In vivo differences between endothelial transcriptional profiles of coronary and iliac arteries revealed by microarray analysis

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Zhang J, Burridge KA, Friedman MH. In vivo differences between endothelial transcriptional profiles of coronary and iliac arteries revealed by microarray analysis. Am J Physiol Heart Circ Physiol 295: H1556–H1561, 2008.—Endothelial cells (ECs) from different vascular beds display a remarkable heterogeneity in both structure and function. Phenotypic heterogeneity among arterial ECs is particularly relevant to atherosclerosis since the disease occurs predominantly in major arteries, which vary in their atherosusceptibility. To explore EC heterogeneity between typical atheroprone and atheroresistant arteries, we used DNA microarrays to compare gene expression profiles of freshly harvested porcine coronary (CECs) and iliac artery (IECs) ECs. Statistical analysis revealed 51 genes that were differentially expressed in CECs relative to IECs at a false discovery rate of 5%. Seventeen of these genes are known to be involved in atherogenesis. Consistent with coronary arteries being more atherosusceptible, almost all putative atherogenic genes were overexpressed in CECs, whereas all atheroprotective genes were downregulated, relative to IECs. A subset of the identified genes was validated by quantitative polymerase chain reaction (PCR). PCR results suggest that the differences in expression levels between CECs and IECs for the HOXA10 and HOXA9 genes were >100-fold. Gene ontology (GO) and biological pathway analysis revealed a global expression difference between CECs and IECs. Genes in twelve GO categories, including complement immune activation, immunoglobulin-mediated response, and system development, were significantly upregulated in CECs. CECs also overexpressed genes involved in several inflammatory pathways, including the classical pathway of complement activation and the IGF-1-mediated pathway. The in vivo transcriptional differences between CECs and IECs found in this study may provide new insights into the factors responsible for coronary artery atherosusceptibility.

CORONARY HEART DISEASE, primarily caused by atherosclerosis, is the single leading cause of death in the United States and other developed countries (29, 42). The relative clinical importance of systemic cardiovascular risk factors differs among arterial beds; e.g., coronary heart disease is mostly affected by cholesterol level, whereas stroke is more associated with hypertension (19). A recent human biopsy study suggested that the initiation, development, and composition of atherosclerotic plaques differ among artery types (9). The coronary arteries had a higher prevalence of atherosclerotic plaques compared with the carotid and femoral arteries. An understanding of the origin of arterial differences in disease development may yield insight into factors that affect atherosusceptibility as well as disease progression. The endothelium that forms the inner lining of the vascular system plays an essential role in the development of atherosclerosis (30); thus, in this context, interarterial differences in endothelial cell (EC) biology are of considerable interest.

ECs from different anatomical locations demonstrate a remarkable heterogeneity in both structure and function under normal and pathological conditions (2, 3). The notion of phenotypic diversity of ECs arises from both in vivo (34, 35) and cell culture (6, 8, 37) studies that suggest there are substantial differences in gene expression between ECs from different vascular beds. For example, microarray studies demonstrate that significant transcriptional differences exist between cultured arterial and venous ECs (8), which have also been shown to respond differently to atherogenic stimuli and shear stress (11, 22).

Phenotypic heterogeneity among arterial ECs is of particular interest in relation to atherosclerosis since the disease occurs predominantly in the major arteries, which vary in their atherosusceptibility (2, 30). Dancu and Tarbell (10) demonstrated the differential expression of two genes in cells scraped from the aorta and coronary arteries of rabbits. The relative importance of, and interplay among, environmental and biological factors in determining arterial EC phenotype is unclear. Here, we seek to determine whether transcriptional differences exist between known atheroprone and atheroresistant arteries in vivo. In this study, we used young and healthy swine as the source of ECs because swine vasculature is one of the best models of the human circulation with respect to normal and pathological physiology (4, 33). We demonstrate that in vivo the endothelial transcriptional profile of a coronary artery (the right coronary artery) is different from that of a major conduit vessel (the external iliac artery) and that this difference is consistent with the former vessel being more prone to atherosclerosis.

MATERIALS AND METHODS

Animal surgery. Animal experiments were performed in accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee. Four commercial juvenile female swine (60–70 kg) were maintained under anesthesia with inhaled isoflurane. Basic physiological parameters, including heart rate and blood pressure, were recorded for at least 20 to 25 min. The animal was then euthanized, and the arterial system was flushed with Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO) to clear the blood from the vessels. The left and right external iliac arteries and right coronary arteries were dissected out. After the adventitial tissue was removed, the arteries were cut open and pinned out flat. A metal spatula was used to scrape ECs from the arterial wall as described previously (20). As shown in Fig. 1, iliac artery ECs

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(IECs) were scraped from the straight portion of the vessel between the circumflex iliac and deep femoral branches. Right coronary artery ECs (CECs) were scraped from the proximal portion, approximately 5 to 8 cm from the ostium. The cells were transferred directly to cell culture media.

ECs (CECs) were scraped from the proximal portion, approximately 5 to 8 cm from the ostium. The cells were transferred directly to cell culture media. The purity of the scraped ECs was further confirmed in this study by using the microarray results; indeed, the expression of the smooth muscle cell-specific genes, calponin and myosin heavy chain, was less in all samples than in a reference sample of cultured ECs.

Microarray experiments. The microarray experiments were performed as described earlier (14). Briefly, RNA samples were amplified and labeled with Cy5 dyes. A reference RNA sample (Cy3 labeled) was derived from cultured porcine aortic ECs (Qiagen, Valencia, CA). The arrays were hybridized to the microarray experiments. For the iliac samples, equal amounts of RNA from the left and right arteries were pooled to maximize the amount of RNA available for PCR, except for one animal for which no right iliac RNA remained after the microarray sample was obtained.

Quantitative real-time PCR. Quantitative real-time PCR was performed with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA), using iQ SYBR Green (Bio-Rad) to detect double-stranded DNA. The $2^{-\Delta \Delta Ct}$ method (21) was used to quantify the fold difference in expression levels, using ribosomal 18S as the internal control gene. Three replicate amplifications were used for each sample, and the melting curves verified the quality of DNA product in each well. The RNA samples used for PCR were isolated at the same time and from the same animals as those used in the microarray measurements. For the iliac samples, equal amounts of RNA from the left and right arteries were pooled to maximize the amount of RNA available for PCR, except for one animal for which no right iliac RNA remained after the microarray sample was obtained. cDNA was synthesized from 50 ng of total RNA using the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA), which includes a DNase treatment step. To verify that the DNase treatment removed genomic DNA, controls were performed on the same plate for each primer pair by substituting diethyl pyrocarbonate-treated water (EMD, San Diego, CA) for reverse transcriptase. For the control sample used with water, the exception of 18S, no gene product was observed even after 50 PCR cycles or was detected much later (26 cycles), indicating that $<1.5 \times 10^{-8}$ of the total product arose from template not dependent on reverse transcriptase.

PCR primers for each gene were designed using Primer3 software (32) after performing BLAST searches to identify longer porcine sequences containing the 70-mer of interest on the microarrays. Primers were synthesized by Integrated DNA Technologies (Coralville, IA) and tested using a 100-fold dilution of either CEC or IEC cDNA. CEC cDNA was used for genes that the microarray analysis had shown to be overexpressed in CECs, whereas IEC cDNA was used for genes overexpressed in IECs. Efficiencies were calculated from plots of C$_T$ versus ln(dilution) for each primer. Primer efficiencies for the seven genes of interest were close to 100% and within 10% of the 18S efficiency. As further evidence that the primer efficiencies were close to the internal reference over the concentration range of interest, the slopes of plots of the differences in threshold cycle (C$_T$) values between the genes of interest and 18S versus ln(dilution) were $<0.1$ (21). The PCR primers used for each gene are listed in supplemental Table 1 (note: all supplemental material for this study may be found at the online version of this article).

RESULTS

Differential gene expression. Fifty-one genes were found to be differentially expressed in CECs relative to IECs at a FDR of $5\%$ using the paired test. These genes are listed in supplemental Table 2. For 42 of them, the expression level ratio (CECs/IECs) was $>2$ or $<0.5$. The paired test was used to avoid confounding by the variation among animals. We further conducted the unpaired test, where the expression level was not normalized within each animal; 43 of the 51 genes were identified at the same FDR. Thus, for 84% of the differentially expressed genes, the difference between CECs and IECs dominates the difference among individual animals. Statistical significance despite normal biological variation among animals provides additional evidence that the transcriptional profiles of coronary and iliac arteries in vivo are different. No gene was found to be differentially expressed between the left and right IECs at FDR values as high as 10%.

Seventeen of the 51 differentially expressed genes are known or have been suggested to be involved in atherogenesis (Fig. 2). Among them are several transcription factors. Early growth response factor-1 (EGR-1), a major vascular patho-
genic transcription factor, was expressed fivefold more in CECs than in IECs. EGR-1 is a stress and injury response gene, which can activate the transcription of several proinflammatory genes, including TNF-α, monocyte chemoattractant protein-1, which can activate the transcription of several proinflammatory genes in IECs. EGR-1 is a stress and injury response gene, the expression values of HOXA10 and DUSP1 were averaged over the microarray spots representing these genes. References are in online data supplement.

Fig. 2. Genes associated with atherogenesis and identified by microarray analysis as differentially expressed between coronary (CECs) and iliac artery (IECs) endothelial cells at a false discovery rate (FDR) of 5%. Expression values of CECs were normalized by the averaged expression values of left and right IECs. The expression values of HOXA10 and DUSP1 were averaged over all microarray spots representing these genes. References are in online data supplement.

Quantitative real-time PCR confirmation of microarray results. To confirm the differences in gene expression observed in the microarray experiments, quantitative real-time PCR was performed on the following seven genes: FABP5, KLF2, IGFR-1, MAOA, EGR-1, HOXA10, and HOXA9. In the microarray experiments, these genes were either over- or underexpressed in CECs over a threefold range. A strong linear correlation (R² = 0.9987) was observed between the expression levels of the first five of the genes measured using the two independent techniques (Fig. 3). Although consistently detected for IECs, HOXA10 and HOXA9 expression was not detected for CECs within 50 PCR cycles run on the same plates, with one exception [HOXA9 was detected in one of 12 wells with a log2 (fold difference) of −16]. Increasing the amount of starting RNA from 50 to 150 ng did not improve PCR detection of either HOX gene in CEC cDNA. As primer efficiencies for both HOXA10 and HOXA9 were determined using a 100-fold dilution of IEC cDNA, the results suggest that the differences in expression levels between CECs and IECs for these two HOX genes are >100-fold.

GO analysis using GoMiner. GoMiner was used as a data mining tool to compare CECs and IECs in the context of gene functions and biological processes. It is beneficial to include more genes than the 51 in supplemental Table 2 so that the transcriptional difference can be seen in a broader context. The FDR restriction was therefore relaxed to 10%, and 171 genes were found to be differentially expressed between CECs and IECs. This list of genes was analyzed by GoMiner, using an FDR of 5%. As shown in Fig. 4 and supplemental Table 4, 12 GO categories or pathways were identified with significant numbers of genes differentially expressed between CECs and IECs. The P values of all the categories were <0.0004, and in all
categories, the majority of identified genes were overexpressed in CECs. Genes involved in inflammatory and wounding response were significantly overexpressed in CECs. Specifically, the complement activation (the classic pathway) and the immunoglobulin-mediated immune response were upregulated in CECs. Complement activation is known to induce EC injury, and it has been associated with early atherosclerosis (25). Genes involved in system development, particularly nervous system development, were also expressed at different levels in CECs compared with IECs. Although the GO category involved in vascular system development was not specifically identified, it has been appreciated that the development of the vascular system shares several common cues and pathways with nervous system development (18, 43).

Three GO categories of particular importance in atherogenesis are immune response, stress response, and cell adhesion. Although these categories were not identified by GoMiner with statistical significance, 11, 29, and 11 genes in the three categories, respectively, were differentially expressed. As shown in Fig. 5, the majority of the genes in the three categories (9, 23, and 9 genes, respectively) were overexpressed in CECs relative to IECs. Consistent with Fig. 2, most putative atherogenic genes were overexpressed in CECs, whereas most atheroprotective genes were underexpressed, relative to IECs. Individual genes and their functions are listed in supplemental Table 5.

Pathway analysis using GSEA. A genomewide comparison of transcriptional profiles was performed to further explore the global expression differences, using GSEA. Instead of using a list of previously selected genes, GSEA uses the transcriptional level of the entire array to determine whether a priori defined gene sets are differentially expressed. At the recommended FDR of 25%, 27 gene sets were identified as significantly overexpressed in CECs (listed in supplemental Table 6), whereas no gene set was overexpressed in IECs. Several amino acid and organic compound metabolism pathways were identified by GSEA, reflecting the overexpression in CECs of the aldehyde dehydrogenase family (ALDH2, ALDH9A1, and ALDH9A1) and monoamine oxidase (MAO) A and B. A study by Narita et al. (24) may link ALDH to atherosclerosis; the authors found carotid plaque scores to be significantly lower in patients deficient in the ALDH2 gene. MAOA and MAOB are mitochondrial enzymes that catalyze the oxidative deamination of monoamines. One important product of MAO catalysis, hydrogen peroxide, can regulate the NF-κB pathway and the proliferation and adhesion of ECs (16). Two inflammatory pathways, IGF-1 and NTHI, were also identified as overexpressed in CECs, suggesting the possible activation of immune responses in the coronary endothelium.

DISCUSSION

Our results demonstrate that in vivo coronary artery endothelium has a transcription profile different from that of a typical systemic vessel. When compared with iliac artery endothelium, coronary endothelium overexpresses a number of proinflammatory, oxidative, and adhesion genes that are involved in atherogenesis. Several immune response pathways, including the NF-κB-, IGF-, and AP-1-mediated pathways, may be activated in normal CECs. Genes in the classic complement activation pathway were highly expressed in CECs. It is noteworthy that these transcriptional differences were found in young, healthy animals maintained on a normal diet. No atherosclerotic lesions were observed during any of the postmortem procedures. Thus these results are relevant to the...
initiation and early stage of atherosclerosis. Since the prevalence of atherosclerotic plaques is known to be higher in coronary vessels compared with other arteries, the transcriptional differences observed in this study may yield insight into molecules or pathways that influence atherosusceptibility.

Although this investigation focused on differences between CECs and ECs from the iliac arteries, we have compared the transcriptional profiles of CECs and thoracic aorta endothelial cells (AECs) harvested from a single animal. Among the 51 genes that were identified as differentially expressed in CECs vis-à-vis IECs, 37 genes were regulated in the same direction in CECs relative to AECs in that animal. This result is consistent with the notion that the transcriptional pattern of CECs is different from that of systemic arterial ECs.

Our study also shows that the transcript levels of HOXA9, HOXA10, and HOXD3 are considerably reduced in CECs compared with IECs (and the AECs referred to above). PCR results suggest that the differences in HOXA9 and HOAX10 expression may be >100-fold between CECs and IECs. Trivedi et al. (39) recently demonstrated the potential role of HOXA9 in maintaining ECs in a “basal” state and negatively regulating EC activation by interacting with the NF-κB pathway. The overexpression of HOXA9 inhibited NF-κB-dependent EC activation by affecting NF-κB DNA binding, thus inhibiting the induction of ICAM-1, VCAM-1, and E-selectin by proinflammatory cytokines. One may speculate that the higher expression level of HOXA9 in IECs, possibly maintained by a positive-feedback autoregulation mechanism (38), may cause IECs to have a higher activation threshold than CECs and therefore to be less susceptible to NF-κB-mediated EC activation.

The expression of HOX genes appears to be sensitive to shear stress. Rossig et al. (31) demonstrated that in vitro shear stress upregulated the expression of HOXA9 in human umbilical vein endothelial cells in a time- and dose-dependent manner. Passerini et al. (26) found that several HOX genes were differentially expressed in endothelial cells from the “disturbed” flow regions of porcine aorta. We have measured in vivo EC gene expression in regions of the porcine iliac artery exposed to different levels of shear, using microarrays. HOXA10 and HOAX9 were upregulated by 1.6-fold ($P < 10^{-4}$, $n = 11$) and 1.5-fold ($P = 0.002$, $n = 11$), respectively, in high shear-stress regions compared with medium shear-stress regions (unpublished data, 2006). When we consider the interaction between HOXA9 and the shear-sensitive NF-κB pathway, the mechanism by which shear stress regulates HOX genes merits further investigation.

Although the origin of the differences in gene expression between CECs and IECs in vivo is uncertain, there are several possible contributing factors. The local mechanical environment, including shear stress and circumferential strain, can affect the EC transcriptional profile as an extrinsic factor (2, 3). Dancu and Tarbell (10) recently reported reduced endothelial nitric oxide synthase (eNOS) expression and increased endothelin-1 expression in left CECs compared with AECs in rabbits. They suggest this is due to the unique asynchrony between shear stress and circumferential strain in coronary arteries. Notably, eNOS and endothelin-1 were not among the genes exhibiting significant interarterial differences in expression in the present study. This inconsistency may be due to a different choice of coronary artery and animal model in the present work. In the human, the asynchrony is less in the right coronary artery than in the left coronary artery (7); the effect of asynchrony could also be species dependent.

“Disturbed” flow or low shear stress has been considered as an atherogenic stimulus to endothelial cells. Passerini et al. (26) measured endothelial transcriptional profiles in putatively disturbed and undisturbed regions of the porcine aorta, and identified 13 of the 51 genes identified in this study as possibly sensitive to shear stress. The curved geometry of the proximal right coronary artery may induce a flow field that is more complex than that in the portion of the iliac artery examined here (15). Johnston et al. (17) demonstrated that the distribution pattern of wall shear stress is highly nonuniform in human proximal right coronary arteries and that several regions are exposed to low shear stress throughout the cardiac cycle. However, it is not clear that the difference between the shear environments in the two vessels is sufficient to explain the differences in gene expression seen here. The motion of the coronary arteries has only a modest effect on the shear exposure in these vessels (27, 45).

The CEC expression pattern may also be influenced by the unique developmental origin of coronary vessels (28, 41). Unlike most embryonic vessels, coronary vessels are derived from the proepicardial organ, a transient structure consisting of mesothelial cells, and start to develop later, at the tubular heart stage. It is reasonable to speculate that the unique origin and developmental pathway of coronary vessels can lead to CECs certain “signature” transcriptional patterns. However, further investigation is necessary to explore the possibility that CECs could constitute a unique endothelial type compared with ECs from other vessels.

As coronary arteries are generally more atherosclerosis prone than systemic arteries, the in vivo transcriptional differences between CECs and IECs found in this study may yield insight into potential molecules or pathways that influence atherosusceptibility. The lists of differentially expressed genes and GO categories presented here may suggest potential avenues for further study. Until more is understood about the origin of the observed differences, in vitro studies of endothelial response employing systemic arterial cells (and almost certainly venous cells as well) should be interpreted with caution as far as their implications with respect to coronary artery disease is concerned. Some of the genes identified here may prove to be essential regulators of atherogenesis and could serve as therapeutic targets.

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GRANTS

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