Transcription Profiles of Aortic Smooth Muscle Cells from Atherosclerosis-Prone and -Resistant Regions in Young Apolipoprotein E-Deficient Mice before Plaque Development

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Key Words

Atherosclerosis · Smooth muscle cells · Microarray · Apolipoprotein E-deficient mice

Abstract

Background/Aims: Site-specific atherosclerosis is generally attributed to differential gene expression in endothelial cells. We investigated whether the transcriptome of smooth muscle cells is different between atherosclerosis-prone and atherosclerosis-resistant regions in apolipoprotein E-deficient (apoE^{-/-}) mice before plaque development, and in C57Bl/6 mice. **Methods:** De-endothelialized aortas (both strains: 3 males, 3 females, age 4 months) were divided into atherosclerosis-prone (AA: ascending aorta, aortic arch and proximal 2 mm of thoracic aorta) and -resistant (CTA: central thoracic aorta, i.e. 6 mm distal from the proximal 2 mm) regions. The transcriptome of these two regions was compared using whole-genome mouse microarrays. **Results:** Microarray analysis revealed differential expression (>2-fold difference) of 70 and 244 genes in C57Bl/6 and apoE^{-/-} mice. This was confirmed for 6 genes using the real-time quantitative polymerase chain reaction. Up- or downregulation in the AA was observed for 33 and 37 genes in C57Bl/6, and for 186 and 58 genes in apoE^{-/-} mice, respectively. The 201

genes that showed exclusively differential expression in apoE^{-/-} mice were related to atherosclerotic processes, such as cell adhesion, proliferation, differentiation, motility, cell death, lipid metabolism and immune responses. **Conclusion:** Our findings indicate that smooth muscle cells display an altered transcriptome at atherosclerosis-prone locations before actual lesion development.

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Introduction

Early development of atherosclerotic plaques is determined by biochemical stimuli, including growth factors, cytokines and levels of cholesterol and (oxidized) lipoproteins, as well as biomechanical stimuli. The latter originate from differences in wall tension or blood flow patterns at branching points, the outer wall of bifurcations, and the inner wall of curvatures in arteries. Regions with disturbed flow patterns are prone to develop atherosclerosis and are therefore called 'atherosclerosis-

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prone', whereas segments with steady laminar unidirectional flow are less susceptible and therefore named 'atherosclerosis-resistant' [1, 2]. The site-specific development of atherosclerotic lesions is also observed in murine models for atherosclerosis [3]. Apolipoprotein E-deficient (apoE^{-/-}) mice preferentially exhibit lesion formation in the aortic root and arch, and the proximal and distal part of the thoracic aorta [4–6].

Human aortic [7] and umbilical vein [8] endothelial cells exposed to disturbed flow patterns showed upregulated mRNA levels of proinflammatory, proapoptotic and procoagulant genes. Therefore, these endothelial cells show a 'proatherogenic phenotype', whereas steady laminar flow raised expression of atheroprotective genes, such as superoxide dismutase 1 and endothelial cell nitric oxide synthase [9]. These findings suggest that the shear stress-dependent pro- or antiatherogenic phenotype of endothelial cells could explain the site specificity of atherosclerosis. In addition to differences in flow, atherosclerosis-prone regions display a larger diameter or a geometry that deviates from a circle (at branch points). As a consequence, endothelial cells and smooth muscle cells (SMCs) endure increased wall tension in these locations as compared to atherosclerosis-resistant regions [10, 11]. Indeed, focal adhesion kinase phosphorylation, which plays a role in cell adhesion, is enhanced in endothelial cells and SMCs of rabbit aortas exposed to a steady stretch of 150 mm Hg for 24 h [12]. This finding indicates that in atherosclerosis-prone regions SMCs could be affected by biomechanical stimuli as well. Some *in vitro* evidence also suggests that SMCs respond to shear stress. Cultured arterial SMCs subjected to increasing shear stress show less cell proliferation, whereas decreasing shear forces [13] or pulsatile turbulent shear [14–16] stimulated SMC mitosis. This suggests that the cell cycle is arrested in SMCs in segments with laminar flow, whereas it is promoted in atherosclerosis-prone regions. Thus, in accordance with the endothelium, the shear-determined phenotype of SMCs correlates with the site specificity of atherosclerosis.

However, the effects of shear stress and wall tension on SMCs have only been studied *in vitro*, and it remains to be determined whether comparable indications can be found *in vivo*. Therefore, we compared SMC gene expression in an atherosclerosis-prone and an atherosclerosis-resistant region. This was performed before plaque development, since subendothelial accumulation of monocytes, foam cells, T cells and SMCs with a synthetic phenotype will inevitably change the 'overall' gene expression profile of the vessel wall as soon as atherosclerosis develops [17]. RNA was collected from the lesion-free,

endothelium-denuded aortic arch (AA; atherosclerosis-prone) and central thoracic aorta (CTA; atherosclerosis-resistant) of young (4 months) apoE^{-/-} and wild-type (C57Bl/6) mice. Each RNA extract was divided into two aliquots: one was pooled for genome-wide microarray analysis; the other half was stored for validation of gene expression by means of the real-time quantitative polymerase chain reaction (RT-qPCR). The impact of the location was studied in C57Bl/6 mice, whereas the combination of location and hypercholesterolemia was evaluated in apoE^{-/-} mice.

Materials and Methods

Animals

The studies were approved by the ethical committee of the University of Antwerp. C57Bl/6 and apoE^{-/-} mice (back-crossed into the C57Bl/6 background for more than 10 generations) were kept on a regular chow diet and given tap water *ad libitum* throughout the study. Plaque size was evaluated in apoE^{-/-} mice at the age of 6 (n = 5), 14 (n = 5), 18 (n = 5) and 22 (n = 9) months. SMC gene expression was analyzed (microarray) and validated (RT-qPCR) at 4 months (both strains, n = 6, 3 males, 3 females); for confirmation, a second sample of both strains (n = 5) was analyzed at 4 months.

Isolation of Aorta

Mice were anesthetized with sodium pentobarbital (Nembutal®, 75 mg/kg, *i.p.*). The aorta was carefully excised and cleaned from adherent tissue. For plaque size analysis, the aorta was systematically sectioned as depicted in figure 1: the first millimeter of the aorta after the heart is defined the aortic root; the thoracic aorta was divided into 5 × 2 mm segments (TA1–5) starting approximately 3 mm from the origin of the left subclavian artery (where the azygos vein crosses over the aorta) down to the diaphragm.

For gene expression studies, the aorta was perfused with 4 ml Krebs-Ringer solution ([mM] NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, CaEDTA 0.025 and glucose 11.1) containing 0.01% TritonX-100 to remove the endothelium and rinsed with 10 ml Krebs-Ringer solution without TritonX-100. The atherosclerosis-prone region (AA: aortic arch and the proximal 2 mm of the thoracic aorta) and atherosclerosis-resistant region (CTA: central thoracic aorta = 6 mm distal from the proximal segment of the thoracic aorta; fig. 1) were placed in 350 µl Trizol reagent within 2 min after starting the perfusion. Successful removal of the endothelium was validated by RT-qPCR of CD31, an endothelial cell marker.

Plaque Size Analysis

Each segment was placed in paraformaldehyde (4%, 24 h), embedded in optimum compound temperature (OCT) and kept at –80°C for further analysis. From each segment, 3 transverse sections (6 µm) – taken at intervals of 150 µm (aortic root) or 250 µm (all other segments) – were stained for elastin (Verhoeff-Van Gieson). The cross-sectional area of the intima was quantified by

Fig. 1. Bar graph representing the age-dependent rise in the cross-sectional area of the intima in the aortic root and the thoracic aorta of apoE^{-/-} mice on regular chow. This shows that atherosclerosis develops preferentially in specific regions. At the age of 6 months, the aorta was plaque free, but at 14 months it showed lesions in the aortic root, the aortic arch (AA) and the proximal 2 mm of the AA, and in the distal thoracic aorta. These regions are therefore designated 'atherosclerosis-prone'. The central thoracic aorta (CTA) remained largely plaque free and is therefore called 'atherosclerosis-resistant'. SMC gene expression of endothelium-denuded atherosclerosis-prone (AA) and -resistant (CTA) regions was compared at the age of 4 months, i.e. before plaque development. Data represent means $\pm$ SEM; * $p < 0.05$, ^s $p < 0.01$, different from zero, one-sample t test.

means of point counting using a grid on the microscope eyepiece [18]. The average of the 3 measurements was used for further analysis.

RNA Isolation

Aortic segments were immediately cut into small pieces and lysed in 350 μ l Trizol reagent (Invitrogen, Merelbeke, Belgium). Subsequently, RNA was purified using the RNeasy MinElute kit (Qiagen, Antwerp, Belgium) and collected in 25 μ l RNase-free water. RNA quality was tested by electrophoresis using the Agilent Bioanalyser. All RNA samples had an RNA integrity number between 7.5 and 7.7. RNA extracts (12 μ l) from the 6 mice were pooled and used for microarray analysis of the atherosclerosis-prone and -resistant region; the remaining RNA from individual mice was stored separately and used to validate the microarray results with RT-qPCR.

RNA Amplification, Labeling and Microarray

Isolated RNA was amplified and labeled in duplicate using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent) at the VIB MicroArray Facility (Leuven, Belgium, www.microarrays.be). It features a single amplification, two-color labeling method to create cyanine 3- and cyanine 5-labeled cRNA. One sample was labeled with cyanine 3 and one with cyanine 5. After labeling, both samples were combined, and cRNA was hybridized to an Agilent-Mouse-Whole Genome Array (Ag-Mo-WG-G4122A). Hybridization of cRNA to the microarray oligos occurred at 65°C while rotating the microarray slide (4 rpm) during 17 h, followed by washing according to the manufacturer's guidelines. Finally, the microarray slide was scanned using an Agilent Scanner, and the resulting two-color TIF image was analyzed using Agilent Feature Extraction Software 8. The light intensity of each gene in the duplicate slides was compared. When intensities differed from each other, the result for that gene was excluded. Otherwise, it was examined whether the mean intensity of that gene in the CTA sample of both microarrays was different from the mean for that gene in the AA sample by means of Student's t test ($\alpha = 0.01$). Finally, only intensity differences greater than 2-fold were considered. Using freely available software (Ease version 2.0), differentially expressed genes were categorized in biological processes.

The microarray data are available to the public via the National Center for Biotechnology Information Gene Expression Omnibus at: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13865>.

Real-Time Quantitative Polymerase Chain Reaction

Relative expression of genes of interest was evaluated using the Two Step RT-qPCR Core kit (Eurogentec, Seraing, Belgium). RNA was converted into cDNA (Reverse Transcriptase Core kit, Eurogentec), then subjected to quantitative PCR (qPCR Core kit, Eurogentec) on an ABI 7300 Instrument (Applied Biosystems, Foster City, Calif., USA) using primers and probes shown in table 1. The relative expression of mRNA species was calculated using the comparative threshold cycle (Ct) method using β -actin and hypoxanthine guanine phosphoribosyl transferase as reference genes; the mean value of the 6 CTA extracts was set to 1 using the $2^{-\Delta\Delta Ct}$ method [19].

Statistical Analysis

All results are expressed as means $\pm$ SEM; n represents the number of mice. A 5% level of significance was selected. A one-sample t test was used to verify whether the cross-sectional area of the intima was different from zero. Differences among regions and sexes were analyzed using a repeated-measures ANOVA of the $-\Delta\Delta Ct$ values with segment (AA vs. CTA) as within-subject factor and gender (male or female) as between-subjects factor. Deming regression on $^2\log$ -transformed microarray and RT-qPCR results were used to assess the relationship between these methods.

Results

Plaque Size Analysis

In apoE^{-/-} mice, plaques were not observed in any segment studied at the age of 6 months. After 14 months, however, atherosclerotic lesions had developed and their size

Table 1. Forward and reverse primers, and fluorogenic probes used in RT-qPCR

Gene	Sequence or code
β -Actin	
Forward primer	GCT CTG GCT CCT AGC ACC AT
Reverse primer	GCC ACC GAT CCA CAC AGA GT
Taqman probe	FAM-ATC AAG ATC ATT GCT CCT CCT GAG CGC-TAMRA
Hypoxanthine guanine phosphoribosyl transferase	
Forward primer	CAT TAT GCC GAG GAT TTG GAA
Reverse primer	TCC TTC ATG ACA TCT CGA GCA A
Taqman probe	FAM-CTT TCA GTC CTG TCC ATA ATC AGT CCA TGA GG-TAMRA
Secreted phosphoprotein 1	Mm00436767_m1 (Applied Biosystems)
Amyloid beta precursor protein	Mm00431827_m1 (Applied Biosystems)
Sodium potassium ATPase, beta 1 subunit	Mm00437612_m1 (Applied Biosystems)
Sarcosin	Mm00481536_m1 (Applied Biosystems)
Homeobox B7	Mm00650702_m1 (Applied Biosystems)
Fatty acid-binding protein 1	Mm00444340_m1 (Applied Biosystems)

Table 2. List of genes that were up- or downregulated in the AA versus the CTA of C57Bl/6 and apoE^{-/-} mice, or both strains (common)

Strain	Up	Down
C57Bl/6	2610027K06Rik; 4833415N18Rik; 4930544G21Rik; 5033428I22Rik; BC049816; D630004D15Rik; Dner; Enpp2; Gdf2; Hrasls; Klf1a; Matn3; Wif1	4921525H12Rik; A1646519; Angptl1; D830007B15Rik; Hoxb8; Junb; LOC620807; LOC635091; Wnt2; XM_620048; Ptpn20; Rgs17
Common	1600029D21Rik; 2210407C18Rik; A930010C08Rik; Atp1b1; Cyt1; Dspg3; Flrt3; Fndc5; Gsta3; Lnx1; Myl7; Pln; Ren1; Slc38a4; Sln; Sncg; Stc2; Tcf21; Thbd; Tnnt2	1600015H20Rik; Alb1; Aldh1a3; Art3; Avpr1a; BC034076; Bmper; Btc; Calcl; Fhl2; Gpsm2; Hoxa5; Hoxa7; Hoxb3; Hoxb5; Hoxb6; Hoxb7; Hoxc6; Inmt; Lgi2; Mup1; Mup3; Mup4; NAP102507-1; Pax1
apoE ^{-/-}	1110008I14Rik; 1500001A10Rik; 1700110N18Rik; 2010002N04Rik; 2810046M22Rik; 5033428I22Rik; 9030611O19Rik; A_52_P517668; A930008A22Rik; A930008K15Rik; Abca1; Abcb1a; Abcg1; Adam23; Adam8; Ahsg; A1549825; AK045479; AK046514; AK046686; AK078599; AK089297; Akp2; Aldh1a2; Amph; App; Aqp1; Arhgap25; Atp6v0d2; AY248756; B230106I24Rik B430119L13Rik; B430320C24Rik; BC023928; BC087964; Bok; Brip1; Bst1; C3; C630002N23Rik; C77080; Cacnb4; Cd5l; Cdkn2b; Celsr1; Clec1b; Clec7a; Clstn2; Cmya4; Cox6a2; Cplx2; Crym; Csf2rb2; Csrp3; Cst9; Cthrc1; Ctss; Cxcl7; Cxcl9; Cyp2s1; D11Ert759e; D130058I21Rik; D330050I23Rik; D3Bwg0562e; Dlgap1; Dnmt3a; E130010M05Rik; Edn1; Efemp1; Esrrg; F730004D16Rik; Fetub; Fgf10; Frzb; Fxyd3; Galnt6; Gas6; Gcnt1; Gpm6a; Gpnmb; Gsta1; Igfbp2; Igfbp3; Igfbp4; Igfbp5; Il18bp; Il7r; Inhbb; Itga6; Itgax; Kdr; Krt1-18; Krt1-19; Krt1-23; Krt2-7; Krt2-8; Lama2; Lars2; Lgals3; Lgals7; Lisch7; LOC224833; LOC433431; Lrat; Lrrn3; Ltbp2; Lum; Lypdc1; Mb; Meox1; Mgat3; Mmp12; Mpeg1; Msln; Myh8; Myl4; Nkx6-2; Npr3; Nt5e; Odz4; Palmd; Pamci; Pappa; Pcdh9; Peg3; Plekha6; Polydom; Popdc2; Prg4; Psap; Ptn; Ptpnb; Ptpnf; Ptx3; Ren2; Rgs1; Rhp2; Rnf128; Rragd; Sdc3; Serpinb6b; Slc15a3; Slc23a3; Slc26a3; Slc37a2; Slc39a8; Slco2a1; Smoc1; Smpx; Sox9; Spp1; Syngri; TC1307854; Tmem26; Tnfrsf11b; Tnni3; Tpm1; Trdn; Trem2; Unc5b; Upk1b; Upk3b; Vcam1; Vil2; Wfdc2; Wt1; Zfp185; Zic1	1100001G20Rik; 2610034M16Rik; 2900093B09Rik; 3110032G18Rik; AB080658; Agxt; AK027978; AK040144; C1qtnf3; Calml4; Col8a2; Cpb1; ENSMUST00000081396; Esm1; F2; Fabp1; Hhip; Hoxc8; Kng1; LOC329416; LOC433053; Lrrn1; Mybpc2; NAP112201-1; Pde6h; Rgs2; Saa3; Sema3a; Sfrp4; Slc22a1; Slc22a2; TC1324209; Zfp60

Fig. 2. Ratio-intensity plots of the average $^2\log$ ratio between the AA and the CTA versus the average $^2\log$ intensity for the microarray results of the the apoE $^{-/-}$ (a) and C57Bl/6 mice (b). In C57Bl/6 mice 70 genes and in ApoE $^{-/-}$ mice 246 genes showed a greater than 2-fold statistically significant ratio difference (Student's t test, $p < 0.01$).

increased further at 18 and 22 months (fig. 1). The largest plaques were found in the aortic root, and the proximal (TA1) and distal (TA5) thoracic aorta segments. Furthermore, macroscopically visible lesions were present in the aortic arch, its side branches and around the carotid bifurcation. The CTA (TA2, TA3 and TA4) was usually not or only minimally affected. This very consistent observation led us to define the aortic root, aortic arch, TA1 and TA5 as 'atherosclerosis-prone' aorta segments, while TA2, TA3

and TA4 can be considered 'atherosclerosis-resistant' aorta segments. The 4-month-old apoE $^{-/-}$ mice used for the gene expression studies did not exhibit any visible lesions.

Microarray

From the 41,174 gene sequences evaluated, only 70 genes in C57Bl/6 and 246 genes in apoE $^{-/-}$ mice showed differential expression between AA and CTA, as shown in ratio-intensity plots [20] (fig. 2). Increased expression

('upregulation', >2-fold) in the AA was seen for 33 genes in C57Bl/6 and for 188 genes in apoE^{-/-} mice, while decreased expression ('downregulation', <0.5-fold) was seen for 37 genes in C57Bl/6 and for 58 genes in apoE^{-/-} mice. Of the 33 genes upregulated in C57Bl/6 mice, 20 genes were upregulated in apoE^{-/-} mice as well, and 25 (of 37) genes downregulated in C57Bl/6 mice were also expressed at a lower level in apoE^{-/-} mice (table 2).

Among the 45 genes showing differential expression in both C57Bl/6 and apoE^{-/-} mice, most genes were related to development (13 genes), cell growth (8 genes) and transcription (10 genes, table 3). Only 7 (of 25) genes that were exclusively up- or downregulated in C57Bl/6 mice could be linked to two specific processes: development (4 genes) and cell growth (3 genes). The other 18 genes are all involved in different biological processes (table 3).

The 33 genes that were only downregulated in the AA of apoE^{-/-} mice were involved in signal transduction (7 genes), metabolism (3 genes) and immune responses (3 genes). The 168 genes that were specifically upregulated in the AA of apoE^{-/-} mice could all be linked to processes involved in atherosclerosis, e.g. lipid metabolism, cell adhesion, immune response, cell death and ossification (table 3).

Validation

Six genes that showed differential expression in apoE^{-/-} mice were selected for validation using the two RNA extracts of each individual mouse (fig. 3). RT-qPCR confirmed the overall increase or decrease in the AA for all genes in the 6 apoE^{-/-} mice. Individual variability (fig. 3a-f) was surprisingly small for amyloid precursor protein (App), ATPase, NA⁺/K⁺ transporting, β_1 polypeptide (Atp1b1) and Homeobox B7 (HoxB7), or rather large for secreted phosphoprotein 1 (Spp1), sarcolipin (Sln), and fatty acid-binding protein 1 (Fabp1). Upregulation of App and Atp1b1 occurred in both genders, but was more pronounced in males, as indicated by the interaction in the repeated-measures ANOVA (fig. 3). Upregulation of Spp1 in the microarray was caused by high expression in the AA of 2 mice, and the mean RT-qPCR values were statistically not different.

RT-qPCR of the selected genes in individual C57Bl/6 mice also confirmed the microarray results and indicated that 3 genes (Atp1b1, HoxB7 and Sln) were differentially expressed, in contrast to the others (fig. 4). Moreover, Deming regression showed a linear relationship between the ²log-transformed microarray and RT-qPCR results. Slope of regression line was 1.30 with a 95% confidence interval from 1.049 to 1.546 (fig. 4).

Table 3. Biological processes linked to the genes up- or downregulated in an atherosclerosis-prone versus an atherosclerosis-resistant region in C57Bl/6 mice only, in both strains (common) or solely in apoE^{-/-} mice

Biological process	Number of genes					
	C57Bl/6		common		apoE ^{-/-}	
	up	down	up	down	up	down
Development	3	1	4	9		3
Cell growth and/or maintenance	2	1	3	5	44	7
Cell proliferation						8
Cell differentiation						3
Transcription	-	1	-	10	9	-
Metabolism	1	-	-	-	16	6
Glycoprotein metabolism						3
Lipid metabolism	-	-	-	-	4	-
Cell adhesion	-	-	-	-	15	-
Immune response	-	-	-	-	12	3
Inflammatory response						4
Lymphocyte activation						3
Proteolysis and peptidolysis	-	-	-	-	7	-
Cell death	-	-	-	-	6	-
Cell motility	-	-	-	-	4	-
Ossification	-	-	-	-	3	-
Sum	6	3	7	24	120	19

Table 4. RT-qPCR of amyloid precursor protein (App), ATPase, NA⁺/K⁺ transporting, beta 1 polypeptide (Atp1b1), fatty acid-binding protein 1 (Fabp1), homeo box B7 (HoxB7), sarcolipin (Sln), and secreted phosphoprotein 1 (Spp1) in CTA and AA in a second sample of apoE^{-/-} and C57Bl/6 mice

Strain	Gene	CTA	AA	p
apoE ^{-/-}	App	1.00 ± 0.25	1.88 ± 0.08	0.015
	Atp1b1	1.00 ± 0.25	6.87 ± 1.30	0.010
	Fabp1	1.00 ± 0.66	0.10 ± 0.04	0.048
	HoxB7	1.00 ± 0.21	0.02 ± 0.00	<0.001
	Sln1	1.00 ± 0.28	18.66 ± 5.65	<0.001
	Spp1	1.00 ± 0.45	2.67 ± 2.30	0.496
C57Bl/6	App	1.00 ± 0.07	1.09 ± 0.12	0.386
	Atp1b1	1.00 ± 0.19	4.49 ± 0.63	0.002
	Fabp1	1.00 ± 0.44	0.39 ± 0.36	0.361
	HoxB7	1.00 ± 0.05	0.02 ± 0.00	<0.001
	Sln1	1.00 ± 0.24	6.37 ± 1.38	0.002
	Spp1	1.00 ± 0.22	1.01 ± 0.23	0.879

Results show $2^{-\Delta\Delta C_t}$, mean ± SEM, n = 5; p: paired Student's t test of $\Delta\Delta C_t$.

Fig. 3. RT-qPCR of genes in individual apoE^{-/-} mice confirmed the microarray results: all 6 genes showed a comparable differential expression in AA and CTA (a-f). The results for amyloid precursor protein (a), ATPase (c) and homeobox B7 (e) were very consistent in all mice. Upregulation of amyloid precursor protein (a) and ATPase (c) was more pronounced in males, whereas upregulation of secreted phosphoprotein 1 (osteopontin, b) was

mainly determined by 1 male (m2) and 1 female (f3), which also showed the largest difference for sarcolipin (d), and the lowest expression of fatty acid binding protein 1 (f). Values are means $\pm$ SEM of duplicate RT-qPCR runs (in individual mice), or means $\pm$ SEM of the 6 mice; m = male; f = female. The significance of the effects of segment (CTA or TA), gender and their interaction in a repeated-measures ANOVA is reported.

Fig. 4. Validation of microarray analysis versus RT-qPCR. Deming regression was performed on $^2\log$ -transformed microarray and RT-qPCR results to assess the relationship between the two methods. WT = C57Bl/6 mice.

In view of the variation in *Spp1*, *Sln* and *Fabp1* expression between mice, we repeated the RT-qPCR analysis in a second sample of both strains (table 4). The results fully corroborated the first assay, including the rather variable expression of *Spp1* in the AA of apoE-/- mice in comparison to C57Bl/6 mice.

Discussion

The present study showed that in contrast to SMCs in the CTA, an atherosclerosis-resistant region, SMCs in the AA, an atherosclerosis-prone region, displayed a proatherogenic phenotype even before the development of atherosclerotic lesions in hypercholesterolemic, but plaque-free apoE-/- mice.

In apoE-/- mice fed on normal chow, plaques were absent in all segments studied at 6 months; they were seen at 14 months and developed further after 18 and 22 months. The regional distribution of lesions within the aorta was very consistent. Apart from lesions in the aortic root and aortic arch, plaques were predominantly found in the most proximal and distal segments of the thoracic aorta, as previously reported [4–6]. The central region of the aorta was only minimally affected, even after 18 and 22 months. This makes the apoE-/- mouse an interesting model to study differences between atherosclerosis-prone and atherosclerosis-resistant regions.

Borang et al. [21] compared gene expression of vascular cells between an atherosclerosis-prone and an atherosclerosis-resistant region in 6- to 8-week-old apoE-/-

and LDL receptor knockout mice using intact aortas. Because endothelial cells can mask genes that are over- or underexpressed by SMCs and vice versa [22], we removed the endothelial cells before RNA isolation. Effective removal of the endothelium was confirmed by analyzing mRNA expression of CD31, an endothelial cell marker. We cannot claim, however, that we only looked at SMCs, since neurons and inflammatory cells are also present in the aortic wall. The number of subendothelial inflammatory cells may differ between atherosclerosis-prone and -resistant regions even in C57Bl/6 wild-type mice [23]. Finally, the AA may have been contaminated by cardiomyocytes. To counteract these limitations as adequately as possible, we compared gene expression between AA and CTA in both apoE-/- and C57Bl/6 mice. This enabled us to discriminate between differential expression due to location in the aorta (C57Bl/6 and apoE-/- mice) and due to the location in combination with hypercholesterolemia (apoE-/- mice). The factor 'location in the aorta' comprises differences in cellular composition, embryological origin, and altered shear stress.

Genes that were differentially expressed in C57Bl/6 and apoE-/- mice could be largely linked to transcription and development processes. This could be due to the different embryological origin of AA and CTA [24], or to biomechanical differences between the two regions [25]. Indeed, SMCs in culture can respond to biomechanical stimuli; disturbed flow decreases cell proliferation [13] while inducing fibroblast growth factor-2 secretion [26], cell contraction [27, 28], cytoplasmic calcium [29], cell

alignment [30] and apoptosis [31]. However, endothelial cells serve as a 'buffer' between blood flow and SMCs, and it is uncertain to what extent SMCs are exposed to shear-induced forces *in vivo*. Nonetheless, it has been demonstrated that SMCs in atherosclerosis-prone regions are subjected to increased wall tension [10, 11]. *In vitro* studies showed that pulsatile stretch can modulate SMC phenotype, and promote proliferation, depending on the degree of stretch [32], and SMC motility [33, 34] and alignment [35, 36], and induce apoptosis [37]. Moreover, stretch of isolated blood vessels stimulates protein synthesis and contributes to the maintenance of the contractile SMC phenotype [38].

It is also possible that the differential gene expression in the medial SMCs is driven by changes in the endothelial cells. It has been reported that endothelial cells in atherosclerosis-prone regions exhibit unique expression patterns [39–42] that shift their phenotype towards a sustained activated, proinflammatory state, and this might affect the underlying SMCs. For example, the transcription of endothelial nitric oxide synthase [43] is decreased in atherosclerosis-prone regions of the mouse aorta, and nitric oxide not only mediates SMC contractile responses but also determines alignment [32] and SMC proliferation in response to remodeling stimuli [44]. To what degree SMCs *in vivo* are influenced by changes in shear stress, wall tension, or paracrine endothelium-derived mediators has still to be elucidated. However, our study clearly demonstrated that gene expression of SMCs *in vivo* is different in the AA and CTA.

Regional differences alone are, however, not sufficient to induce atherosclerosis. Therefore, differences in gene expression in SMCs caused by the combination of hypercholesterolemia and location in the aorta are more likely to be involved in the onset of atherosclerosis. Indeed, classification of genes that were solely differentially expressed in apoE^{-/-} mice revealed that SMCs located in the AA showed increased expression of genes involved in proatherogenic processes, such as cell proliferation, differentiation, motility and adhesion, immune response and cell death. Moreover, the fact that genes involved in protein metabolism were upregulated suggests that SMCs in an atherosclerosis-prone region may already be shifting from a contractile to a synthetic phenotype [45, 46]. It can be concluded that, in analogy to the endothelium, SMCs also showed a proatherogenic phenotype in the atherosclerosis-prone region before the actual formation of atherosclerotic lesions.

Microarray Validation

RT-qPCR of each individual mouse confirmed the microarray data for 6 genes in apoE^{-/-} mice, though 3 genes [fatty acid binding protein 1, secreted phosphoprotein 1 (osteopontin) and sarcolipin] exhibited large interindividual variation. For the other genes, the variation was much smaller. This stresses the importance of validating gene expression in each individual mouse that was used to create the RNA pool for the microarray to establish whether the mean expression is rather homogeneous or highly variable. It appeared that the value of the pooled population sample can be upregulated, while downregulation is apparent in some or even most individuals. Yet, in view of the interindividual variability of some genes, a second analysis of different mice was used to confirm the original data.

The microarray results for these 6 genes were also validated in the C57Bl/6 mice using RT-qPCR of the original RNA extracts and in the second sample. Moreover, Deming regression showed that the microarray results were accurate compared to the RT-qPCR results. The slope of the regression, however, was slightly higher than 1. This is probably explained by the increased dynamic range of RT-qPCR in comparison with microarray analysis.

The fact that 3 genes (Atp1b1, HoxB7 and Sln) were similarly up- or downregulated in C57Bl/6 and apoE^{-/-} mice indicates that their expression is mainly controlled by the location in the aorta rather than by the combination of location and biochemical stimuli (hypercholesterolemia, oxidative stress) in apoE^{-/-} mice.

Selected Genes

Sarco/endoplasmic Ca²⁺ ATPase (SERCA) [47] is an enzyme that is important for Ca²⁺ homeostasis and contractile function in SMCs, but it has also been implicated in phenotypic switching [45, 46]. Two regulators of SERCA (sarcolipin, phospholamban) showed increased expression in the AA. Sln was selected because it has been reported that mechanical stress (pressure overload through transverse aortic constriction) significantly decreased protein expression of Sln in mouse hearts [48]. In accordance with this finding, both mouse strains showed increased mRNA levels in the AA, demonstrating that the localization in the aorta, and therefore mechanical stress, differentially regulated Sln expression. However, the effect was greater in apoE^{-/-} mice, and in this respect it is interesting that SERCA function appeared to decrease in aortic SMCs of old apoE^{-/-} mice and could be restored by paraoxonase-1 transfection [49].

Atp1b1 is an ion transporter that showed large upregulation in the AA of both strains. Na/K-ATPase has two major subunits (α_1 and β_1) that are both located in caveolae of cardiac myocytes [50]. It has been reported that Na/K-ATPase from the duodenum of spontaneously hypertensive rats is inhibited compared to that of Wistar-Kyoto controls [51]. The physiological relevance of the differential expression of this ATPase in AA and CTA remains, however, unknown.

Six homeobox genes, a family of transcription factors that are linked to the establishment of a correct body plan [52], were differently expressed in AA and CTA of both strains. HoxB7 was selected, since Miano et al. [53] demonstrated that restricted expression of HoxB7 and -C9 distinguishes fetal from adult human SMCs. Moreover, overexpression of HoxB7 in C3H10T1/2 cells is able to differentiate this multipotent cell line into vascular SMCs or osteogenic or chondrogenic lineages [54], further evidence supporting the function of HoxB7 as a regulator of SMC phenotype. In addition, HoxB7 overexpression led to increased cell proliferation, a vascular SMC-like morphology and expression of early SMC markers calponin and smooth muscle protein 22- α (smoothelin), but not of the intermediate SMC marker smooth muscle myosin heavy chain [55]. HoxB7 mRNA has been detected at higher levels in human atherosclerotic plaques than in normal arteries, implying that it might be involved in the switch of SMCs to a more immature phenotype during atherosclerosis [55]. However, our results showed reduced expression of HoxB7 in SMCs of the AA of both apoE $^{-/-}$ and C57Bl/6 mice. Whether HoxB7 is upregulated after plaque development remains to be determined.

App was selected because we previously showed that this protein is involved in the uptake and processing of platelets by macrophages around hemorrhagic microvessels in human plaques. App is stored in α -granules of human platelets, and phagocytosis of blood platelets represents an alternative, non-LDL-dependent pathway of foam cell formation during atherosclerosis [56]. Moreover, during platelet phagocytosis, macrophages become activated, as indicated by the upregulation of proinflammatory genes, such as inducible nitric oxide synthase. App was essential for the activation of macrophages, since blood platelets of App-deficient mice were phagocytosed, but failed to activate macrophages [57]. The present study shows that App is expressed at a higher level in SMCs in the atherosclerosis-prone AA of apoE $^{-/-}$ mice, and this warrants further study of its role in atherogenesis.

Spp1, better known as osteopontin, has emerged as a key factor in both vascular remodeling and development of atherosclerosis [58]. Osteopontin transgenic mice fed a high-cholesterol diet develop atherosclerotic lesions [59, 60], whereas osteopontin deficiency reduces atherosclerosis in apoE $^{-/-}$ [61] and apoE/LDL receptor-deficient mice [62]. In vitro overexpression of osteopontin promotes vascular SMC proliferation [63], and in vivo overexpression is associated with increased thickening of the media with aging and of the intima after arterial injury [64]. Finally, Iizuka et al. [65] reported that atmospheric pressure promotes osteopontin expression in human aortic SMCs. The microarray analysis demonstrated increased gene expression of osteopontin at the atherosclerosis-prone location, but both validation assays pointed to a large interindividual variance between mice. This is potentially interesting because it might be that apoE $^{-/-}$ mice with higher expression of osteopontin in the atherosclerosis-prone region develop atherosclerosis sooner than mice with lower osteopontin expression. Indeed, it has been proposed that osteopontin may be a potential marker for predicting atherosclerosis in patients with essential hypertension [66].

Fatty acid-binding proteins (FABPs) are members of the superfamily of lipid-binding proteins. The primary role of all FABP family members is the regulation of fatty acid uptake and intracellular transport [67]. FABP1 has been shown to be involved in vascular SMC foam cell formation, and its gene expression increases in human SMCs grown in adipocyte differentiation medium for 21 days [68]. It has been reported, however, that hypertension suppressed the expression of Fabp mRNA. Our data also showed downregulation of FABP1 expression in the atherosclerosis-prone AA before plaque formation.

Conclusion

In 4-month-old C57Bl/6 mice, the differential expression in the AA and CTA consisted only of a limited number of genes. In age-matched hypercholesterolemic but plaque-free apoE $^{-/-}$ mice, most of these genes showed the same tendency. However, apoE $^{-/-}$ mice showed a larger number of genes that were up- or downregulated in the atherosclerosis-prone AA. Those supplementary genes were related to processes involved in atherosclerosis, such as cell adhesion, proliferation, differentiation, motility and death, lipid metabolism and immune responses. It has previously been demonstrated that endo-

thelial cells display a proatherogenic transcription profile in the mouse aorta before lesion development. This study shows that SMCs of an atherosclerosis-prone segment demonstrated a proatherogenic phenotype before the onset of atherosclerosis, and this could possibly facilitate local plaque development.

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