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Regional determinants of arterial endothelial phenotype dominate the impact of gender or short-term exposure to a high-fat diet

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Abstract

Regional arterial hemodynamics correlates with distinct endothelial phenotypes that may be modified by risk factors to influence focal and regional susceptibility to atherosclerosis. We compared endothelial transcript profiles from hemodynamically distinct arterial regions in 15 mature pigs: males and females fed a normal diet, and males fed a high-fat diet (15% lard, 1.5% cholesterol) for two weeks. Hierarchical clustering analysis showed preferential grouping of arrays by region over risk factor. A set of differentially expressed genes was identified which clearly distinguished regions of disturbed flow from undisturbed flow; however, few differences were observed within the same region based on gender or diet. Consistent with previous results in the absence of risk factors, the balance in gene expression was not inherently pathological at this early time-point. The results implicate regional hemodynamics as a predominant epigenetic determinant of endothelial phenotypic heterogeneity underlying atherosusceptibility in vivo.

Keywords: Atherosclerosis; Regional arterial hemodynamics; Microarray; Pig; Endothelial cell heterogeneity; In vivo; Cholesterol

There is a growing appreciation for the importance of vascular endothelial heterogeneity across multiple biological scales in normal physiology and cardiovascular disease. In the arterial system, there is a strong correlation between flow characteristics and focal susceptibility to atherogenesis, suggesting that regional hemodynamics is an important determinant of endothelial cell (EC) phenotype [1]. In humans [2,3] and pigs [4,5], susceptible regions of curvature, branches, and bifurcations experience significant flow separations, reversals, and secondary helical flows (collectively, *disturbed flow*, DF) while relatively resistant regions of straight unbranching arteries are exposed to pulsatile, laminar, unidirectional *undisturbed flow* (UF). The coordinated regulation of multiple endothelial genes in response to differential hemodynamics is proposed to result in differing functional phenotypes which promote net susceptibility to, or protection against, atherosclerosis [1].

Our recent work profiling steady-state differential gene expression between hemodynamically distinct regions of the adult pig aorta revealed pro-inflammatory transcript profiles in lesion-susceptible regions of DF that were balanced by protective biological mechanisms,

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including enhanced expression of anti-oxidant pathways [6]. We proposed that this balance would be modified by the introduction of risk factors such as gender and diet to favor a pathological outcome. In humans, gender is an intrinsic risk factor for atherosclerosis, males being more susceptible than pre-menopausal females and, possibly, females on hormonal supplementation [7]. Diets high in fat and cholesterol contribute to a pathologic lipid profile which is a well-recognized risk factor for vascular disease. We have investigated gender and a short-term exposure to a high fat diet in the context of differential hemodynamics using global analysis of endothelial gene expression. Hierarchical clustering analysis based on the most variable genes across all animals revealed that arrays grouped preferentially by arterial region (DF or UF) rather than by gender or diet. Thus, regional determinants of phenotype dominated the influence of these risk factors.

Methods

Detailed methods are provided as supplementary material online. Protocols were approved by the Institutional Animal Care and Use Committee. Fifteen gonadally intact domestic swine raised to sexual maturity (~6 mo, ~250 lbs) on a normal (standard commercial) diet were assigned to the following treatment groups for two weeks: females (NF, $n = 5$) and males (NM, $n = 5$) maintained on the normal diet, and males (HCM, $n = 5$) fed a diet high in fat (15%) and cholesterol (1.5%). At tissue harvest $\sim 10^4$ endothelial cells (EC) were immediately isolated from the following ~ 1 cm^2 regions as previously described [6]: a DF region of the aortic arch (DF-A), and UF regions of the descending thoracic aorta (UF-A) and of the common carotid artery (UF-C).

Total RNA (100 ng) was extracted and linearly amplified [6,8]. Microarray probes were prepared from 5 μg amplified aRNA by an indirect (aminoallyl-Cy dye) labeling method. Cy5-labeled arterial endothelial samples and a Cy3-labeled pig common reference probe (processed simultaneously) were hybridized to Agilent Human 1 cDNA microarrays (12,684 genes). Array images were quantified with Agilent's Feature Extraction software (v. A.7.1.1). Data pre-processing and global lowess normalization were performed using the R (v. 1.8.1)-Statistics for Microarray Analysis (SMA) package (v. 0.5.14) (http://cran.r-project.org/). Differential expression analysis was performed with PaGE (v. 5.1) (http://www.cbil.upenn.edu/PaGE), which uses a permutation-based approach to estimate the false discovery rate (FDR), and reports gene-by-gene confidences corrected for multiple testing [9,10]. Hierarchical clustering analysis was performed using XCluster (http://genetics.stanford.edu/~sherlock/cluster.html) [11] with the centered Pearson correlation coefficient as a similarity measurement. Gene lists were additionally mined for biological themes using GeneSpring (Silicon Genetics), EASE [12] (http://david.niaid.nih.gov/david/ease.htm), and Pathways Analysis (Ingenuity Systems). The complete annotated study is publicly available in accordance with MIAME standards through the RNA Abundance Database (http://www.cbil.upenn.edu/RAD/) [13].[1]

EC purity was assessed by cell-specific staining using standard immunohistochemical techniques. Tissue samples were characterized by en face nuclear staining (Hoechst 33258) and cross-sectional vessel histology (H&E, oil red-O) according to standard protocols. Relative expression of selected genes was validated by quantitative real-time PCR (QRT-PCR).

Results

Supporting information including interactive versions of figures and tables with links to fully annotated gene lists and the results of biological pathway mining are maintained online at the supplementary website (open-access upon publication; http://www.cbil.upenn.edu/RAD/extra/AtherogenicRiskFactors/).

Sample characterization

In previous studies using this model, we have demonstrated that EC are isolated from regions where they display characteristic morphological differences in shape and alignment consistent with adaptation to differential hemodynamic environments [6]. Furthermore, periodic monitoring of representative EC isolates using cell-specific immunohistochemical markers has demonstrated that pure EC populations (>99%) are rapidly and routinely achieved for transcript analysis (Supplementary Fig. 1).

Plasma cholesterol levels were measured in blood samples obtained at tissue harvest. Both total cholesterol and HDL cholesterol levels were found to be elevated in the HCM animals relative to the NM or NF groups while triglyceride levels remained unchanged (Table 1). Histological analysis (H&E) of representative sections from each flow region showed no evidence of pathological changes in the vessel walls and staining by oil red-O (not shown) was negative for lipid deposition in each of the experimental groups.

Clustering analysis

Hierarchical clustering analysis was first performed based on a subset of features with the greatest variance (top 25%) across all arrays (i.e., without regard to region, gender or diet). Strikingly, much of the variance in this set of features appeared to be attributable to regional differences (DF, UF); arrays grouped preferentially by region over gender or diet (Figs. 1A and B). Specifically, prominent clusters were visible for regions of DF and UF as indicated by the colored bars in Fig. 1A. In contrast, the groupings appeared random when arrays were identified by gender or diet (Fig. 1B) or by litter (not shown).

Regional differences were examined more closely by clustering based on a set of 1495 features identified as differentially expressed (DF-A vs UF-A) across all 15 animals at a FDR = 10% as shown in Figs. 1C and D.

[1] Open-access upon publication; accessible to reviewers at the above website. Follow link to instructions on how to query RAD. Study, "Atherogenic risk factors and regional endothelial cell gene expression."

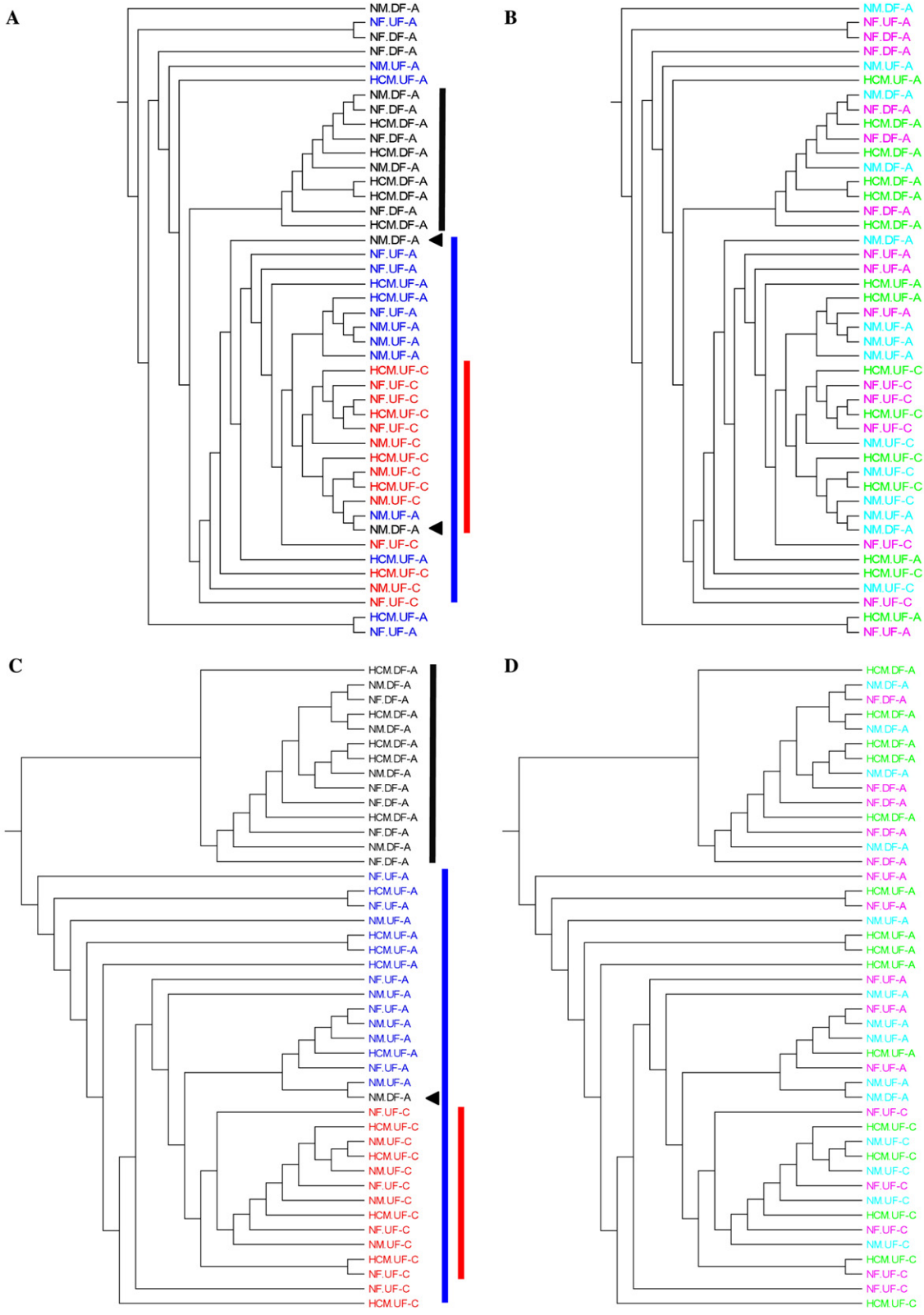

Fig. 1. Hierarchical clustering analysis of arrays: (A,B) based on the subset of 3231 features which exhibited the greatest variance (top 25%) across all assays, (C,D) based on the subset of 1475 features identified as differentially expressed (DF-A vs. UF-A) across all animals ($n = 15$, FDR = 10%). Coloring is by (A,C) region (black, DF-A; blue, UF-A; and red, UF-C), or (B,D) risk factor (pink, NF; cyan, NM; and green, HCM). Prominent clusters are illustrated by colored bars with exceptions noted by arrowheads. All results were based upon a subset of normalized log ratios (sample/reference) using the centered Pearson correlation coefficient as a similarity measure.

Table 1
Plasma total cholesterol, HDL, and triglyceride levels by animal group

Group	Total cholesterol (mg/dl)	HDL (mg/dl)	Triglycerides (mg/dl)
NM	57.8 ± 4.8	20.8 ± 1.6	19.0 ± 6.7
NF	63.8 ± 7.2	23.0 ± 3.3	22.0 ± 8.3
HCM	345.6 ± 81.1	80.4 ± 9.3	17.0 ± 8.6

Values are average ± SD. NM, normal male; NF, normal female; and HCM, high cholesterol male. $n = 5$ per group.

This set of features clearly distinguished the region of DF in the aortic arch (black bar) from both regions of UF (blue bar, Fig. 1C). Interestingly, samples from the undisturbed flow region in the carotid (UF-C, red bar) also clustered more closely with each other than with samples from the undisturbed flow region in the thoracic aorta (UF-A) based on the same set of features. There were no trends observed in sub-grouping by risk factors within the context of the regional differences (Fig. 1D). Although similar trends were observed for various subsets of differentially expressed genes, no set of features was identifiable that resulted in clustering by gender or diet. These results lead us to conclude that regional determinants of endothelial phenotype were predominant in these samples.

Differential gene expression

The relative importance of regional contributions to phenotype over that of risk factors in the cluster analysis was also reflected by differential gene expression. Differentially expressed genes were identified for each regional comparison (Table 2) both across all animals ($n = 15$) and within each experimental group ($n = 5$). The table emphasizes the reproducibility and importance of the regional differences irrespective of gender or diet. Although more genes were identified when comparing DF-A to either UF-A or UF-C, a moderate number was also found when comparing UF-A to UF-C, indicating that regions of similar UF can also exhibit substantially different gene expression profiles. Of the modest number of differentially expressed genes identified for each of the animal groups (NM, NF, and HCM) for a particular regional comparison (e.g., DF-A/UF-A), many genes were found to be common to all three groups and also overlapped substantially with the set of features identified as differentially expressed across all animals (e.g., Supplementary Fig. 2). However, even at a very liberal FDR (50%), very few genes were identified as differentially expressed within a region based on gender or diet (Table 3). The online version of these tables links to fully annotated gene lists.

Table 3
Differentially expressed genes by risk factor (gender or diet) within region

	DF-A ($n = 5$) FDR = 50%	UF-A ($n = 5$) FDR = 50%	UF-C ($n = 5$) FDR = 50%
HCM vs NM	0	0	0
NM vs NF	2	4	10

DF-A, disturbed flow region (aortic arch); UF-A, undisturbed flow region (descending thoracic aorta); UF-C, undisturbed flow region (common carotid); HCM, high cholesterol males; NM, normal males; NF, normal females; and FDR, false discovery rate.

Biological pathway mining

For the purpose of comparison to our previous results in normal animals, sets of genes identified as differentially expressed (DF-A/UF-A) are presented in the following results of data mining. Genes were annotated using information available in public databases and further examined in the context of gene categories and pathways determined a priori to be of putative interest to the mechanisms of atherogenesis (Table 4). The online version of this table links to fully annotated gene lists. Many of the genes have annotations associated with these biological categories. However, consistent with our previous work in the normal animal [6] in the absence of risk factors, the balance between pro- and anti-atherosclerotic mechanisms evident in the gene expression appears to have been retained. Key to this conclusion is the observation that no consistent differences in regional expression for the inflammation-induced adhesion molecules VCAM1 or E-selectin were seen either by microarray analysis or by QRT-PCR (Supplementary Table I). Furthermore, there was no evidence of NFκB activation by immunohistochemical analysis. Similarity between the experimental groups was reflected in the substantial overlap in the gene lists associated with the categories in Table 4. In support of the observations made herein, similar biological themes

Table 2
Differentially expressed genes by region within animal group

	ALL ($n = 15$) FDR = 10%	HCM ($n = 5$) FDR = 15%	NM ($n = 5$) FDR = 15%	NF ($n = 5$) FDR = 15%
DF-A vs UF-A	1475	364	259	220
DF-A vs UF-C	1842	604	48	506
UF-A vs UF-C	1158	130	16	241

DF-A, disturbed flow region (aortic arch); UF-A, undisturbed flow region (descending thoracic aorta); UF-C, undisturbed flow region (common carotid); ALL, across all animals; HCM, high cholesterol males; NM, normal males; NF, normal females; and FDR, false discovery rate.

Table 4
Differentially expressed genes (DF-A vs UF-A) by biological classification

Biological classification	# Genes represented	# Differentially expressed (DF-A/UF-A)			
		ALL ($n = 15$) FDR = 10%	HCM ($n = 5$) FDR = 15%	NM ($n = 5$) FDR = 15%	NF ($n = 5$) FDR = 15%
All genes	12,684	1475	364	259	220
Adhesion	498	69	18	20	17
Apoptosis	305	32	6	9	2
Cell cycle	326	34	6	6	6
Coagulation	75	7	3	1	1
Complement	55	15	6	4	4
Extracellular matrix	181	32	10	9	8
Growth factor	263	46	19	17	15
Immune response	321	48	18	12	9
Inflammation	169	19	6	2	4
Lipid/cholesterol/F. A. metabolism	337	37	14	10	3
NF-κB	17	1	0	0	0
Oxidative mechanisms	176	22	7	4	6
Oxidative stress	20	5	1	0	0
Proliferation	337	39	7	7	7
Signal transduction	924	116	34	22	20
TNF signaling	81	9	2	2	1
Transcription factor	759	83	31	19	28

DF-A, disturbed flow region (aortic arch); UF-A, undisturbed flow region (descending thoracic aorta); ALL, across all animals; HCM, high cholesterol males; NM, normal males; NF, normal females; and FDR, false discovery rate.

were associated with the differentially expressed genes (DF-A/UF-A) identified across all animals and within each treatment group (Supplementary Tables II and III).

Discussion

The study confirms differential expression of multiple endothelial genes reflective of steady state differences in vivo which are associated with multiple biological functions and pathways. Notably, a set of genes was identified by which a region of DF in the aortic arch that correlates with atherosusceptibility was clearly distinguishable from atheroprotected regions of UF both in the descending thoracic aorta and the common carotid artery. Importantly, regional differences dominated differences introduced by gender or by short-term feeding of a diet high in fat and cholesterol.

The apparent lack of gender-specific differences in these sexually mature animals is rather surprising in light of the differential susceptibility to atherosclerosis by gender observed in humans [7]. Although it is possible that this can be attributed in part to the animal model, swine have proven to be an excellent model for atherosclerosis in humans, and female pigs appear to be protected from atherosclerosis [4]. Furthermore, we have observed differences in the healing response following device intervention between male and female pigs (W.F.P., J.W.K., unpublished results). Some gender-specific effects with implications for atherosclerosis have been observed in vascular smooth muscle cells and may be non-genomic in nature [14]. These results may indicate that multiple risk factors act in the context of gender differences to produce the differential pathologic response observed in humans. Increasing the statistical power of the study using larger cohorts of pigs may reveal additional differences between males and females. However, it is apparent from the current study that regional effects dominate gender effects in endothelial cells.

In a previous work, we demonstrated that distinct endothelial phenotypes correlated with differential hemodynamics within the aortas of normal animals [6]. Although there appeared to be nothing inherently pathological about a susceptible region of disturbed flow, there was indication of a priming of inflammatory pathways balanced by protective compensatory mechanisms. This raises an important consideration as to whether there is a definable point at which a stimulus or risk factor causes sufficient insult to shift the gene expression profile towards a pro-pathological phenotype within the context of non-modifiable factors, thus overriding the compensatory mechanisms. We postulated that an elevated cholesterol level, a clinically important risk factor [7] induced by a high fat diet, would induce changes in regional endothelial gene expression that might be apparent very early-on in HCM, and that might cause a shift towards a pathological outcome. For example, an inflammatory response involving NFκB activation and the subsequent downstream expression of adhesion molecules for inflammatory cells is involved in early atherogenesis [15]. However, the evidence presented indicates that the short-term exposure to the high fat diet in this study did not dramatically influence the

underlying gene expression profile. The balance in gene expression observed prior to the introduction of risk factors appears to be retained upon short-term (two-week) elevation of plasma cholesterol levels.

Characterization of endothelial gene expression in response to hemodynamics has benefited greatly from in vitro studies using simulated flow conditions, and it has been largely through extrapolation of these findings that the susceptible or protective phenotype has been defined [16]. A merit of an in vivo approach is that we have demonstrated distinct endothelial phenotypes, characterized by the steady-state differential expression of multiple endothelial genes, in small numbers of cells isolated from representative regions in the context of their biological milieu. Analysis of regional susceptibility in vivo, however, is complicated by determinants of phenotype beyond the local hemodynamic environment. Here for example, we have also shown distinct phenotypes associated with two regions of UF from different arteries of the same animal, and other influences (e.g., developmental origins) likely contribute to these differences. Recent studies on cultured cells derived from different tissues have also contributed to an appreciation of EC heterogeneity across vascular beds [17].

Our approach to identifying differentially expressed genes (using PaGE) relies on sufficient replication to conduct a gene-by-gene statistical analysis while applying the FDR correction for multiple testing, thus controlling for the proportion of false positives within the predictions. This is superior to the heuristic approach which sets an arbitrary cutoff in expression ratios to determine differential expression. A merit of our approach, where each gene is evaluated based on its own variance, is that we routinely identify relatively small changes in gene expression which are *statistically* significant with high probability. The question arises as to whether or not these small changes are *biologically* significant. It is likely that the answer to this question is different for each individual gene, given the levels of complexity involved in biological regulation. A very small difference in transcript levels may have a large impact in one case, while relatively large differences may not translate to a meaningful effect in another. An additional level of confidence for the biological importance of selected differentially expressed genes is provided by the pathway level data where patterns involving many genes are identified. These methods are valuable for identifying targets for validation, and pathways for functional analysis, using tools such as QRT-PCR, RNAi, and immunoassays.

The characterization of regional differences in endothelial phenotype in vivo and the elucidation of the represented functions and pathways are expected to provide insight into the predisposition of certain regions of the arterial system to occlusive atherosclerotic disease. The observations made here establish a baseline of regional EC phenotypic heterogeneity in normal animals both within and across vascular beds. The study implicates regional hemodynamics as an important determinant of endothelial heterogeneity and contributes to ongoing efforts to elucidate the mechanisms underlying atherosusceptibility in vivo. Ultimately in characterizing the susceptible phenotype, it will be important to determine the relative contributions of intrinsic and environmental risk factors acting in the context of this regional heterogeneity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.04.103.

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