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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."