Differential gene expression in pulmonary artery endothelial cells exposed to sickle cell plasma

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ALTHOUGH PATIENTS WITH SICKLE cell disease (SCD) share a common β-globin gene mutation, their clinical variability suggests that extra-erythrocytic factors may modulate the pathophysiology of disease. Vasooclusive episodes (VOC) are the hallmark of SCD pathophysiology. Among the many VOC complications of this disease, acute chest syndrome (ACS) is the most common pulmonary complication, occurring in up to 45% of patients (27, 28), and pulmonary disease is the major cause of death in adults. Key to the pathology of ACS is pulmonary VOC, the etiology of which is unknown and likely multifactorial (5).

Modifications of the endothelial cell (EC) phenotype can occur by exposure to sickle erythrocytes (RBCs) and/or plasma (7, 8, 16, 19, 23). Exposure of cultured bovine or human pulmonary artery and human lung microvascular EC (BPAEC, HPAEC, and HMVEC-L, respectively) to plasma from ACS patients resulted in upregulation of mRNA expression of the vasoconstrictor endothelin-1 (ET-1) and dysregulation of nitric oxide (NO) metabolism (7, 8). Exposure of EC to sickle RBCs from SCD patients without a recent acute VOC event (steady state) results in expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin (19, 23). These data suggest that alterations in the EC phenotype occur during exposure to blood components from SCD patients even when they appear clinically well and that this provasoconstrictive and proadhesive phenotype may play an important role in the pathogenesis of VOC. The cellular mechanisms responsible for sickle VOC have yet to be defined. Moreover, the role that other, unidentified, pathways play in the development of ACS or other VOC remains unclear. We hypothesized that EC gene expression may be altered in SCD, possibly due to perturbation by noncellular circulating blood components, leading to an increased propensity toward VOC during ACS and nonpulmonary VOC.

To study this, we used synthetic oligonucleotide microarrays (U133A-B GeneChips, Affymetrix) to analyze gene expression in HPAEC exposed to plasma from SCD patients at steady state, more than 4 wk post-VOC event (steady state) results in differential expression of 50 genes in HPAEC. These genes, molecules involved in cholesterol biosynthesis and lipid transport, the cellular stress response, and extracellular matrix proteins were most prominent. Another 58 genes were differentially expressed in HPAEC exposed to plasma from ACS patients. The pattern of altered gene expression suggests that plasma from SCD patients induces an EC phenotype which is anti-apoptotic and favors cholesterol biosynthesis. An altered EC phenotype elicited by SCD plasma may contribute to the pathogenesis of sickle vasooocclusion.

Although patients with sickle cell disease (SCD) share a common β-globin gene mutation, their clinical variability suggests that extra-erythrocytic factors may modulate the pathophysiology of disease. Vasooclusive episodes (VOC) are the hallmark of SCD pathophysiology. Among the many VOC complications of this disease, acute chest syndrome (ACS) is the most common pulmonary complication, occurring in up to 45% of patients (27, 28), and pulmonary disease is the major cause of death in adults. Key to the pathology of ACS is pulmonary VOC, the etiology of which is unknown and likely multifactorial (5).

Materials and Methods

Patient selection and plasma preparation. Blood samples were obtained from 1) eight patients with sickle cell anemia (HbSS; homozygosity for the HbS gene) and one patient with HbSC disease (compound heterozygosity for HbS and HbC genes), either a) upon admission to the Medical Intensive Care Unit at Boston Medical Center with a diagnosis of ACS (n = 9) or b) at routine clinic visits (steady-state baseline, more than 4 wk post-VOC event) (n = 9); 2) healthy age-matched and racially matched volunteers without evidence of cardiopulmonary disease or SCD (n = 9). Samples were
obtained from two HbSS patients during both ACS and steady state; the remaining samples were obtained from different subjects. ACS was defined as the presence of dyspnea, cough, fever, and new pulmonary infiltrates on chest radiograph (27, 28). Peripheral blood samples were drawn into heparinized syringes and placed on ice. Platelet-poor plasma was prepared as previously described (7, 8) and was stored at −70°C until used. These studies were approved by the Institutional Review Board at Boston Medical Center.

Cell cultures. HPAEC were grown to confluence in EBM-2 media containing 10% FBS and growth supplements (Clonetics, San Diego, CA). Once confluent, the cells were trypsinized, and plated onto 100-mm culture dishes. All experiments used cells of passage 6–10. For control and patient studies, cells were incubated with serum-free media with growth supplements, media plus 10% platelet-poor plasma from normal volunteers, or media plus 10% platelet-poor plasma from either ACS patients or SCD patients at baseline. Based on prior studies demonstrating that incubation of HPAEc with 10% plasma in EBM-2 for 24 h was necessary to induce maximal NOx production, a 24-h incubation was chosen for these experiments (7); at that time, EC were well spread and RNA isolated.

RNA isolation. Total RNA was isolated using RNeasy method (Qiagen). Approximately 20 µg of total RNA was obtained from each 100-mm plate; purity was verified by agarose gel electrophoresis. After concentration determination, RNA was used to generate probes for microarray hybridization.

Microarrays. cDNA synthesis from total RNA used poly-dT primers incorporating the T7 promoter. Biotin-labeled cRNA was transcribed using cDNAs as templates, incorporating biotinylated CTP and UTP. The biotinylated cRNA was purified using RNeasy columns (Qiagen), fragmented, and used as a probe along with control probes supplied by the manufacturer (Affymetrix). Affymetrix microarrays (HG-U133A and HG-U133B) were hybridized for 16 h at 45°C, washed, stained with streptavidin-phycoerythrin, and then incubated with an anti-streptavidin antibody, according to the manufacturers’ instructions. Arrays were scanned at 488 nm using a gene array scanner (model G2500A; Agilent, Palo Alto, CA).

Data quality. Scanned images were quantified using Microarray Suite 5.0 software (MAS, Affymetrix). Gene expression levels were scaled to an average intensity of 500 U on each microarray. Detection of P values >0.04 was used to identify probes that were only marginally present or absent, meaning that no hybridizations had occurred. Of 44,928 probes, 19,606 (44%) were absent throughout all samples, 10,243 (23%) were present in all samples, and more than 50% of probes were absent in more than 40% of samples. The median expression of absent probes was 123, and 75% of absent probes had expression below 151. These values were used to determine the low-intensity differentially expressed genes. Repeated hybridizations (3 repeated samples of HPAEC incubated with plasma from SCD patients and 2 samples of HPAEC incubated with plasma from ACS patients) were performed to assure the reproducibility of the hybridization. Correlation analysis of the repeated hybridizations confirmed the high reproducibility of the results (average correlation 0.98); averages of repeated hybridizations were used for the differential analysis.

Bayesian analysis of differential gene expression (BADGE). We identified genes with differential expression by comparing three data sets: 1) EC gene expression in “steady-state” SCD patients vs. normal volunteers; 2) EC gene expression in ACS patients vs. steady-state SCD patients; 3) EC gene expression in ACS patients vs. normal volunteers. Because of the inherent problems of analyzing large data sets derived from small numbers of patients by standard statistical approaches, gene expression measurements of all the plasma samples were analyzed using the program BADGE 1.0 (http://genomethods.org/badge), which employs a Bayesian approach to analysis (17). For each gene, BADGE computes the probability of differential expression (pde), defined as the probability that the fold change of expression between two conditions A and B is greater than 1. Given the gene expression data. This probability is used as evidence for differential expression, and a probability close to 1 implies evidence of overexpression in condition A, whereas a probability close to 0 implies evidence of overexpression in condition B. BADGE computes the pde as a weighted average of two pdes derived under the assumption that the gene expression data follow a gamma and a log-normal distribution. The mixing weights are given by the probability of the two distributions, given the data. Furthermore, BADGE provides a robust estimate of the fold change of expression between the two conditions by averaging the estimates of the fold changes that are computed under the same two modeling assumptions for the gene expression data (17). In our initial analysis we selected only those genes with a pde >99.9% and an estimated relative change >1.5-fold for further analysis. This threshold was shown to produce ~90% sensitivity and specificity in experiments with ~10 samples per condition (17). The genes selected by this first analysis were ranked by selecting the largest set of genes able to correctly classify the samples by using a Bayesian classification model (17). The classification accuracy was measured by leave-one-out cross-validation.

Briefly, one sample at a time was removed from each data set, and the remaining samples were used to train the classification model that was used to label the removed sample. Classification accuracy was measured by the proportion of samples correctly classified by this procedure. The genes selected by this second analysis were further scored by their predictive score that was computed by robust leave-one-out cross-validation in which, for each sample removed, data from the remaining samples were used to select those genes with a pde >99.9% and an estimated relative change >1.5-fold that were used to classify the removed sample. By repeating this procedure for each sample, each gene was assigned a predictive score given by the proportion of times it was used as predictor. Furthermore, the selected genes were annotated by the percentage of absent calls, to identify those genes with more reliable hybridization. All data were submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession no. GSE1849.

Reverse transcriptase-polymerase chain reaction. To support the results observed by microarray analysis, the reverse transcriptase-polymerase chain reaction (RT-PCR) was performed utilizing primers for five differentially expressed genes: three of which were downregulated and two of which were upregulated. Three different gene expression sets derived from small numbers of patients by standard statistical approaches, gene expression measurements of all the plasma samples were analyzed using the program BADGE 1.0 (http://genomethods.org/badge), which employs a Bayesian approach to analysis (17). For each gene, BADGE computes the probability of differential expression (pde), defined as the probability that the fold change of expression between two conditions A and B is greater than 1. Given the gene

RESULTS

Patient characteristics. The clinical characteristics of the SCD patients and normal volunteers are summarized in Table 1. There was no significant difference in age or sex between the

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Table 1. Patient characteristics and laboratory values

<table>
<thead>
<tr>
<th></th>
<th>ACS</th>
<th>SCD</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>29.7±16.6</td>
<td>28.6±7.0</td>
<td>34.2±6.1</td>
</tr>
<tr>
<td>% Women</td>
<td>40%</td>
<td>78%</td>
<td>78%</td>
</tr>
<tr>
<td>WBC</td>
<td>18.9±5.8</td>
<td>10.0±3.3</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>9.0±1.7</td>
<td>8.8±0.8</td>
<td></td>
</tr>
<tr>
<td>Platelet count, × 10^3</td>
<td>279.9±111.9</td>
<td>370.2±105.7</td>
<td></td>
</tr>
<tr>
<td>HbF, g/dl</td>
<td>4.2±4.9</td>
<td>8.9±2.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. WBC, white blood cell count; ACS, sickle acute chest syndrome patients; SCD, sickle cell disease patients; HbF, fetal hemoglobin. *P = 0.001. †P = 0.26.

SCD patients studied at baseline and the normal volunteers, although there were fewer women in the ACS group. There was a significant increase in the white blood cell count observed in ACS patients compared with SCD patients at baseline (18.94 ± 5.8 vs. 10.0 ± 3.3, P = 0.001). There was a trend toward reduced platelet counts in the ACS patients (P = 0.07) compared with the SCD patients at baseline. Hemoglobin concentration, hematocrit, and fetal hemoglobin (HbF) levels were similar in the two groups.

Differential EC gene expression in “steady-state” SCD patients. Compared with incubation of HPAEC with plasma from normal volunteers, incubation with plasma from SCD patients at baseline resulted in differential expression of 642 genes (Supplemental Table A1; the Supplemental Material for this article is available at the Physiological Genomics web site). Examination of genes with high intensity differential expression decreased this list to 72 genes (Supplemental Table A2); 50 remained after elimination of duplicate genes and open reading frames of genes without known function (Fig. 1). Of these genes, 20 were upregulated in SCD and 30 were downregulated. Upregulated genes included those involved in cholesterol biosynthesis (HMGCR, SLCO2A1, SCD, INSIG1), extracellular matrix formation (COLIA2), and cellular proliferation (INSIG1). Downregulated genes include those involved in regulation of cellular proliferation (SAS) and the cellular stress response (HSPA4).

Differential EC gene expression in ACS patients. Compared with coincubation of HPAEC with plasma from SCD patients at baseline, coincubation with ACS plasma resulted in differential expression of 234 genes (Supplemental Table A3). Examination of genes with high-intensity differential expression decreased this list to 142 genes; 75 of which had a predicted score >70 (Supplemental Table A5). After removal of duplicate genes, ESTs and open reading frames of genes without known function, 58 differentially expressed genes remained (Fig. 2). Thirty-five of these genes were upregulated; 23 were downregulated. Thirty-one of the 58 genes have no known function. Compared with coincubation with plasma from SCD patients during the steady state, coincubation with plasma from ACS patients results in upregulation of genes involved in the response to cellular stress and/or inflammation (MMP14, HSPA4, GIP3, CALR, TXN) and downregulation of several transcription factors (CREBL1, RERE).

Comparison of gene expression during ACS with steady state baseline (Table 2). Comparison of the list of genes that were differentially expressed between SCD steady-state and normal volunteers with those differentially expressed between ACS patients and normal volunteers revealed that 24 genes overlapped both lists (Supplemental Table A7). Four of these genes were upregulated in both including COLIA2, important in collagen metabolism and extracellular matrix deposition, and SLCO2A1, involved in lipid transport and functioning of the plasma membrane. Eight genes are upregulated in HPAEC coincubated with SCD steady-state plasma and downregulated in HPAEC coincubated with ACS plasma including INSIG1, important in cell proliferation. Thirteen genes are upregulated.
in HPAEC exposed to ACS plasma and downregulated in HPAEC exposed to SCD steady-state plasma. Five of these 13 genes (38.5%) encode for ribosomal genes, and an additional 5 encode for hypothetical proteins, open reading frames, and those with no known function. The three remaining genes have a role in cell proliferation (SS18, SAS) or cAMP-mediated signal transduction (PDE4C).

One concern with a study such as ours is the differential effect that plasma from different patients could have on cultured EC. To investigate this possibility, we performed an additional analysis of data derived when plasma samples were obtained from the same individuals (n = 9) at steady state and during ACS. This analysis demonstrated that 914 genes changed expression by at least 1.5-fold. Twenty-five of the high intensity genes identified in our prior groups were differentially expressed in these samples as well, confirming the reliability of our findings (Supplemental Table A8).

Sex-specific gene expression. In our study set, there was a predominance of women, raising concerns that sex-specific gene expression may have falsely altered the results. To investigate this possibility, we created a data set in which HPAEC coincubated with plasma samples from SCD men (n = 9) were compared with HPAEC coincubated with plasma from women (n = 9). This analysis included samples from both the SCD and ACS data sets. This analysis (data available at http://www.bu.edu/sicklecell/downloads/HPAEC/) demonstrated that none of the genes identified from our previous analyses were sex specific.

RT-PCR. To support the microarray findings, RT-PCR was performed utilizing primers for 5 selected genes from the original list of 642 genes (Fig. 3). mRNA expression of ADAMTS1, CNN1, CYP1A1, CTSZ, and INSIG1 was determined in HPAEC exposed to SCD steady-state and normal plasma. Although a quantitative comparison of gene expression cannot be established by RT-PCR, similar expression patterns were
observed in all five genes by both RT-PCR and microarray. These data provide general confirmation of our microarray results.

DISCUSSION

The hallmark pathological event of SCD is VOC, a complex process with a multifactorial etiology (5). Abnormal interactions between the endothelium and blood components in SCD patients suggest that endothelial dysfunction may contribute to the pathology of sickle VOC. Our gene expression data support the hypothesis that sickle cell plasma induces phenotypic changes within the endothelium of patients. It is likely that the resultant alterations in endothelial function contribute to the pathophysiology of VOC processes such as ACS.

Increased endothelial proliferation. These studies support the development of an EC phenotype favoring proliferation. Pulmonary hypertension (PH), a common complication of SCD, may develop in part because of dysregulated angiogenesis (3, 6). Furthermore, studies evaluating expression of the key angiogenic molecule, VEGF, in SCD have demonstrated increased immunoreactivity in proliferative and nonproliferative retinal vessels. Compared with circulating EC from normal volunteers, circulating EC obtained from SCD patients have decreased apoptosis that correlates inversely with plasma VEGF levels (21). In our studies, there was a clustering of gene expression in the HPAEC exposed to SCD steady state and, particularly, ACS plasma that favored EC proliferation. MMP14 and HSPA4, genes upregulated in HPAEC exposed to ACS plasma, are both cellular stress response genes that are protective against apoptosis (10, 12). Additionally, the MMP14 knockout mouse, although embryonically lethal, demonstrates decreased EC migration and tube formation, suggesting that this molecule is critical for vasculogenesis (15). SAS and SS18 are genes implicated in tumorigenesis (14, 22, 24, 26), suggesting that their upregulation in HPAEC exposed to ACS plasma may contribute to increases in cellular proliferation. These data suggest that in SCD there are phenotypic changes in the endothelium favoring dysregulated angiogenesis. Although the etiological link between recurrent episodes of ACS and PH of SCD is not clear, dysregulated angiogenesis may play an important role in both processes. Future studies will focus on identifying genes that may provide a common link between ACS and PH of SCD.

Cholesterol biosynthesis. An unexpected finding of our study was a clustering of genes important in cholesterol biosynthesis in the HPAEC exposed to SCD steady-state plasma. The role of altered endothelial cholesterol biosynthesis or function is unknown in SCD. Although SCD patients typically have low serum total cholesterol levels, their plasma low-density lipoprotein is more susceptible to oxidation than normal volunteers (2, 20). This suggests that altered cholesterol metabolism may be important in SCD. At a cellular level, cholesterol and lipid metabolism has been best studied in the sickle RBC. Cholesterol content of the sickle RBC is increased, compared with normal volunteers, and de-oxygenation induces an alteration of the membrane cholesterol structure with increased spicule formation and membrane sterol exchange (11). It is thought that these conformational changes in the membrane may contribute to the deformability of the sickle RBC. In our model, coincubation of HPAEC from plasma from steady-state SCD patients resulted in upregulation of a number of genes implicated in cholesterol biosynthesis and lipid transport (HMGCR, HMGCS1, SLC02A1, SCD, INSIG1) (4, 13, 25). Additionally, SLC02A1 was further upregulated in HPAEC exposed to ACS plasma. Although the role of increased endothelial cholesterol biosynthesis in SCD is unknown, it may be indicative of increased cellular turnover and formation of plasma membranes. Recent studies have demonstrated that, during VOC, microparticles are shed from the plasma membrane of the endothelium, supporting this theory (18). Further studies are necessary to clarify the role of this pathway in the pathogenesis of VOC of SCD.

Limitations. There are several limitations of this study. One concern is that many genes with altered endothelial expression in other models of SCD did not change significantly in this study. There are several possible explanations for this. First, since we only evaluated mRNA expression, changes in protein expression and posttranslational protein modifications are not reflected in the results. In fact, our previous work demonstrated that, in HPAEC, although endothelial nitric oxide synthase protein and enzymatic activity were upregulated by coincubation with ACS plasma, mRNA expression was unaltered (7). Second, the current experiments were conducted at a single time point, 24 h. Thus other genes of interest in which the level of expression changes earlier, such as VCAM-1 in which the level of expression increases 8–12 h after EC stimulation (9), would have been missed under the current protocol.

The greatest changes in gene expression were seen in the comparison of SCD patients at baseline with normal volunteers. Differential gene expression occurred in a smaller number of genes (58) when comparisons were made between HPAEC coincubated with ACS plasma and plasma from SCD patients during the steady state. Once again, the issues of using one time point and the lack of protein and functional data may contribute to this. The use of plasma samples from different patients in the ACS and SCD baseline group further limits our ability to assess changes between the groups. Moreover, as many SCD patients experience subclinical crises, the distinction between steady state and crisis may be difficult to assess (1).

The changes in gene expression that occur when HPAEC are exposed to plasma from patients with SCD suggest a phenotype that is anti-apoptotic and favors cholesterol biosynthesis. During VOC, perturbations occur which may be responsible for the observed endothelial cell dysfunction thought to play a role in the pathogenesis of sickle VOC events. Moreover, the proangiogenic EC phenotype observed during ACS may predispose to chronic complications of SCD. A more complete understanding of the interactions between the endothelium and the blood components is essential to understand the pathophysiology of sickle VOC and developing more novel treatment strategies.

GRANTS

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