancer Cell* 3, 317-325.
- Lockhart, D.J., Dong, H., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E.L. (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* 14, 1675-1680.
- Lockhart, D.J. and Winzler, E.A. (2000) Genomics, gene expression and DNA arrays. *Nature* 405, 827-836.
- Lueking, A., Horn, M., Eickhoff, H., Bussow, K., Lehrach, H., and Walter, G. (1999) Protein microarrays for gene expression and antibody screening. *Anal. Biochem.* 270, 103-111.
- Luo, L., Salunga, R.C., Guo, H., Bittner, A., Joy, K.C., Galindo, J.E., Xiao, H., Rogers, K.E., Wan, J.S., Jackson, M.R., and Erlander, M.G. (1999) Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat. Med.* 5, 117-122.
- Luzzi, V., Holtschlag, V., and Watson, M.A. (2001) Expression profiling of ductal carcinoma in situ by laser capture microdissection and high-density oligonucleotide arrays. *Am. J. Pathol.* 158, 2005-2010.
- Ma, X.J., Salunga, R., Tuggle, J.T., Gaudet, J., Enright, E., McQuary, P., Payette, T., Pistone, M., Stecker, K., Zhang, B.M.,

- Zhou, Y.X., Varnholt, H., Smith, B., Gadd, M., Chatfield, E., Kessler, J., Baer, T.M., Erlander, M.G., and Sgroi, D.C. (2003) Gene expression profiles of human breast cancer progression. *Proc. Natl. Acad. Sci. U. S. A* *100*, 5974-5979.
- MacBeath, G. and Schreiber, S.L. (2000) Printing proteins as microarrays for high-throughput function determination. *Science* *289*, 1760-1763.
- Mallat, Z., Besnard, S., Duriez, M., Deleuze, V., Emmanuel, F., Bureau, M.F., Soubrier, F., Esposito, B., Duez, H., Fievet, C., Staels, B., Duverger, N., Scherman, D., and Tedgui, A. (1999) Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* *85*, e17-e24.
- Marian, A.J. and Gollob, M.H. (2003) Genomics Perspective for Drug Discovery. In *Proteomic and Genomic Analysis of Cardiovascular Disease*, J.E. Van Eyk and M.J. Dunn, eds. (Darmstadt, Germany: WILEY-VCH Verlag GmbH & Co.), pp. 341-358.
- Martinet, W., Knaapen, M.W., De Meyer, G.R., Herman, A.G., and Kockx, M.M. (2003) Overexpression of the anti-apoptotic caspase-2 short isoform in macrophage-derived foam cells of human atherosclerotic plaques. *Am. J. Pathol.* *162*, 731-736.
- Martinet, W., Schrijvers, D.M., De Meyer, G.R., Thielemans, J., Knaapen, M.W., Herman, A.G., and Kockx, M.M. (2002) Gene expression profiling of apoptosis-related genes in human atherosclerosis: upregulation of death-associated protein kinase. *Arterioscler. Thromb. Vasc. Biol.* *22*, 2023-2029.
- Marx, N., Sukhova, G., Murphy, C., Libby, P., and Plutzky, J. (1998) Macrophages in human atheroma contain PPARgamma: differentiation-dependent peroxisomal proliferator-activated receptor gamma (PPARgamma) expression and reduction of MMP-9 activity through PPARgamma activation in mononuclear phagocytes in vitro. *Am. J. Pathol.* *153*, 17-23.
- Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asaoka, H., Hayakawa, I., Kanamori, H., Aburatani, H., Takaku, F., Suzuki, H., and . (1990) Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions. *Proc. Natl. Acad. Sci. U. S. A* *87*, 9133-9137.
- McCaffrey, T.A. (2000) TGF-beta and TGF-beta receptors in atherosclerosis. *Cytokine Growth Factor Rev.* *11*, 103-114.
- McCaffrey, T.A., Fu, C., Du, B., Eksinar, S., Kent, K.C., Bush, H., Jr., Kreiger, K., Rosengart, T., Cybulsky, M.I., Silverman, E.S., and Collins, T. (2000) High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J. Clin. Invest.* *105*, 653-662.
- McCormick, S.M., Eskin, S.G., McIntire, L.V., Teng, C.L., Lu, C.M., Russell, C.G., and Chittur, K.K. (2001) DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc. Natl. Acad. Sci. U. S. A* *98*, 8955-8960.
- McEver, R.P., Moore, K.L., and Cummings, R.D. (1995) Leukocyte trafficking mediated by selectin-carbohydrate interactions. *J. Biol. Chem.* *270*, 11025-11028.
- Meager, A. (1999) Cytokine regulation of cellular adhesion molecule expression in inflammation. *Cytokine Growth Factor Rev.* *10*, 27-39.
- Meyer, R., Hatada, E.N., Hohmann, H.P., Haiker, M., Bartsch, C., Rothlisberger, U., Lahm, H.W., Schlaeger, E.J., van Loon, A.P., and Scheidereit, C. (1991) Cloning of the DNA-binding subunit of human nuclear factor kappa B: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor alpha. *Proc. Natl. Acad. Sci. U. S. A* *88*, 966-970.
- Mietus-Snyder, M., Gowri, M.S., and Pitas, R.E. (2000) Class A scavenger receptor up-regulation in smooth muscle cells by oxidized low density lipoprotein. Enhancement by calcium flux and concurrent cyclooxygenase-2 up-regulation. *J. Biol. Chem.* *275*, 17661-17670.
- Mii, S., Ware, J.A., and Kent, K.C. (1993) Transforming growth factor-beta inhibits human vascular smooth muscle cell growth and migration. *Surgery* *114*, 464-470.
- Mikita, T., Porter, G., Lawn, R.M., and Shiffman, D. (2001) Oxidized low density lipoprotein exposure alters the transcriptional response of macrophages

- to inflammatory stimulus. *J. Biol. Chem.* 276, 45729-45739.
- Murakami, M. and Kudo, I. (2003) New phospholipase A(2) isozymes with a potential role in atherosclerosis. *Curr. Opin. Lipidol.* 14, 431-436.
- Muzykantov, V.R. (2001) Targeting of superoxide dismutase and catalase to vascular endothelium. *J. Control Release* 71, 1-21.
- Nakahashi, T.K., Hoshina, K., Tsao, P.S., Sho, E., Sho, M., Karwowski, J.K., Yeh, C., Yang, R.B., Topper, J.N., and Dalman, R.L. (2002) Flow loading induces macrophage antioxidative gene expression in experimental aneurysms. *Arterioscler. Thromb. Vasc. Biol.* 22, 2017-2022.
- Nakata, A., Nakagawa, Y., Nishida, M., Nozaki, S., Miyagawa, J., Nakagawa, T., Tamura, R., Matsumoto, K., Kameda-Takemura, K., Yamashita, S., and Matsuzawa, Y. (1999) CD36, a novel receptor for oxidized low-density lipoproteins, is highly expressed on lipid-laden macrophages in human atherosclerotic aorta. *Arterioscler. Thromb. Vasc. Biol.* 19, 1333-1339.
- Napoli, C., de Nigris, F., Welch, J.S., Calara, F.B., Stuart, R.O., Glass, C.K., and Palinski, W. (2002) Maternal hypercholesterolemia during pregnancy promotes early atherogenesis in LDL receptor-deficient mice and alters aortic gene expression determined by microarray. *Circulation* 105, 1360-1367.
- Nasmyth, K. (1996) Viewpoint: putting the cell cycle in order. *Science* 274, 1643-1645.
- Nelken, N.A., Coughlin, S.R., Gordon, D., and Wilcox, J.N. (1991) Monocyte chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest* 88, 1121-1127.
- Newman, P.J., Berndt, M.C., Gorski, J., White, G.C., Lyman, S., Paddock, C., and Muller, W.A. (1990) PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science* 247, 1219-1222.
- Nicosia, R.F., Nicosia, S.V., and Smith, M. (1994) Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. *Am. J. Pathol.* 145, 1023-1029.
- Norata, G.D., Pirillo, A., Callegari, E., Hamsten, A., Catapano, A.L., and Eriksson, P. (2003) Gene expression and intracellular pathways involved in endothelial dysfunction induced by VLDL and oxidised VLDL. *Cardiovasc. Res.* 59, 169-180.
- Nordestgaard, B.G. (1996) The vascular endothelial barrier--selective retention of lipoproteins. *Curr. Opin. Lipidol.* 7, 269-273.
- O'Toole, L. and Grech, E.D. (2003) Chronic stable angina: treatment options. *BMJ* 326, 1185-1188.
- Oesterreich, S., Allred, D.C., Mohsin, S.K., Zhang, Q., Wong, H., Lee, A.V., Osborne, C.K., and O'Connell, P. (2001) High rates of loss of heterozygosity on chromosome 19p13 in human breast cancer. *Br. J. Cancer* 84, 493-498.
- Ohura, N., Yamamoto, K., Ichioka, S., Sokabe, T., Nakatsuka, H., Baba, A., Shibata, M., Nakatsuka, T., Harii, K., Wada, Y., Kohro, T., Kodama, T., and Ando, J. (2003) Global analysis of shear stress-responsive genes in vascular endothelial cells. *J. Atheroscler. Thromb.* 10, 304-313.
- Ohyama, H., Zhang, X., Kohno, Y., Alevizos, I., Posner, M., Wong, D.T., and Todd, R. (2000) Laser capture microdissection-generated target sample for high-density oligonucleotide array hybridization. *Biotechniques* 29, 530-536.
- Okulicz, W.C., Ace, C.I., and Torres, M.S. (2003) Gene expression in the rhesus monkey endometrium: differential display and laser capture microdissection. *Front Biosci.* 8, d551-d558.
- Palinski, W. and Napoli, C. (2002) Unraveling pleiotropic effects of statins on plaque rupture. *Arterioscler. Thromb. Vasc. Biol.* 22, 1745-1750.
- Paoni, N.F., Peale, F., Wang, F., Errett-Baroncini, C., Steinmetz, H., Toy, K., Bai, W., Williams, P.M., Bunting, S., Gerritsen, M.E., and Powell-Braxton, L. (2002) Time course of skeletal muscle repair and gene expression following acute hind limb ischemia in mice. *Physiol Genomics* 11, 263-272.
- Pentikainen, M.O., Oorni, K., Ala-Korpela, M., and Kovanen, P.T. (2000) Modified LDL - trigger of atherosclerosis and inflammation in the arterial intima. *J. Intern. Med.* 247, 359-370.

- Pober, J.S. and Cotran, R.S. (1990) Cytokines and endothelial cell biology. *Physiol Rev.* 70, 427-451.
- Pollman, M.J., Naumovski, L., and Gibbons, G.H. (1999) Vascular cell apoptosis: cell type-specific modulation by transforming growth factor-beta1 in endothelial cells versus smooth muscle cells. *Circulation* 99, 2019-2026.
- Preli, R.B., Klein, K.P., and Herrington, D.M. (2002) Vascular effects of dietary L-arginine supplementation. *Atherosclerosis* 162, 1-15.
- Price, D.T. and Loscalzo, J. (1999) Cellular adhesion molecules and atherogenesis. *Am. J. Med.* 107, 85-97.
- Rader, D.J. (2003) Regulation of reverse cholesterol transport and clinical implications. *Am. J. Cardiol.* 92, 42J-49J.
- Radomski, M.W. and Moncada, S. (1993) The biological and pharmacological role of nitric oxide in platelet function. *Adv. Exp. Med. Biol.* 344, 251-264.
- Ramprasad, M.P., Terpstra, V., Kondratenko, N., Quehenberger, O., and Steinberg, D. (1996) Cell surface expression of mouse macrophage receptors for oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A* 93, 14833-14838.
- Randi, A.M., Biguzzi, E., Falciani, F., Merlini, P., Blakemore, S., Bramucci, E., Lucreziotti, S., Lennon, M., Faioni, E.M., Ardissino, D., and Mannucci, P.M. (2003) Identification of differentially expressed genes in coronary atherosclerotic plaques from patients with stable or unstable angina by cDNA array analysis. *J. Thromb. Haemost.* 1, 829-835.
- Ranganna, K., Yousefipour, Z., Yatsu, F.M., Milton, S.G., and Hayes, B.E. (2003) Gene expression profile of butyrate-inhibited vascular smooth muscle cell proliferation. *Mol. Cell Biochem.* 254, 21-36.
- Reape, T.J., Rayner, K., Manning, C.D., Gee, A.N., Barnette, M.S., Burnand, K.G., and Groot, P.H. (1999) Expression and cellular localization of the CC chemokines PARC and ELC in human atherosclerotic plaques. *Am. J. Pathol.* 154, 365-374.
- Renier, G., Clement, I., Desfaits, A.C., and Lambert, A. (1996) Direct stimulatory effect of insulin-like growth factor-I on monocyte and macrophage tumor necrosis factor-alpha production. *Endocrinology* 137, 4611-4618.
- Resnick, N., Yahav, H., Schubert, S., Wolfowitz, E., and Shay, A. (2000) Signalling pathways in vascular endothelium activated by shear stress: relevance to atherosclerosis. *Curr. Opin. Lipidol.* 11, 167-177.
- Reunanen, A. (2000) Kroonisen sepelvaltimotautaudin epidemiologia. In *Kardiologia*, J.Heikkilä, H.Huikuri, K.Luomanmäki, M.S.Nieminen, and K.Peuhkurinen, eds. *Duodecim*, pp. 377-383.
- Risau, W., Drexler, H., Mironov, V., Smits, A., Siegbahn, A., Funa, K., and Heldin, C.H. (1992) Platelet-derived growth factor is angiogenic in vivo. *Growth Factors* 7, 261-266.
- Rissanen, T. T. (2003) Gene Transfer for Blood and Lymphatic Vessel Growth.
- Rissanen, T.T., Vajanto, I., and Yla-Herttuala, S. (2001) Gene therapy for therapeutic angiogenesis in critically ischaemic lower limb - on the way to the clinic. *Eur. J. Clin. Invest* 31, 651-666.
- Robinson, W.H., DiGennaro, C., Hueber, W., Haab, B.B., Kamachi, M., Dean, E.J., Fournel, S., Fong, D., Genovese, M.C., de Vegvar, H.E., Skriver, K., Hirschberg, D.L., Morris, R.I., Muller, S., Pruijn, G.J., van Venrooij, W.J., Smolen, J.S., Brown, P.O., Steinman, L., and Utz, P.J. (2002) Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat. Med.* 8, 295-301.
- Rodriguez, C., Raposo, B., Martinez-Gonzalez, J., Casani, L., and Badimon, L. (2002) Low density lipoproteins downregulate lysyl oxidase in vascular endothelial cells and the arterial wall. *Arterioscler. Thromb. Vasc. Biol.* 22, 1409-1414.
- Rogers, Y.H., Jiang-Baucom, P., Huang, Z.J., Bogdanov, V., Anderson, S., and Boyce-Jacino, M.T. (1999) Immobilization of oligonucleotides onto a glass support via disulfide bonds: A method for preparation of DNA microarrays. *Anal. Biochem.* 266, 23-30.

- Roses,A.D. (2000) Pharmacogenetics and the practice of medicine. *Nature* 405, 857-865.
- Ross,R. (1986) The pathogenesis of atherosclerosis--an update. *N. Engl. J. Med.* 314, 488-500.
- Ross,R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362, 801-809.
- Ross,R. (1999) Atherosclerosis is an inflammatory disease. *Am. Heart J.* 138, S419-S420.
- Rosseau,S., Selhorst,J., Wiechmann,K., Leissner,K., Maus,U., Mayer,K., Grimminger,F., Seeger,W., and Lohmeyer,J. (2000) Monocyte migration through the alveolar epithelial barrier: adhesion molecule mechanisms and impact of chemokines. *J. Immunol.* 164, 427-435.
- Rutanen,J., Rissanen,T.T., Kivela,A., Vajanto,I., and Yla-Herttua,S. (2001) Clinical applications of vascular gene therapy. *Curr. Cardiol. Rep.* 3, 29-36.
- Sack,M. (2002) Tumor necrosis factor-alpha in cardiovascular biology and the potential role for anti-tumor necrosis factor-alpha therapy in heart disease. *Pharmacol. Ther.* 94, 123-135.
- Sawamura,T., Kume,N., Aoyama,T., Moriwaki,H., Hoshikawa,H., Aiba,Y., Tanaka,T., Miwa,S., Katsura,Y., Kita,T., and Masaki,T. (1997) An endothelial receptor for oxidized low-density lipoprotein. *Nature* 386, 73-77.
- Schena,M., Shalon,D., Davis,R.W., and Brown,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467-470.
- Schinke,M., Riggi,L., Butte,A.J., and Izumo,S. (2003) Large Scale Expression Profiling in Cardiovascular Disease Using Microarrays: Prospects and Pitfalls. In *Proteomic and Genomic Analysis of Cardiovascular Disease*, J.E. Van Eyk and M.J.Dunn, eds. (Darmstadt, Germany: WILEY-VCH Verlag GmbH & Co.), pp. 3-26.
- Schmitz,G., Langmann,T., and Heimerl,S. (2001) Role of ABCG1 and other ABCG family members in lipid metabolism. *J. Lipid Res.* 42, 1513-1520.
- Schoen,F.J. and Cotran,R.S. (1999) Blood vessels. In *Pathologic basis of disease*, R.S.Cotran, K.V.Kumar, and T.Collins, eds. W.B. Saunders Company), pp. 493-541.
- Schwartz,S.M., Majesky,M.W., and Murry,C.E. (1995) The intima: development and monoclonal responses to injury. *Atherosclerosis* 118 Suppl, S125-S140.
- Schweitzer,B. and Kingsmore,S.F. (2002) Measuring proteins on microarrays. *Curr. Opin. Biotechnol.* 13, 14-19.
- Shaulian,E. and Karin,M. (2002) AP-1 as a regulator of cell life and death. *Nat. Cell Biol.* 4, E131-E136.
- Shibata,D., Hawes,D., Li,Z.H., Hernandez,A.M., Spruck,C.H., and Nichols,P.W. (1992) Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. *Am. J. Pathol.* 141, 539-543.
- Shiffman,D., Mikita,T., Tai,J.T., Wade,D.P., Porter,J.G., Seilhamer,J.J., Somogyi,R., Liang,S., and Lawn,R.M. (2000) Large scale gene expression analysis of cholesterol-loaded macrophages. *J. Biol. Chem.* 275, 37324-37332.
- Sidawy,A.N., Mitchell,M.E., and Neville,R.F. (1998) Peptide growth factors and signal transduction. *Semin. Vasc. Surg.* 11, 149-155.
- Siegbahn,A., Hammacher,A., Westermarck,B., and Heldin,C.H. (1990) Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. *J. Clin. Invest* 85, 916-920.
- Southern,E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98, 503-517.
- Sporn,M.B., Roberts,A.B., Wakefield,L.M., and de Crombrughe,B. (1987) Some recent advances in the chemistry and biology of transforming growth factor-beta. *J. Cell Biol.* 105, 1039-1045.
- Springer,T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76, 301-314.
- Sreekumar,A., Nyati,M.K., Varambally,S., Barrette,T.R., Ghosh,D., Lawrence,T.S., and Chinnaiyan,A.M. (2001) Profiling of cancer cells using protein microarrays: discovery of novel

- radiation-regulated proteins. *Cancer Res.* 61, 7585-7593.
- Stanton,L.W. (2001) Methods to profile gene expression. *Trends Cardiovasc. Med.* 11, 49-54.
- Stanton,L.W., Garrard,L.J., Damm,D., Garrick,B.L., Lam,A., Kapoun,A.M., Zheng,Q., Protter,A.A., Schreiner,G.F., and White,R.T. (2000) Altered patterns of gene expression in response to myocardial infarction. *Circ. Res.* 86, 939-945.
- Stary,H.C., Blankenhorn,D.H., Chandler,A.B., Glagov,S., Insull,W., Jr., Richardson,M., Rosenfeld,M.E., Schaffer,S.A., Schwartz,C.J., Wagner,W.D., and . (1992) A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler. Thromb.* 12, 120-134.
- Stary,H.C., Chandler,A.B., Dinsmore,R.E., Fuster,V., Glagov,S., Insull,W., Jr., Rosenfeld,M.E., Schwartz,C.J., Wagner,W.D., and Wissler,R.W. (1995) A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 92, 1355-1374.
- Stary,H.C., Chandler,A.B., Glagov,S., Guyton,J.R., Insull,W., Jr., Rosenfeld,M.E., Schaffer,S.A., Schwartz,C.J., Wagner,W.D., and Wissler,R.W. (1994) A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler. Thromb.* 14, 840-856.
- Steinberg,D. (1983) Lipoproteins and atherosclerosis. A look back and a look ahead. *Arteriosclerosis* 3, 283-301.
- Steinberg,D., Parthasarathy,S., Carew,T.E., Khoo,J.C., and Witztum,J.L. (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* 320, 915-924.
- Steinberg,D. and Witztum,J.L. (1999) Lipoproteins, Lipoprotein Oxidation and Atherogenesis. In *Molecular Basis of Cardiovascular Disease*, K.R.Chien, ed. (Philadelphia: W.B. Saunders Company), pp. 458-471.
- Stoeckert,C.J., Jr., Causton,H.C., and Ball,C.A. (2002) Microarray databases: standards and ontologies. *Nat. Genet.* 32 *Suppl.*, 469-473.
- Sukhanov,S., Hua,S.Y., and Delafontaine,P. (2003) Global analysis of differentially expressed genes in oxidized LDL-treated human aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* 306, 443-449.
- Svensson,P.A., Englund,M.C., Markstrom,E., Ohlsson,B.G., Jernas,M., Billig,H., Torgerson,J.S., Wiklund,O., Carlsson,L.M., and Carlsson,B. (2003) Copper induces the expression of cholesterologenic genes in human macrophages. *Atherosclerosis* 169, 71-76.
- Tall,A.R., Dammerman,M., and Breslow,J.L. (1999) Disorders of lipoprotein metabolism. In *Molecular Basis of Cardiovascular Disease*, K.R.Chien, ed. (Philadelphia: W.B. Saunders Company), pp. 413-425.
- Tamayo,P., Slonim,D., Mesirov,J., Zhu,Q., Kitareewan,S., Dmitrovsky,E., Lander,E.S., and Golub,T.R. (1999) Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc. Natl. Acad. Sci. U. S. A* 96, 2907-2912.
- Tavazoie,S., Hughes,J.D., Campbell,M.J., Cho,R.J., and Church,G.M. (1999) Systematic determination of genetic network architecture. *Nat. Genet.* 22, 281-285.
- Tedgui,A. and Mallat,Z. (2001) Anti-inflammatory mechanisms in the vascular wall. *Circ. Res.* 88, 877-887.
- Terpstra,V., van Amersfoort,E.S., van Velzen,A.G., Kuiper,J., and van Berkel,T.J. (2000) Hepatic and extrahepatic scavenger receptors: function in relation to disease. *Arterioscler. Thromb. Vasc. Biol.* 20, 1860-1872.
- Thomas,K.A. (1996) Vascular endothelial growth factor, a potent and selective

- angiogenic agent. *J. Biol. Chem.* 271, 603-606.
- Todd,R. and Margolin,D.H. (2002) Challenges of single-cell diagnostics: analysis of gene expression. *Trends Mol. Med.* 8, 254-257.
- Tontonoz,P., Nagy,L., Alvarez,J.G., Thomazy,V.A., and Evans,R.M. (1998) PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 93, 241-252.
- Topper,J.N., Cai,J., Falb,D., and Gimbrone,M.A., Jr. (1996) Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc. Natl. Acad. Sci. U. S. A* 93, 10417-10422.
- Toronen,P., Kolehmainen,M., Wong,G., and Castren,E. (1999) Analysis of gene expression data using self-organizing maps. *FEBS Lett.* 451, 142-146.
- Trogan,E., Choudhury,R.P., Dansky,H.M., Rong,J.X., Breslow,J.L., and Fisher,E.A. (2002) Laser capture microdissection analysis of gene expression in macrophages from atherosclerotic lesions of apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* 99, 2234-2239.
- Tseng,G.C., Oh,M.K., Rohlin,L., Liao,J.C., and Wong,W.H. (2001) Issues in cDNA microarray analysis: quality filtering, channel normalization, models of variations and assessment of gene effects. *Nucleic Acids Res.* 29, 2549-2557.
- Tsuchiya,S., Kobayashi,Y., Goto,Y., Okumura,H., Nakae,S., Konno,T., and Tada,K. (1982) Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.* 42, 1530-1536.
- Tsukamoto,K., Kinoshita,M., Kojima,K., Mikuni,Y., Kudo,M., Mori,M., Fujita,M., Horie,E., Shimazu,N., and Teramoto,T. (2002) Synergically increased expression of CD36, CLA-1 and CD68, but not of SR-A and LOX-1, with the progression to foam cells from macrophages. *J. Atheroscler. Thromb.* 9, 57-64.
- Tsunawaki,S., Sporn,M., Ding,A., and Nathan,C. (1988) Deactivation of macrophages by transforming growth factor-beta. *Nature* 334, 260-262.
- Tuimala,J. (2003) Basic Statistics. In *DNA Microarray Data Analysis*, J.Tuimala and M.M.Laine, eds. (Helsinki, Finland: CSC-Scientific Computing Ltd.), pp. 42-64.
- Tung,W.S., Lee,J.K., and Thompson,R.W. (2001) Simultaneous analysis of 1176 gene products in normal human aorta and abdominal aortic aneurysms using a membrane-based complementary DNA expression array. *J. Vasc. Surg.* 34, 143-150.
- Tuomisto,T.T. and Yla-Herttuala,S. (2003) Genes Involved in Atherosclerosis and Plaque Formation. In *Proteomic and Genomic Analysis of Cardiovascular Disease*, J.E.Van Eyk and M.J.Dunn, eds. (Weinheim, Germany: WILEY-VCH Verlag GmbH & Co.), pp. 141-151.
- Ungvari,Z., Csiszar,A., Edwards,J.G., Kaminski,P.M., Wolin,M.S., Kaley,G., and Koller,A. (2003) Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of tumor necrosis factor-alpha, NAD(P)H oxidase, and inducible nitric oxide synthase. *Arterioscler. Thromb. Vasc. Biol.* 23, 418-424.
- Vajanto,I., Rissanen,T.T., Rutanen,J., Hiltunen,M.O., Tuomisto,T.T., Arve,K., Narvanen,O., Manninen,H., Rasanen,H., Hippelainen,M., Alhava,E., and Yla-Herttuala,S. (2002) Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. *J. Gene Med.* 4, 371-380.
- Venter,J.C., Adams,M.D., Myers,E.W., Li,P.W., Mural,R.J., Sutton,G.G., Smith,H.O., Yandell,M., Evans,C.A., Holt,R.A., Gocayne,J.D., Amanatides,P., Ballew,R.M., Huson,D.H., Wortman,J.R., Zhang,Q., Kodira,C.D., Zheng,X.H., Chen,L., Skupski,M., Subramanian,G., Thomas,P.D., Zhang,J., Gabor Miklos,G.L., Nelson,C., Broder,S., Clark,A.G., Nadeau,J., McKusick,V.A., Zinder,N., Levine,A.J., Roberts,R.J., Simon,M., Slayman,C., Hunkapiller,M.,

- Bolanos,R., Delcher,A., Dew,I., Fasulo,D., Flanigan,M., Florea,L., Halpern,A., Hannenhalli,S., Kravitz,S., Levy,S., Mobarry,C., Reinert,K., Remington,K., Abu-Threideh,J., Beasley,E., Biddick,K., Bonazzi,V., Brandon,R., Cargill,M., Chandramouliswaran,I., Charlab,R., Chaturvedi,K., Deng,Z., Di,F., V, Dunn,P., Eilbeck,K., Evangelista,C., Gabrielian,A.E., Gan,W., Ge,W., Gong,F., Gu,Z., Guan,P., Heiman,T.J., Higgins,M.E., Ji,R.R., Ke,Z., Ketchum,K.A., Lai,Z., Lei,Y., Li,Z., Li,J., Liang,Y., Lin,X., Lu,F., Merkulov,G.V., Milshina,N., Moore,H.M., Naik,A.K., Narayan,V.A., Neelam,B., Nusskern,D., Rusch,D.B., Salzberg,S., Shao,W., Shue,B., Sun,J., Wang,Z., Wang,A., Wang,X., Wang,J., Wei,M., Wides,R., Xiao,C., Yan,C., Yao,A., Ye,J., Zhan,M., Zhang,W., Zhang,H., Zhao,Q., Zheng,L., Zhong,F., Zhong,W., Zhu,S., Zhao,S., Gilbert,D., Baumhueter,S., Spier,G., Carter,C., Cravchik,A., Woodage,T., Ali,F., An,H., Awe,A., Baldwin,D., Baden,H., Barnstead,M., Barrow,I., Beeson,K., Busam,D., Carver,A., Center,A., Cheng,M.L., Curry,L., Danaher,S., Davenport,L., Desilets,R., Dietz,S., Dodson,K., Doup,L., Ferreira,S., Garg,N., Gluecksmann,A., Hart,B., Haynes,J., Haynes,C., Heiner,C., Hladun,S., Hostin,D., Houck,J., Howland,T., Ibegwam,C., Johnson,J., Kalush,F., Kline,L., Koduru,S., Love,A., Mann,F., May,D., McCawley,S., McIntosh,T., McMullen,I., Moy,M., Moy,L., Murphy,B., Nelson,K., Pfannkoch,C., Pratts,E., Puri,V., Qureshi,H., Reardon,M., Rodriguez,R., Rogers,Y.H., Romblad,D., Ruhfel,B., Scott,R., Sitter,C., Smallwood,M., Stewart,E., Strong,R., Suh,E., Thomas,R., Tint,N.N., Tse,S., Vech,C., Wang,G., Wetter,J., Williams,S., Williams,M., Windsor,S., Winn-Deen,E., Wolfe,K., Zaveri,J., Zaveri,K., Abril,J.F., Guigo,R., Campbell,M.J., Sjolander,K.V., Karlak,B., Kejariwal,A., Mi,H., Lazareva,B., Hatton,T., Narechania,A., Diemer,K., Muruganujan,A., Guo,N., Sato,S., Bafna,V., Istrail,S., Lippert,R., Schwartz,R., Walenz,B., Yooseph,S., Allen,D., Basu,A., Baxendale,J., Blick,L., Caminha,M., Carnes-Stine,J., Caulk,P., Chiang,Y.H., Coyne,M., Dahlke,C., Mays,A., Dombroski,M., Donnelly,M., Ely,D., Esparham,S., Fosler,C., Gire,H., Glanowski,S., Glasser,K., Glodek,A., Gorokhov,M., Graham,K., Gropman,B., Harris,M., Heil,J., Henderson,S., Hoover,J., Jennings,D., Jordan,C., Jordan,J., Kasha,J., Kagan,L., Kraft,C., Levitsky,A., Lewis,M., Liu,X., Lopez,J., Ma,D., Majoros,W., McDaniel,J., Murphy,S., Newman,M., Nguyen,T., Nguyen,N., and Nodell,M. (2001) The sequence of the human genome. *Science* 291, 1304-1351.
- Verhoeckx,K.C., Bijlsma,S., de Groene,E.M., Witkamp,R.F., van der,G.J., and Rodenburg,R.J. (2004) A combination of proteomics, principal component analysis and transcriptomics is a powerful tool for the identification of biomarkers for macrophage maturation in the U937 cell line. *Proteomics*. 4, 1014-1028.
- Vihinen,M. and Tuimala,J. (2003) Cluster Analysis of Microarray Information. In *DNA Microarray Data Analysis*, J.Tuimala and M.M.Laine, eds. (Helsinki, Finland: CSC-Scientific Computing Ltd.), pp. 108-119.
- Virgili,F., Ambra,R., Muratori,F., Natella,F., Majewicz,J., Minihane,A.M., and Rimbach,G. (2003) Effect of oxidized low-density lipoprotein on differential gene expression in primary human endothelial cells. *Antioxid. Redox. Signal.* 5, 237-247.
- Vuong,H., Patterson,T., Adisheshaiah,P., Shapiro,P., Kalvakolanu,D.V., and Reddy,S.P. (2002) JNK1 and AP-1 regulate PMA-inducible squamous differentiation marker expression in Clara-like H441 cells. *Am. J. Physiol Lung Cell Mol. Physiol* 282, L215-L225.
- Walczak,R. and Tontonoz,P. (2002) PPARadigms and PPARadoxes: expanding roles for PPARgamma in the control of lipid metabolism. *J. Lipid Res.* 43, 177-186.
- Waltenberger,J. (1997) Modulation of growth factor action: implications for the

- treatment of cardiovascular diseases. *Circulation* 96, 4083-4094.
- Waltenberger, J., Lundin, L., Oberg, K., Wilander, E., Miyazono, K., Heldin, C.H., and Funa, K. (1993a) Involvement of transforming growth factor-beta in the formation of fibrotic lesions in carcinoid heart disease. *Am. J. Pathol.* 142, 71-78.
- Waltenberger, J., Wanders, A., Fellstrom, B., Miyazono, K., Heldin, C.H., and Funa, K. (1993b) Induction of transforming growth factor-beta during cardiac allograft rejection. *J. Immunol.* 151, 1147-1157.
- Walter, G., Bussow, K., Lueking, A., and Glokler, J. (2002) High-throughput protein arrays: prospects for molecular diagnostics. *Trends Mol. Med.* 8, 250-253.
- Wang, X., Reape, T.J., Li, X., Rayner, K., Webb, C.L., Burnand, K.G., and Lysko, P.G. (1999) Induced expression of adipophilin mRNA in human macrophages stimulated with oxidized low-density lipoprotein and in atherosclerotic lesions. *FEBS Lett.* 462, 145-150.
- Wang, X., Yue, T.L., Ohlstein, E.H., Sung, C.P., and Feuerstein, G.Z. (1996) Interferon-inducible protein-10 involves vascular smooth muscle cell migration, proliferation, and inflammatory response. *J. Biol. Chem.* 271, 24286-24293.
- Wang, X.Q., Panousis, C.G., Alfaro, M.L., Evans, G.F., and Zuckerman, S.H. (2002) Interferon-gamma-mediated downregulation of cholesterol efflux and ABC1 expression is by the Stat1 pathway. *Arterioscler. Thromb. Vasc. Biol.* 22, e5-e9.
- Wasserman, B.A. (2002) Clinical carotid atherosclerosis. *Neuroimaging Clin. N. Am.* 12, 403-419.
- Wilson, A.S., Hobbs, B.G., Speed, T.P., and Rakoczy, P.E. (2002) The microarray: potential applications for ophthalmic research. *Mol. Vis.* 8, 259-270.
- Wiltshire, S., O'Malley, S., Lambert, J., Kukanskis, K., Edgar, D., Kingsmore, S.F., and Schweitzer, B. (2000) Detection of multiple allergen-specific IgEs on microarrays by immunoassay with rolling circle amplification. *Clin. Chem.* 46, 1990-1993.
- Woodside, K.J., Hernandez, A., Smith, F.W., Xue, X.Y., Hu, M., Daller, J.A., and Hunter, G.C. (2003) Differential gene expression in primary and recurrent carotid stenosis. *Biochem. Biophys. Res. Commun.* 302, 509-514.
- Wuttge, D.M., Sirsjo, A., Eriksson, P., and Stemme, S. (2001) Gene expression in atherosclerotic lesion of ApoE deficient mice. *Mol. Med.* 7, 383-392.
- Xie, B., Dong, Z., and Fidler, I.J. (1994) Regulatory mechanisms for the expression of type IV collagenases/gelatinases in murine macrophages. *J. Immunol.* 152, 3637-3644.
- Xu, B.J., Caprioli, R.M., Sanders, M.E., and Jensen, R.A. (2002) Direct analysis of laser capture microdissected cells by MALDI mass spectrometry. *J. Am. Soc. Mass Spectrom.* 13, 1292-1297.
- Yamada, Y., Doi, T., Hamakubo, T., and Kodama, T. (1998) Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. *Cell Mol. Life Sci.* 54, 628-640.
- Yap, A.S. and Kovacs, E.M. (2003) Direct cadherin-activated cell signaling: a view from the plasma membrane. *J. Cell Biol.* 160, 11-16.
- Yla-Herttuala, S. (1994) Role of lipid and lipoprotein oxidation in the pathogenesis of atherosclerosis. *Drugs of Today* 30, 507-514.
- Yla-Herttuala, S. and Martin, J.F. (2000) Cardiovascular gene therapy. *Lancet* 355, 213-222.
- Yla-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L., and Steinberg, D. (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest* 84, 1086-1095.
- Yla-Herttuala, S., Rosenfeld, M.E., Parthasarathy, S., Glass, C.K., Sigal, E., Witztum, J.L., and Steinberg, D. (1990) Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc. Natl. Acad. Sci. U. S. A* 87, 6959-6963.

- Yla-Herttuala,S., Rosenfeld,M.E., Parthasarathy,S., Sigal,E., Sarkioja,T., Witztum,J.L., and Steinberg,D. (1991) Gene expression in macrophage-rich human atherosclerotic lesions. 15-lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts. *J. Clin. Invest* 87, 1146-1152.
- Yla-Herttuala,S., Solakivi,T., Hirvonen,J., Laaksonen,H., Mottonen,M., Pesonen,E., Raekallio,J., Akerblom,H.K., and Nikkari,T. (1987) Glycosaminoglycans and apolipoproteins B and A-I in human aortas. Chemical and immunological analysis of lesion-free aortas from children and adults. *Arteriosclerosis* 7, 333-340.
- Yoshida,M., Sawada,T., Ishii,H., Gerszten,R.E., Rosenzweig,A., Gimbrone,M.A., Jr., Yasukochi,Y., and Numano,F. (2001) Hmg-CoA reductase inhibitor modulates monocyte-endothelial cell interaction under physiological flow conditions in vitro: involvement of Rho GTPase-dependent mechanism. *Arterioscler. Thromb. Vasc. Biol.* 21, 1165-1171.
- Zhao,N., Hashida,H., Takahashi,N., Misumi,Y., and Sakaki,Y. (1995) High-density cDNA filter analysis: a novel approach for large-scale, quantitative analysis of gene expression. *Gene* 156, 207-213.
- Zhou,X., Paulsson,G., Stemme,S., and Hansson,G.K. (1998) Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J. Clin. Invest* 101, 1717-1725.
- Zhu,H., Klemic,J.F., Chang,S., Bertone,P., Casamayor,A., Klemic,K.G., Smith,D., Gerstein,M., Reed,M.A., and Snyder,M. (2000) Analysis of yeast protein kinases using protein chips. *Nat. Genet.* 26, 283-289.
- Zhu,H. and Snyder,M. (2003) Protein chip technology. *Curr. Opin. Chem. Biol.* 7, 55-63.
- Zingg,J.M., Ricciarelli,R., Andorno,E., and Azzi,A. (2002) Novel 5' exon of scavenger receptor CD36 is expressed in cultured human vascular smooth muscle cells and atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* 22, 412-417.
- Zohlhofer,D., Klein,C.A., Richter,T., Brandl,R., Murr,A., Nuhrenberg,T., Schomig,A., Baeuerle,P.A., and Neumann,F.J. (2001) Gene expression profiling of human stent-induced neointima by cDNA array analysis of microscopic specimens retrieved by helix cutter atherectomy: Detection of FK506-binding protein 12 upregulation. *Circulation* 103, 1396-1402.
- Zubay,G.L., Parson,W.W., and Vance,D.E. (1995) Regulation of Gene Expression in Eukaryotes. In *Principles of Biochemistry*, G.L.Zubay, W.W.Parson, and D.E.Vance, eds. (Dubuque, United States of America: Wm. C. Brown Publisher), pp. 800-829.