Vascular Biology, Atherosclerosis and Endothelium Biology

Distinctive Expression of Chemokines and Transforming Growth Factor- β Signaling in Human Arterial Endothelium during Atherosclerosis

Oscar L. Volger,* Joost O. Fledderus,*
Natasja Kisters,[†] Ruud D. Fontijn,*
Perry D. Moerland,[‡] Johan Kuiper,[§]
Theo J. van Berkel,[§] Ann-Pascale J.J. Bijlens,[†]
Mat J.A.P. Daemen,[†] Hans Pannekoek,* and
Anton J.G. Horrevoets*

From the Departments of Medical Biochemistry* and Clinical Epidemiology, Biostatistics, Bioinformatics,[†] Academic Medical Center, University of Amsterdam, Amsterdam; the Division of Biopharmaceutics,[§] Gorlaeus Laboratories, Leiden University, Leiden; and the Department of Pathology,[‡] Cardiovascular Research Institute Maastricht, University of Maastricht, Maastricht, The Netherlands

Knowledge about the *in vivo* role of endothelium in chronic human atherosclerosis has mostly been derived by insights from mouse models. Therefore, we set out to establish by microarray analyses the gene expression profiles of endothelium from human large arteries, as isolated by laser microbeam microdissection, having focal atherosclerosis of the early or the advanced stage. Within individual arteries, the endothelial transcriptomes of the lesional and unaffected sides were compared pairwise, thus limiting genetic and environmental confounders. Specific endothelial signature gene sets were identified with changed expression levels in either early ($n = 718$) or advanced atherosclerosis ($n = 403$), relative to their paired plaque-free controls. Gene set enrichment analysis identified distinct sets of chemokines and differential enrichments of nuclear factor- κ B-, p53-, and transforming growth factor- β -related genes in advanced plaques. Immunohistochemistry validated the discriminative value of corresponding endothelial protein expression between early (fractalkine/CX3CL1, IP10/CCL10, TBX18) or advanced (BAX, NF κ B2) stages of atherosclerosis and versus their plaque-free controls. The functional involvement of transforming growth factor- β signaling in directing its downstream gene repertoire was substantiated by a consistent detection of activated SMAD2 in advanced lesions. Thus, we identified truly common, local

molecular denominators of pathological changes to vascular endothelium, with a marked distinction of endothelial phenotype between early and advanced plaques. (Am J Pathol 2007, 171:326–337; DOI: 10.2353/ajpath.2007.061196)

Inflammatory activation of vascular endothelium plays a central role in the development of atherosclerosis.¹ This occurs via trafficking and retention of leukocytes into the vessel wall by an enhanced expression of chemokines and adhesion molecules, such as CCR2, E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1).^{2,3} The inflammatory status of the endothelium can be enhanced by a diverse set of local and systemic stimuli, such as oxidized low-density lipoprotein (oxLDL), interleukin-1, tumor necrosis factor- α , and C-reactive protein.^{4–6} Nuclear factor (NF)- κ B, which can be activated by several of these stimuli, including cytokines and oxidized lipids,^{7,8} has shown to be an important transcription factor responsible for modulating this enhanced proinflammatory status.⁹ In addition, hemodynamic forces have been shown to modulate endothelial inflammation and the development of atherosclerotic plaques.^{10,11} Throughout the past decade, inbred mouse models of accelerated atherosclerosis have also significantly contributed to the large body of pathophysiological knowledge on the development of atherosclerosis.¹² In addition to these mouse studies

Supported by the Netherlands Organization for Scientific Research, (NWO) genomics grant 050-110-1014, the European Union, the European Vascular Genomics Network (grant LSHM-CT-2003-1503254), the Netherlands Heart Foundation, The Hague (Molecular Cardiology Program grant M93.007).

Accepted for publication March 26, 2007.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Dr. A.J.G. Horrevoets, Department of Medical Biochemistry, Room K1-114, Academic Medical Center, University of Amsterdam, Meibergdreef 15, NL-1105 AZ, Amsterdam, The Netherlands. E-mail: a.j.horrevoets@amc.uva.nl.

and single-gene approaches, a number of recent studies have assessed the role of transcriptome changes in the development of human atherosclerosis.¹³ However, there is little direct knowledge about the *in vivo* role of the endothelium in the development of chronic atherosclerosis in humans.

Previous human gene expression profiling studies, in which atherosclerotic tissues were compared with plaque-free tissues from different individuals, have been complicated by confounding effects of interindividual genetic variation, differences in vessel-specific transcriptomes, and differences in systemic parameters.¹³ One of the distinctive properties of human atherosclerosis is, however, the focality of its lesions, probably related to differences in local hemodynamics.^{10,11} Hence, within a single artery, distinct regions with early or advanced plaques coexist with morphologically normal looking vascular wall, despite the fact that both individual genetic background as well as exposure to systemic, circulating risk factors such as high low-density lipoprotein (LDL) levels or proinflammatory cytokines are identical. Apparently, because this is observed in all major arteries affected by the disease, there must be common functional denominators that lie at the basis of pathological changes of the vessel wall, irrespective of genetic background, vascular origin, or systemic factors. We chose to study this by applying laser microbeam microdissection (LMM) for the isolation of the arterial endothelial cells. Since the introduction of LMM, it has become possible to obtain cell type-enriched isolates from vascular material.¹⁴ In addition, T7 RNA polymerase-mediated mRNA amplification has enabled reliable microarray gene expression profiling from small quantities of input mRNA, both *in vitro* and *ex vivo*.^{15–20} To identify only the genes that are potentially functionally involved in lesion formation, irrespective of arterial location and individual differences, we used a diverse set of human large arteries of which the types as well as the locations were randomly selected. To minimize the individual or local confounders, we envisioned a paired comparative approach thereby enabling us to study gene expression differences in a limited set of arteries. Thus, we compared within individual arterial sections with focal atherosclerosis, the endothelial microarray expression profiles of endothelium from plaque-free regions with endothelium overlying atherosclerotic plaques of either the early or the advanced stage. Strikingly, we observed that there was little overlap between the genes with changed microarray intensities in the early and advanced stages of atherosclerosis, as compared with the plaque-free controls. At the molecular pathway level, these plaque stage-specific differences in the microarray data, as assessed by gene set enrichment analysis (GSEA),²¹ revealed important roles for transforming growth factor (TGF)- β -signaling in endothelium during advanced atherosclerosis. In addition, the present study confirmed the involvement of NF- κ B and p53 signaling and of chemokines during plaque progression in human patients.

Materials and Methods

Collection of Human Vasculature and Classification of Atherosclerosis

Human large arteries were collected postmortem after disease in compliance with institutional guidelines, being an informed consent as approved by the medical ethical committees of the Academic Medical Center (Amsterdam, The Netherlands) or the Academic Hospital Maastricht (Maastricht, The Netherlands), in full compliance with the principles and ethical considerations of the Declaration of Helsinki (1989). Samples collected in the Department of Pathology were stored in the Maastricht Pathology Tissue Collection, and use of tissue and patient data was performed in agreement with the "Code for Proper Secondary Use of Human Tissue in The Netherlands" (<http://www.federa.org/?s=1&m=78&p=&v=4>). The atherosclerotic plaques were classified according to Virmani and colleagues.²² The main criterion for discriminating between early and advanced plaques was the presence of a fibrous cap.

Endothelial Cell Isolation, RNA Isolation, Microarray Analysis, Statistical Analysis

Human arteries were fixed in Zinkfix (24 hours at 4°C). Zinkfix was prepared as described by Scheidl and colleagues.²⁰ For the isolation of endothelial cells, 10- μ m-thick cross sections were cut by using a microtome (no. 340E; Microm International, Walldorf, Germany) and attached to UV-crosslinked PET membrane frame slides (no. 11505151; Leica, Wetzlar, Germany) by using 0.001% (w/v) poly-L-lysine (P8920; Sigma-Aldrich, St. Louis, MO). After deparaffinization (100% xylene, 1 minute; 100% ethanol, 1 minute) and subsequent air-drying for 2 hours at room temperature, LMM was applied for isolation of the endothelial monolayer from four serial cross sections (P.A.L.M. Systems, Bernried, Germany). CD31 immunopositivity was used as a marker for endothelial integrity. RNA was isolated after proteinase K digestion (2 hours at 45°C) by using a paraffin block kit (no. 1902; Ambion, Austin, TX), including the digestion with DNase I. The RNAs were then linearly amplified for two rounds (RiboAmp kit, kit0102; Arcturus, Mountain View, CA), followed by a third-round amplification (MessageAmp aRNA kit, no. 1750; Ambion), synthesizing anti-sense cRNAs with an average base length of 500 nucleotides (see Supplemental Figure 1 at <http://ajp.amjpathol.org>). Within these cRNAs the molar ratio of incorporated aminoallyl-rUTP (A5660; Sigma-Aldrich) to rUTP was 1:1. Cy3 or Cy5 monoreactive dyes (PA23001, PA25001; Amersham Biosciences, Piscataway, NJ) were coupled according to the manufacturer's instructions. Labeled cRNA was purified using the RNeasy purification kit (Qiagen GmbH, Hilden, Germany). Each endothelial cRNA sample (1.5 μ g; Cy5-labeled) was hybridized in duplicate against a common reference cRNA sample (1.5 μ g; Cy3-labeled) on glass-based microarrays representing 18,649 unique oligonucleotide sequences (Micro Array

Table 1. Human Donor Arteries Used in LMM and Immunohistochemistry

No.	Type	Atherosclerosis	Classification	Sex	Age	Cause of death
1	I	Plaque-free, early	IX	Female	43	SAH
2	C	Plaque-free, early	IX	Female	70	Pneumonia and AML
3	C	Plaque-free, early	IX	Female	30	OC
4	C	Early (GSEA)	IX	Male	58	Aortic rupture
5	C	Plaque-free, early, advanced	IT, IX, FCA	Male	85	CH (trauma)
6	C	Plaque-free, early, advanced	PIT, FCP	Male	85	Fatal MI, liver failure
7	A	Plaque-free, early, advanced	IX, FCP	Male	43	CH (trauma)
8	I	Plaque-free, advanced	FCA			
9	C	Plaque-free, advanced	FCA	Female	62	VC
10	I	Plaque-free, advanced	FCA	Female	51	CH (trauma)
11	El	Plaque-free, early	IT	Male	30	Acute trauma
12	C	Plaque-free, early	IX	Male	36	Unknown
13	C	Plaque-free, early	IX	Female	77	Unknown
14	A	Plaque-free, early	IX	Female	41	SAH
15	Cl	Plaque-free, early	IX	Male	41	Sepsis
16	Cl	Plaque-free, early	IX	Male	55	Fatal MI
17	C	Plaque-free, advanced	FCA	Male	60	Unknown
18	C	Plaque-free, advanced	FCA	Male	70	Unknown
19	Cl	Plaque-free, advanced	FCA	Male	49	Alcohol intoxication
20	AB	Plaque-free, advanced	FCP	Male	48	Brain infarction
21	C	Plaque-free, advanced	FCP	Female	62	Unknown

AML, myeloid leukemia; CH, cerebral hemorrhage; OC, ovary carcinoma; MI, myocardial infarction; SAH, subarachnoid hemorrhage; VC, vulva carcinoma; Virmani-classified²² lesions: early plaques: IT, intimal thickening; PIT, pathological intimal thickening; IX, intimal xanthoma; advanced plaques: FCA, fibrous cap atheroma; FCP, fibro-calcific plaques; arteries: A, abdominal aorta; AB, aortic bifurcation; C, carotid; Cl, common iliac; El, external iliac.

Department, University of Amsterdam, Amsterdam, The Netherlands; <http://www.microarray.nl/libraries.html>). The common reference cRNA sample comprised equal quantities of the following three components 1) human umbilical cord endothelial cells, stimulated with tumor necrosis factor- α (50 μ g/ml, 6 hours); 2) THP-1 cell line incubated for 24 hours with phorbol myristate acetate (100 μ g/ml), subsequently for 6 hours with lipopolysaccharide (1 μ g/ml); and 3) a mix of whole-mount human aorta and iliac artery. The microarray intensity data were acquired and imported in the Rosetta Resolver database after Loess normalization (limma package; Bioconductor; <http://www.bioconductor.org>) and were statistically analyzed by a paired Cyber-T test, essentially as previously described,²³ and analyzed at the functional group level by using GSEA, of which the methodology has been described.²¹

Statistical Analyses

After combining the duplicate microarray data per sample, a paired Cyber-T test was applied to the microarray intensity data, as previously described.²³ Criteria for significant differences: Bayesian *P* value <0.05 and an average microarray intensity >20. The genome-wide microarray expression profiles were analyzed at the functional group level, by using GSEA, of which the methodology has been described.²¹ The dataset that was used for GSEA comprised 8574 unique gene symbols. Filtering criteria for the microarray dataset gene set were the presence of a gene symbol and an average microarray intensity >20. The molecular signature database that was used for the GSEA was a modified version of the original set

named c2.symbols.gmt (available on-line at http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html) that was extended with the most relevant KEGG pathways and consisted of 594 gene sets. Analysis settings were 100 permutations on phenotype; gene sets with more than 50 genes were included in the analysis. The gene sets named NF- κ B-induced, p53-signaling, and TGF- β -induced consisted of 61, 77, and 89 genes, respectively, that were present in the microarray dataset.

Immunohistochemistry

A separate set of human arteries from different individuals with focal atherosclerosis was used for immunohistochemical validation (nos. 11 to 20, Table 1). Individual cross sections were incubated with the listed antibodies (Table 2). All antibodies were diluted in Tris-buffered saline containing bovine serum albumin [1% (w/v); Sigma] and Tween 20 [0.01% (w/v)]. The incubations with the listed secondary biotin-conjugated antibodies were followed by amplification with a streptavidin-horseradish peroxidase complex (no. K0377; DAKO, Glostrup, Denmark) and a peroxidase-substrate staining (SK-4800, Nova Red kit; Vector Laboratories, Burlingame, CA).

Results

Endothelial Gene Expression Profiling in Atherosclerosis

The atherosclerotic plaques were classified according to Virmani and colleagues.²² The main criterion for

Table 2. Antibodies Used for Immunohistochemistry

Antibody reactivity	Host species	Code	Clone	Manufacturer	Incubation
CD31	Mouse mAb	M0823	JC70A	DakoCytomation Denmark A/S	Overnight at 4°C
SMC α -actin	M0851	1A4	1 hour at room temperature		
Monocyte/macrophage	M063201	HAM56	2 hours at room temperature		
BAX	Mouse mAb	18-0218	2D2	Zymed, South San Francisco, CA	Overnight at 4°C
CD81	Mouse mAb	MCA1847	1D6	Serotec, Oxford, UK	
CX3CL1	Mouse mAb	mAB3651	81513	R&D Systems, Minneapolis, MN	
CYP1B1	Rabbit pAb	CYP1B11-A		Alpha Diagnostics, San Antonio, TX	
ICAM-1	Mouse mAb	18-0173	My13	Zymed	
IP10	Goat pAb	AF266		R&D Systems	
NF- κ B2	Rabbit pAb	ARP32043		Aviva Systems Biology, San Diego, CA	
Phosphorylated-SMAD2*	Rabbit pAb			Persson et al	
TBX18	Rabbit pAb	PAB-11145		Orbigen, San Diego, CA	
Mouse IgG-biotin	Goat pAb	E0433		DakoCytomation Denmark A/S	30 minutes at room temperature
Rabbit IgG-biotin	Goat pAb	E0432		DakoCytomation Denmark A/S	
Goat IgG-biotin	Donkey pAb	BAF109		R&D Systems	

*For details about the antibodies raised against the active phosphorylated forms of SMAD1 and SMAD2, respectively, see Persson et al.⁵⁷
mAb, monoclonal antibody; pAb, polyclonal antibody.

discriminating between early and advanced plaques was the presence of a fibrous cap. The transcriptome differences between the endothelium of plaque-free sections and sections having initial plaques or plaques of the advanced stage were probed by microarray gene expression profiling. LMM was applied for the

isolation of arterial endothelium (Figure 1, A and B). To find the truly common local molecular denominators of pathological changes to the vascular wall, a pairwise comparison was made within individual arteries. A representative example of this approach in an individual human carotid artery with a lateral plaque of the advanced stage is

Figure 1. LMM to identify differential endothelial genes within a single carotid artery with focal atherosclerosis. Representative photomicrographs of arterial cross sections show the isolation of intimal endothelium from an atherosclerotic arterial cross section by LMM (A, B) and show a carotid artery (no. 5, Table 1) having a lateral advanced plaque (fibrous cap atheroma), opposite to a plaque-free artery section (C). An overview (A) and an enlargement of the white squared part in (A), showing the microdissected intimal layer denuded from endothelium, are given in B. Here, the laser track surrounding the dissected area is indicated by the black arrows with a dotted line. Two separate endothelial samples were isolated from serial cross sections of the carotid artery (C), being endothelial cells of the plaque-free vessel wall and of the advanced plaque. The M-versus-A plot compares the endothelial microarray intensity profiles from the plaque with the plaque-free section (reratio experiment; Rosetta Resolver) (D). M, Log10-ratio (advanced/early); A, log10 (advanced + early/2). Advanced plaque, 165 genes increased intensity (red dots); plaque-free: 113 genes increased intensity (green dots) ($P < 0.05$, Rosetta Resolver). Error bars: SD of the intensities. M-versus-A plots depict the endothelial microarray data for a set of 13 SMC-marker genes (red dots), based on Nelander et al.²⁴ for the paired comparisons between the plaque-free situation and either early (E) or advanced (F) atherosclerosis, respectively. The microarray intensities of these SMC markers are depicted for all of the individual LMM-endothelium samples (G, red lines); their average values are given for the plaque-free situation (white bars) and early (gray bars) and advanced atherosclerosis (black bars), respectively (H).

Figure 2. Paired Cyber-T analyses reveal genes with differential microarray intensity ratios in atherosclerosis. These analyses compared within arteries the endothelium from plaques of either the early ($n = 7$ arteries) or the advanced stage ($n = 6$ arteries) to their plaque-free controls, respectively. In the early plaques, 762 sequences had differential microarray intensities ($n = 375$ up, $n = 387$ down), whereas 447 sequences had differential intensities in the advanced stage ($n = 252$ up; $n = 195$ down) compared with their plaque-free controls. **A:** The genes that were significantly changed in these separate paired statistical analyses are compared in a Venn diagram. Two-way hierarchical clustering of these genes, comparing the microarray intensity ratios of early atherosclerosis divided by plaque-free (**B**) and advanced atherosclerosis divided by plaque-free (**C**), are also given. Clustering method, WPGMA (weighted average); similarity measure, Euclidean distance. **Colored arrows**, compared with plaque-free controls: red, increased; green, decreased.

shown (Figure 1C). Furthermore, within this artery the endothelial microarray intensity data of the sections without and with atherosclerosis can be directly compared, using Rosetta Resolver statistics as shown in the scatterplot (Figure 1D).

Currently, it is technically impossible to isolate 100% pure endothelium by LMM from human arterial walls because, in contrast to mice, the intimal layers of human large arteries without histological signs of atherosclerosis often contain smooth muscle cells (SMCs). To verify whether the level of SMC contamination was comparable in our LMM endothelium samples, we checked these samples for whether there were differences between the microarray intensities of 13 SMC-specific genes, based on a publication of Nelander et al²⁴ (Figure 1, E–H). Paired statistical Cyber-T tests, comparing the plaque-free situation with either the early or the advanced stage of atherosclerosis, respectively, showed that there were no differences between the microarray intensities (see Supplemental Table 1 at <http://ajp.amjpathol.org>).

Differential Gene Expression Profiles in Early and Advanced Plaques

The individual paired analyses for a diverse set of large human arteries (Table 1) were collectively analyzed by

paired Cyber-T statistical analysis, corrected for false discovery rate by the Benjamini-Hochberg method.²⁵ A large set of reproducibly differential genes were detected at high statistical significance, which were commonly and reproducibly differential between lesional endothelium versus the endothelium overlying the apparently normal sites of the same arteries. Intriguingly, these signature sets revealed a clear distinction between the sets of genes showing differential microarray intensities in endothelium overlying plaques of the early or the advanced stage relative to their plaque-free controls, with a very limited overlap (Figure 2A). Heat maps show the reproducibility of these findings between the different individual arteries that constitute the two distinct groups (Figure 2, B and C). The corresponding lists of genes are given in Supplemental Table 2 (available at <http://ajp.amjpathol.org>). Manual inspection revealed that the absence in these sets of a number of established marker genes derived from murine models was caused by a lack of statistical significance mainly attributable to low-intensity levels on microarray, the relatively small sample size, or lack of consistency between all of the samples analyzed. Thus, increased levels of ICAM-1 and VCAM-1 and decreased levels of KLF2 were detected, although not at statistically significant levels. Still, we could confirm increases at the level of ICAM-1 protein expression, for

Table 3. Paired Evaluation of the Microarray Data by Immunohistochemistry in Endothelium from Large Human Arteries with Focal Atherosclerosis of the Early or the Advanced Stage

Antibody	Early versus plaque-free		Advanced versus plaque-free	
	Plaque-free	Early	Plaque-free	Advanced
CX3CL1	0/6	5/6	0/5	3/5
CYP1B1	1/6	3/6	2/5	2/5
ICAM-1	0/6	5/6	0/5	4/5
IP10	3/6	6/6	4/5	4/5
TBX18	0/6	3/6	1/5	1/5
BAX	0/6	1/6	0/5	3/5
NFKB2	0/6	1/6	0/5	5/5
pSMAD2	0/6	1/6	0/5	5/5

Immunohistochemistry was performed on cross sections from human arteries (nos. 11 to 21 in Table 1) with focal atherosclerosis of the early ($n = 6$) or the advanced stage ($n = 5$). Ratio scores: the number of artery sections with clusters of immunopositive endothelial cells over the total number of evaluated sections. A cluster is defined as more than three adjacent cells with immunopositivity (Figures 5 and 6).

example, in atherosclerotic plaques of the early and the advanced stage by paired semiquantitative immunohistochemical analysis (see below, Table 3). These data corroborate earlier findings that microarray mRNA expression profiling analyses are not suitable to detect

all expected known genes, especially in the low-intensity range in which background noise can be an issue. Rather, microarray expression profiling is typically suited for detection of large numbers of novel (panels of) genes and of genome-wide signatures.

Figure 3. GSEA of the microarray data identifies differential genes and pathways between plaque-free, early, and advanced atherosclerosis. **A:** Hierarchical clustering of the top 100 of most differential genes between plaque-free, early, and advanced atherosclerosis, as defined by GSEA, is given. GSEA, directly comparing atherosclerotic plaques of the advanced stage with the early stage, identified three gene sets with specific enrichments in advanced atherosclerosis, being NF- κ B-induced (**B**), p53 signaling (**C**), and TGF- β -induced (**D**), compared with early atherosclerosis having 4.0, 0.01, and 8.2% of false positives (Benjamini false discovery rate, correction), respectively. Gene symbol colors, comparing the intensity ratios of advanced with early atherosclerosis; red, increased in advanced plaques; green, increased in early plaques. Although this GSEA did not identify enriched gene sets in early atherosclerosis, individual genes within the NF- κ B and TGF- β sets were enriched in this early stage. These early-enriched genes are depicted as green symbols (**B** and **D**). Values at the right side of each gene symbol represent the GSEA ranking. A one-way average linkage clustering of the core genes with enrichments within the NF- κ B, p53, and TGF- β sets, as shown in **B–D**, is given in **E**. Hierarchical clustering (**A** and **E**): colors of the squares represent the relative expression of the genes in the artery section given in the column heading as established after median centering of normalized hybridization signals. Red and green represent higher and lower expression than the median for that particular gene, respectively. Color intensity is related to the difference with the median (black).

Distinctive Gene Programs and Pathways in Early and Advanced Plaques

The transcriptome differences between the normal plaque-free situation and the early and advanced stages of atherosclerosis, respectively, were further analyzed by pathway-based GSEA. The resulting identification of corresponding top 100 signature gene panels rank-ordered according to the intensity differences between these three situations were arranged by hierarchical clustering (Figure 3A). Corresponding GSEA lists of the top 100 of most differential genes are shown in Supplemental Table 3 at <http://ajp.amjpathol.org>.

A further in-depth analysis of the expression profiles was performed with gene set and pathway analysis software to detect specific functional or diagnostic signatures. At the molecular pathway level, we first focused on the differences between plaques of the early stage and the plaque-free situation. GSEA showed that seven gene sets were enriched in early atherosclerosis with nominal *P* values <0.01 (data not shown), whereas one gene set, named leukocyte chemotaxis adhesion and diapedesis, was enriched with high significance (nominal *P* value <0.001; percent false positives = 22), including MCP-1 (CCL2) and fractalkine (CX3CL1) (see Supplemental Table 4 at <http://ajp.amjpathol.org>). Indeed, by immunohistochemical analyses we specifically confirmed elevated protein expression of CX3CL1 (see below, Figure 5 and Table 3) in early and advanced atherosclerotic lesions, compared with the plaque-free control sections. Next, our novel finding of the distinct differences in atherosclerosis-related transcriptome in early versus advanced atherosclerosis was analyzed at the pathway level by GSEA. This revealed that three gene sets, named NF- κ B-induced, p53 signaling, and TGF- β pathway, were enriched in the advanced situation (nominal *P* values <0.0001). In contrast, no specific gene sets or functional pathways were enriched in early atherosclerosis, despite the large number of individual genes found differential with either normal looking or advanced stage atherosclerosis endothelium. The early or late atherosclerosis-enriched core genes within these sets, as defined by GSEA, are given (Figure 3, B–D), showing the increased expression of NF- κ B, p53, and the TGF- β gene sets specifically in advanced atherosclerosis. After combining the microarray profiles of the core genes from these three gene sets, hierarchical clustering visualizes the two distinct groups of genes with an elevated relative expression level in either early or advanced atherosclerosis, respectively (Figure 3E).

Chemokine Involvement in Human Arterial Endothelium

Remarkably, there was a considerable overlap at the individual gene level between the NF- κ B- and TGF- β -induced gene sets (Figure 3, B and D), including several chemokines, being CXCL-2, -3, -6, -10, interleukin-8,

A colored map (GenMAPP)

B M versus A plot of genes enriched after GSEA

Figure 4. Microarray data of genes involved in leukocyte endothelial cell interactions in advanced versus early atherosclerosis. **A:** A scheme showing the GSEA results is presented, separating these genes into specific functional categories, being the chemotaxis, rolling, firm adhesion to the arterial endothelium, diapedesis, and the activation of leukocytes. Symbol colors: red, core-enriched in advanced atherosclerosis; green, core-enriched in early atherosclerosis; yellow, expressed by the endothelium (intensity, >20); gray, below the detection limit (intensity, <20). Numbers of enriched genes: chemotaxis, advanced (*n* = 9); early (*n* = 2); firm adhesion, advanced (*n* = 6), early (*n* = 2); diapedesis, advanced (*n* = 0), early (*n* = 2); and leukocyte activation, advanced (*n* = 4), early (*n* = 0). A one-way average linkage clustering of the genes that were core-enriched in either early (*n* = 6) or advanced (*n* = 19) atherosclerosis, depicted as green or red symbols in **A**, is shown in **B**. Colors are as explained in the legend of Figure 3.

fractalkine (CX3CL1), and regulated on activation normal T cell expressed and secreted [RANTES (CCL5)]. These chemokines have specific, differential chemotactic, and adhesive properties for leukocytes to the endothelium. Therefore, we made a scheme of the most important genes involved in interactions between endothelial cells and leukocytes, showing the GSEA results comparing early with advanced atherosclerosis (Figure 4A). See Supplemental Table 4 at <http://ajp.amjpathol.org> for the individual GSEA ranks of these genes. We found that a greater number of genes involved in leukocyte activation, adhesion, and chemotaxis was identified in advanced than in early atherosclerosis (advanced, *n* = 19 genes; early, *n* = 6 genes). These genes form two separate panels as visualized by hierarchical clustering (Figure 4B).

Validation of Microarray Identifiers at Protein Level by Immunohistochemistry

Next, we validated our transcriptome analysis for a small set of genes by immunohistochemical analysis, again in a

Figure 5. Representative photomicrographs of differential protein expression in focal atherosclerosis of the early and advanced stage, based on transcriptome data. Photomicrographs depict two human arteries (nos. 14 and 20, Table 1) with early (A, C–R) and advanced atherosclerosis (B, S–AH). Overviews of macrophage HAM56 immunohistochemistry are shown for these two arteries (A and B). Rectangles within these pictures indicate the magnified areas used for the paired comparisons of the plaque-free sections (C–J, S–Z) with early (K–R) or advanced atherosclerosis (AA–AH). Black arrowheads indicate immunopositive endothelial cells. Shown are immunohistochemical stainings for PECAM-1 (C, K, S, AA), CX3CL1 (D, L, T, AB), CYP1B1 (E, M, U, AC), ICAM-1 (F, N, V, AD), IP10 (G, O, W, AE), TBX18 (H, P, X, AF), BAX (I, Q, Y, AG), and NFKB2 (J, R, Z, AH). All cross sections had endothelium, as indicated by their PECAM-1 immunopositivity (C, K, S, AA). Immunopositive endothelium: CX3CL1 (L, AB), CYP1B1 (M), ICAM-1 (N, AD), IP10 (G, O, W, AE), TBX18 (P), BAX (Q, AG), and NFKB2 (AH). See Table 2 for antibody details. Scale bars: 3.0 mm (A, B); 100 μ m (C–AH).

paired manner within individual arteries. This should verify that differential microarray intensities could be translated to potential functional differences at the level of actual protein expression. A separate set of arteries from different individuals, not included in the initial microarray analysis, was used for this immunohistochemical validation (Table 1) to prevent potential bias caused by the limited number of samples and human patients. Representative photomicrographs are shown for all of the proteins assessed, comparing within two separate arteries the endothelial immunopositivity in atherosclerotic plaques of the early and the advanced stage with their plaque-free control sections (Figure 5). The combined results of the immunohistochemical evaluation are sum-

marized in Table 3, showing that for all of the assessed genes immunopositive clusters of endothelial cells can be found specifically in at least half of either the initial or the advanced atherosclerotic plaques. In the plaque-free sections, with the exception of IP10, little or no endothelial immunopositivity was found. The distribution of endothelial immunopositivity among the different stages of atherosclerosis corresponded well to the microarray data for the majority of the genes, being BAX, CX3CL1, ICAM-1, NFKB2, and TBX18. Only for CYP1B1 the GSEA and the immunohistochemistry data seemed in conflict as the increased expression level is only reflected by endothelial immunopositivity in a minority of the atherosclerotic plaques.

Figure 6. Representative endothelial pSMAD2 immunostainings in different stages of atherosclerosis. Composite photomicrographs of immunohistochemistry for macrophage-detecting HAM56 are shown for three human large arteries having focal atherosclerosis of the early (**A** and **B**) and advanced stage (**C**), respectively. Artery donor information: Table 1, donors 15, 12, and 19, respectively. Rectangles within these overview pictures indicate the magnified areas of the plaque-free sections (**D**), of the sections early (**E**), or advanced plaques (**F**). **Black arrowheads** mark pSMAD2-immunopositive endothelial cells. Scale bars: 3.0 mm (**A–C**); 100 μ m (**D–F**).

Distinct Involvement of TGF- β Signaling during Advanced Atherosclerosis

A strong involvement of TGF- β signaling molecules and downstream proteins was noted in the transcriptome data (Figure 3D). TGF- β signaling, like most signal transduction cascades, is marked by phosphorylation of the distinct pathway intermediates SMAD1 and SMAD2 rather than by altered mRNA expression levels. Therefore, we tested specifically the presence of phosphorylated forms of these key effector molecules. In the human donor arteries, we only identified scattered individual lesional endothelial cells positive for phosphorylated SMAD1 (data not shown). In contrast, the antibody against phosphorylated SMAD2 (pSMAD2) stained large clusters of pSMAD2-immunopositive endothelial cells, preferentially in plaques of the advanced stage, whereas little or no clusters of pSMAD2-immunopositive endothelium were observed in early plaques or in the healthy arterial wall, respectively (Table 3). Representative photomicrographs are given (Figure 6).

Discussion

Our combined use of LMM with linear RNA amplification and microarray expression profiling has enabled an unprecedented comparison of global gene expression profiles from human arterial endothelial cells in different stages of human atherosclerosis. For the first time, the presence of distinct repertoires of genomic endothelial expression in different stages of the disease is detected,

dominant functional roles for chemokines and the NF- κ B, p53, and TGF- β signaling pathways in advanced atherosclerosis are shown. The sensitivity and specificity of our approach are illustrated by the protein validation for the majority of the selected genes. We tested several different approaches because the statistical treatment of microarray expression data has been a topic of much debate. We found that in most cases specificity goes at the expense of sensitivity; ie, in trying to exclude false positives, many false negatives are created. Thus, most of the genes for which we were able to validate differential expression at the protein level were considered as non-significant by statistical application of the Bonferroni correction for multiple testing and the paired significance analysis of microarrays test at a false discovery rate of 5%. A paired Cyber-T test with Benjamini-Hochberg multiple testing correction, which uses a Bayesian estimate of the expression variance,²⁶ produced best results as shown also in a previous *in vitro* study in which we reported a 100% validity of identified candidates.²³ Similarly, the used pathway-oriented GSEA has proven to be highly suitable for analyzing microarray data to identify more subtle variations in human *in vivo* material because it detected statistical significance in crucial genes in diabetic muscle, masked by conventional statistics.²⁷

A large number of genes are found to be consistently differentially expressed between lesional endothelium versus endothelium overlying normal looking vessel wall, thus validating our initial hypothesis that consistent, specific functional signatures do exist, irrespective of differences of individual genetic make-up, systemic risk factors, or vascular origin. One of the most distinguished novel findings is the difference in endothelial transcriptomes between early and advanced stages of atherosclerosis, indicating that one is dealing with quite distinct cellular phenotypes. This has significant implications for future applications such as site-specific lesional imaging or therapy. We identified specific sets of chemokines and leukocyte adhesive proteins as having increased microarray intensities in either the advanced or the early stages of atherosclerosis. Remarkably, the two chemokines we show to predominate in early lesions are fractalkine/CX3CL1 and IP10/CCL10, which are known to be endothelial-associated rather than soluble cytokines. Thus they can locally enhance the adhesion of monocytes and T cells, whereas at later stages soluble chemokines for a variety of leukocytes are detected. The endothelial glyocalyx-binding IP10 was expressed specifically in lesional endothelium, but its corresponding protein was also detected in artery sections without plaques, consistent with locally enhanced plasma levels.²⁸ In contrast, fractalkine/CX3CL1 is truly endothelium-anchored by a transmembrane domain, a protein module that is expressed specifically in endothelial cells.²⁹ Indeed, because of this property its protein was detected much more discriminatively in endothelium overlying early lesions only. Therefore, when considering the suitability of these chemokines as markers for early atherosclerosis, our data indicate that fractalkine/CX3CL1 is more suitable than IP10/CCL10.

In line with our microarray intensity data, several single-gene studies in humans have shown that the expression levels of the genes fractalkine/CX3CL1, ICAM-1, RANTES (CCL5), and IP10/CCL10 were elevated in atheroma, as compared with nonatherosclerotic tissue.^{30–34} In mouse models, elevated expression levels of these genes have also been identified in atherosclerotic plaques, functional blocking of the majority of these genes has been shown to reduce lesion size and leukocyte recruitment.^{31,35–38} However, the plaque stage-specific expression patterns of most of these genes differ between our human data and the data from the mice. Important factors that possibly explain these interspecies discrepancies are the species differences in time span of plaque development and the differences in the methodology, analysis, interpretation of the microarray data, as has recently been discussed in detail.¹³

Members of the NF- κ B family play distinctive roles in endothelial physiology, numerous studies have addressed this issue in cultured cells and mouse models. We found by GSEA that sets consisting of NF- κ B-induced and NF- κ B-activating genes were enriched in advanced human atherosclerosis. These NF- κ B-induced genes mainly consisted of proinflammatory chemokines and cytokines, whereas the NF- κ B-activating genes, being present also in the p53-signaling set, comprised RelA, TP53BP2, TRAF1, and TRAF5. In addition, the increased expression of the inflammation-induced NF- κ B subunit NFKB2³⁹ was validated by an increased immunopositivity, specifically in advanced atherosclerotic plaques. These data provide support that especially in advanced atherosclerosis NF- κ B-activation is involved in the induction of proinflammatory genes, mainly involved in the chemotaxis and adhesion of leukocytes.^{9,10} Furthermore, our analyses also confirm the key involvement of the molecules NF- κ B and p53 in apoptosis-related processes during atherosclerotic plaque progression.^{40,41} Our observation of the increased endothelial BAX immunoreactivity in advanced plaques, which may be crucial in the induction of endothelial cell apoptosis by oxidative stress,⁴² indicates that in this late stage of atherosclerosis the endothelial cell tend toward a proapoptotic status. Clearly, this is balanced by anti-apoptotic mechanisms of antioxidant oxidative stress-response genes such as heme oxygenase^{43,44} because we did not find evidence of endothelial erosion in our samples. Other NF- κ B-directed anti-apoptotic mechanisms are also present in endothelium of the human atherosclerotic vessel wall, in agreement with our previous reports on expression of proteins like cIAP (inhibitor of apoptosis protein).⁴⁵

GSEA showed specific enrichments of sets of TGF- β downstream genes, mainly in advanced atherosclerosis. We confirmed activation of the intracellular TGF- β -signaling pathway intermediate SMAD2, by showing a wide, sustained presence of phosphorylated SMAD2 in endothelium overlying advanced atherosclerotic plaques in a diverse set of human arteries. The activation of SMAD2 mainly occurs through phosphorylation by the ubiquitously expressed TGF- β receptor ALK5,⁴⁶ whereas active SMAD1 was only infrequently detected (data not shown), arguing against a dominant role of the endothelial-spe-

cific receptor ALK1. In many cell types, ALK5-driven TGF- β signaling is involved in the regulation of proliferation, differentiation, migration, and survival.⁴⁷ However, it is still debated whether TGF- β signaling plays a pro- or an antiatherogenic role in the endothelium. In mouse models of accelerated atherosclerosis, there is evidence for an antiatherogenic role for TGF- β , for its partial disruption has been shown to induce expression levels of the proatherogenic leukocyte adhesive molecules ICAM-1 and VCAM-1⁴⁸ and to result in a more inflammatory and less fibrotic lesion type.⁴⁹ Recent evidence from *in vitro* studies indicates that TGF- β activation leads to the induction of proatherogenic responses in the human endothelium by aggravated endothelial permeability through SMAD2-dependent p38 activation^{50,51} and by induction of proatherogenic genes such as lectin-like oxidized LDL receptor-1,⁵² the atherothrombotic factor PAI-1,⁵³ and the leukocyte adhesive proteins ICAM-1^{54,55} and MCP-1.⁵⁶

In summary, the present study provides evidence that distinct genome expression profiles distinguish endothelium overlying early versus advanced atherosclerotic plaques. Furthermore, we establish the involvement of specific chemokines and NF- κ B during human atherosclerosis *in vivo*, as previously identified based on mouse models of accelerated atherogenesis and cultured human cells. Most specifically, we are the first to present consistent evidence for a widespread activation of the TGF- β pathway in endothelium overlying advanced human atherosclerotic lesions, which was not previously identified in this cell type in murine models for atherosclerosis.

Acknowledgments

We thank Dr. Allard C. van der Wal, José Popma, Fred W.F. Ultee (Academic Medical Center, Amsterdam, The Netherlands) for providing the artery sections; and Prof. Peter ten Dijke (Molecular Cell Biology, Leiden, The Netherlands) for providing the anti-phosphorylated-SMAD-1 and -2 antibodies.

References

1. Hansson GK: Inflammation, atherosclerosis, coronary artery disease. *N Engl J Med* 2005, 352:1685–1695
2. Charo IF, Taubman MB: Chemokines in the pathogenesis of vascular disease. *Circ Res* 2004, 95:858–866
3. Muller WA: Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* 2003, 24:327–334
4. Ross R: Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999, 340:115–126
5. Libby P, Sukhova G, Lee RT, Galis ZS: Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. *J Cardiovasc Pharmacol* 1995, 25(Suppl 2):S9–S12
6. Venugopal SK, Devaraj S, Jialal I: Effect of C-reactive protein on vascular cells: evidence for a proinflammatory, proatherogenic role. *Curr Opin Nephrol Hypertens* 2005, 14:33–37
7. Matsunaga T, Hokari S, Koyama I, Harada T, Komoda T: NF-kappa B activation in endothelial cells treated with oxidized high-density lipoprotein. *Biochem Biophys Res Commun* 2003, 303:313–319

8. Yeh M, Leitinger N, de Martin R, Onai N, Matsushima K, Vora DK, Berliner JA, Reddy ST: Increased transcription of IL-8 in endothelial cells is differentially regulated by TNF- α and oxidized phospholipids. *Arterioscler Thromb Vasc Biol* 2001, 21:1585–1591
9. Brand K, Page S, Walli AK, Neumeier D, Baeuerle PA: Role of nuclear factor- κ B in atherogenesis. *Exp Physiol* 1997, 82:297–304
10. Lehoux S, Castier Y, Tedgui A: Molecular mechanisms of the vascular responses to haemodynamic forces. *J Intern Med* 2006, 259:381–392
11. Cheng C, Tempel D, van Haperen R, van der Baan A, Grosveld F, Daemen MJ, Krams R, de Crom R: Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. *Circulation* 2006, 113:2744–2753
12. Lusis AJ, Fogelman AM, Fonarow GC: Genetic basis of atherosclerosis: part I: new genes and pathways. *Circulation* 2004, 110:1868–1873
13. Bijlens AP, Lutgens E, Ayoubi T, Kuiper J, Horrevoets AJ, Daemen MJ: Genome-wide expression studies of atherosclerosis. Critical issues in methodology, analysis, interpretation of transcriptomics data. *Arterioscler Thromb Vasc Biol* 2006, 26:1226–1235
14. Tuomisto TT, Yla-Herttuala S: What have we learnt about microarray analyses of atherogenesis? *Curr Opin Lipidol* 2005, 16:201–205
15. Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH: Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc Natl Acad Sci USA* 1990, 87:1663–1667
16. Polacek DC, Passerini AG, Shi C, Francesco NM, Manduchi E, Grant GR, Powell S, Bischof H, Winkler H, Stoeckert Jr CJ, Davies PF: Fidelity and enhanced sensitivity of differential transcription profiles following linear amplification of nanogram amounts of endothelial mRNA. *Physiol Genomics* 2003, 13:147–156
17. Passerini AG, Polacek DC, Shi C, Francesco NM, Manduchi E, Grant GR, Pritchard WF, Powell S, Chang GY, Stoeckert Jr CJ, Davies PF: Coexisting proinflammatory and antioxidative endothelial transcription profiles in a disturbed flow region of the adult porcine aorta. *Proc Natl Acad Sci USA* 2004, 101:2482–2487
18. Tuomisto TT, Korkeala A, Rutanen J, Viita H, Brasen JH, Riekkinen MS, Rissanen TT, Karkola K, Kiraly Z, Kolbe K, Yla-Herttuala S: 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, interleukin receptors. *Arterioscler Thromb Vasc Biol* 2003, 23:2235–2240
19. Trogan E, Choudhury RP, Dansky HM, Rong JX, Breslow JL, Fisher EA: Laser capture microdissection analysis of gene expression in macrophages from atherosclerotic lesions of apolipoprotein E-deficient mice. *Proc Natl Acad Sci USA* 2002, 99:2234–2239
20. Scheidl SJ, Nilsson S, Kalen M, Hellstrom M, Takemoto M, Hakansson J, Lindahl P: mRNA expression profiling of laser microbeam microdissected cells from slender embryonic structures. *Am J Pathol* 2002, 160:801–813
21. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005, 102:15545–15550
22. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM: Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2000, 20:1262–1275
23. Dekker RJ, Boon RA, Rondaij MG, Kragt A, Volger OL, Elderkamp YW, Meijers JC, Voorberg J, Pannekoek H, Horrevoets AJ: KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. *Blood* 2006, 107:4354–4363
24. Nelander S, Mostad P, Lindahl P: Prediction of cell type-specific gene modules: identification and initial characterization of a core set of smooth muscle-specific genes. *Genome Res* 2003, 13:1838–1854
25. Reiner A, Yekutieli D, Benjamini Y: Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* 2003, 19:368–375
26. Long AD, Mangalam HJ, Chan BY, Toller L, Hatfield GW, Baldi P: Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. Analysis of global gene expression in *Escherichia coli* K12. *J Biol Chem* 2001, 276:19937–19944
27. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 2003, 34:267–273
28. Kawamura A, Miura S, Fujino M, Nishikawa H, Matsuo Y, Tanigawa H, Tomita S, Tsuchiya Y, Matsuo K, Saku K: CXCR3 chemokine receptor-plasma IP10 interaction in patients with coronary artery disease. *Circ J* 2003, 67:851–854
29. Umehara H, Bloom ET, Okazaki T, Nagano Y, Yoshie O, Imai T: Fractalkine in vascular biology: from basic research to clinical disease. *Arterioscler Thromb Vasc Biol* 2004, 24:34–40
30. Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P, Luster AD: Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin Invest* 1999, 104:1041–1050
31. Bursill CA, Channon KM, Greaves DR: The role of chemokines in atherosclerosis: recent evidence from experimental models and population genetics. *Curr Opin Lipidol* 2004, 15:145–149
32. van der Wal AC, Das PK, Tigges AJ, Becker AE: Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am J Pathol* 1992, 141:1427–1433
33. DeGraba TJ, Siren AL, Penix L, McCarron RM, Hargraves R, Sood S, Pettigrew KD, Hallenbeck JM: Increased endothelial expression of intercellular adhesion molecule-1 in symptomatic versus asymptomatic human carotid atherosclerotic plaque. *Stroke* 1998, 29:1405–1410
34. Nuotio K, Lindsberg PJ, Carpen O, Soinne L, Lehtonen-Smeds EM, Saimanen E, Lassila R, Sairanen T, Sarna S, Salonen O, Kovanen PT, Kaste M: Adhesion molecule expression in symptomatic and asymptomatic carotid stenosis. *Neurology* 2003, 60:1890–1899
35. Veillard NR, Kwak B, Pelli G, Mulhaupt F, James RW, Proudfoot AE, Mach F: Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* 2004, 94:253–261
36. Heller EA, Liu E, Tager AM, Yuan Q, Lin AY, Ahluwalia N, Jones K, Koehn SL, Lok VM, Aikawa E, Moore KJ, Luster AD, Gerszten RE: Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. *Circulation* 2006, 113:2301–2312
37. Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R: Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol* 1998, 18:842–851
38. Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL: P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. *J Exp Med* 2000, 191:189–194
39. Shu HB, Agranoff AB, Nabel EG, Leung K, Duckett CS, Neish AS, Collins T, Nabel GJ: Differential regulation of vascular cell adhesion molecule 1 gene expression by specific NF- κ B subunits in endothelial and epithelial cells. *Mol Cell Biol* 1993, 13:6283–6289
40. Chen F, Castranova V, Shi X, Demers LM: New insights into the role of nuclear factor- κ B, a ubiquitous transcription factor in the initiation of diseases. *Clin Chem* 1999, 45:7–17
41. Geng YJ: Molecular signal transduction in vascular cell apoptosis. *Cell Res* 2001, 11:253–264
42. Choy JC, Granville DJ, Hunt DW, McManus BM: Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* 2001, 33:1673–1690
43. Basuroy S, Bhattacharya S, Tcheranova D, Qu Y, Regan RF, Leffler CW, Parfenova H: Heme oxygenase-2 provides endogenous protection against oxidative stress and apoptosis caused by TNF α in cerebral vascular endothelial cells. *Am J Physiol* 2006, 291:C897–C908
44. Parfenova H, Basuroy S, Bhattacharya S, Tcheranova D, Qu Y, Regan RF, Leffler CW: Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial cells: contributions of HO-1 and HO-2 to cytoprotection. *Am J Physiol* 2006, 290:C1399–C1410
45. Horrevoets AJ, Fontijn RD, van Zonneveld AJ, de Vries CJ, ten Cate JW, Pannekoek H: 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, two novel genes. *Blood* 1999, 93:3418–3431

46. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P: Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* 2002, 21:1743-1753
47. Bertolino P, Deckers M, Lebrin F, ten Dijke P: Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. *Chest* 2005, 128:585S-590S
48. Grainger DJ, Mosedale DE, Metcalfe JC, Bottinger EP: Dietary fat and reduced levels of TGFbeta1 act synergistically to promote activation of the vascular endothelium and formation of lipid lesions. *J Cell Sci* 2000, 113:2355-2361
49. Lutgens E, Gijbels M, Smook M, Heeringa P, Gotwals P, Koteliansky VE, Daemen MJ: Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression. *Arterioscler Thromb Vasc Biol* 2002, 22:975-982
50. Lu Q, Harrington EO, Jackson H, Morin N, Shannon C, Rounds S: Transforming growth factor-beta1-induced endothelial barrier dysfunction involves Smad2-dependent p38 activation and subsequent RhoA activation. *J Appl Physiol* 2006, 101:375-384
51. Goldberg PL, MacNaughton DE, Clements RT, Minnear FL, Vincent PA: p38 MAPK activation by TGF-beta1 increases MLC phosphorylation and endothelial monolayer permeability. *Am J Physiol* 2002, 282:L146-L154
52. Minami M, Kume N, Kataoka H, Morimoto M, Hayashida K, Sawamura T, Masaki T, Kita T: Transforming growth factor-beta(1) increases the expression of lectin-like oxidized low-density lipoprotein receptor-1. *Biochem Biophys Res Commun* 2000, 272:357-361
53. Sawdey M, Podor TJ, Loskutoff DJ: Regulation of type 1 plasminogen activator inhibitor gene expression in cultured bovine aortic endothelial cells. Induction by transforming growth factor-beta, lipopolysaccharide, tumor necrosis factor-alpha. *J Biol Chem* 1989, 264:10396-10401
54. Suzuki Y, Tanigaki T, Heimer D, Wang W, Ross WG, Murphy GA, Sakai A, Sussman HH, Vu TH, Raffin TA: TGF-beta 1 causes increased endothelial ICAM-1 expression and lung injury. *J Appl Physiol* 1994, 77:1281-1287
55. Kang YH, Brummel SE, Lee CH: Differential effects of transforming growth factor-beta 1 on lipopolysaccharide induction of endothelial adhesion molecules. *Shock* 1996, 6:118-125
56. Wu X, Ma J, Han JD, Wang N, Chen YG: Distinct regulation of gene expression in human endothelial cells by TGF-beta and its receptors. *Microvasc Res* 2006, 71:12-19
57. Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engstrom U, Heldin CH, Funa K, ten Dijke P: The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett* 1998, 434:83-87