GENE EXPRESSION PROFILE OF HYPOXIA-INDUCED KIDNEY PATHOLOGY IN SICKLE TRANSGENIC MICE

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INTRODUCTION

A number of tissue abnormalities, including renal defects, have been identified in homozygous sickle cell anemia (SCA) patients (1). In addition, a urine concentrating defect, also affecting sickle trait patients, is common (2). These abnormalities are secondary, in large part, to a lack of structural integrity in the loop of Henle, resulting from microcirculatory obstruction by deoxygenated sickle cells.

Patients with SCA are clinically heterogeneous (3) and may also present with one or more of the following renal complications: tubular Na or K transport abnormalities, paradoxically low plasma creatinine, microalbuminuria, glomerular capillary congestion and occlusion, renal enlargement from overall congestion, proteinuria, hematuria, medullary vaso-occlusion, papillary necrosis, medullary fibrosis and medullary carcinoma. Hypoxia, as well as dehydration and infection, are well known inducers of RBC sickling and vaso-occlusion. The kidney medulla is particularly sensitive to vaso-occlusion due to its high osmolality and hypoxic environment.

The human end organ pathophysiology of SCA is re-enacted in a number of sickle transgenic mouse models including the $S+S^{Antilles}$ mouse in which the urine-concentrating defect is constitutive, and the NY1DD mouse in which the urine concentration defect is inducible by hypoxia (4). In a previous publication, we studied the $S+S^{Antilles}$ mouse model exhibiting chronic renal damage (5) and, in this present study, the NY1DD mouse exposed to hypoxia was the model for acute renal damage.

We characterized kidney damage, as measured by urine concentrating ability and histology, with changes in gene expression. We used cDNA microarrays to identify genes up or down regulated after 2 and 4 days of hypoxia and then analyzed the data using hierarchical clustering to potentially identify metabolic pathways that may be targets for therapeutic intervention.

METHODS

RNA isolation: Mice were sacrificed by an overdose of ketamine/xylazine injection, the kidneys rapidly excised, and then either immediately processed with TRIzol reagent (GIBCO BRL, Rockville, MD) to isolate total RNA, or frozen at -135^0C until use. **Microarray hybridization and scanning**: The cDNA microarrays (27 K microarray contained 28704 sequence verified mouse IMAGE consortium clones) were prepared in the Albert Einstein College of Medicine (AECOM) Microarray Facility with a custom made robot described previously (6). **Data analysis**: Data were quantified using GenePix Pro 4.1 software (Axon Instruments, Foster City, CA), normalized using the R package software with Lowess normalization (7) and filtered to remove flagged entries. Significance was determined using

the SAM (Significance Analysis of Microarrays) program (8) with FDR (false discovery rate) set at 5% (the FDR is the median number of genes falsely called significant/total number of significant genes X 100). **Validation of the microarray expression**: Total RNA was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA) using 5 μg RNA and anchored oligo dT. Real-time PCR (RT-PCR) using the Sybr green dye incorporation method (9) was performed in an ABI 7900HT Sequence Detection System with a 384 well set-up (Applied Biosystems, Foster City, CA). **Cluster analysis**: Genes were clustered using a hierarchical clustering program (http://rana.stanford.edu). Significantly regulated genes from 0, 2, and 4 day hypoxic kidneys were compared using this program (10). **Immunohistochemistry**: Kidneys from normoxic and hypoxic C57 and NY1DD mice were split longitudinally, fixed in neutral buffered formalin, and processed for standard paraffin imbedding. Embedded kidneys were sectioned at 5 microns and mounted on slides, which were used for either standard hematoxylin and eosin (H&E) staining or for immunostaining.

RESULTS

Microarray analysis was performed using kidney RNA from 3 different time points (0, 2 and 4 days of hypoxia; n=6 at each time point). Using SAM with an estimated FDR of 5%, we identified 153 significant genes that changed at least 2-fold after 4 days of hypoxia. Of these 153 genes, 124 were up-regulated and 29 were down-regulated.

Hierarchical clustering of 1-fold up and down regulated genes in the normoxic vs hypoxic kidney (**figure 1**) identified patterns of gene expression with most of the differences defined by normoxia vs hypoxia as opposed to increasing time of hypoxia. Differences were most dramatic in the node (cluster 1) containing heme oxygenase-1 (HO-1).

Changes in gene expression shown by microarray analysis were validated by RT-PCR for the up-regulated genes (lipocalin, granulin, myosin Vb, ajuba, calmin, PAM, matrix metalloproteinase 9 {MMP9}, GSTα4 and ceruloplasmin) and down-regulated genes (calbindin, solute carrier 8 and clusterin) (**figure 2**). These genes were chosen as representatives of each of the different into which all of the differentially regulated genes were divided. Categories were defined in terms of function and included mediators of oxidant stress, regulators of ion transport, regulators of cell division, maintenance of cell structure, mediators and regulators of metabolism and tissue repair. Gene expression of MMP 9 and calmin, although decreased on day 2 hypoxia as measured by RT-PCR, was up-regulated on day 4, as measured by both RT-PCR and microarray analysis. Also, the gene expression of both ajuba and PAM, although higher on day 2 compared to day 4 as measured by RT-PCR, was up regulated in hypoxic kidney as determined by both microarray analysis and RT-PCR.

Microarray results for HO-1, a key gene in the expression pattern, were confirmed and further characterized for its protein expression by immunocytochemistry in normoxic and hypoxic kidneys of both C57 and NY1DD mice.

Increased HO-1 staining was present in both C57 and NY1DD kidneys exposed to 4 days of hypoxia (**figure 3B and 3D**), with more intense staining present in the NY1DD kidneys (**fig.3D**). Kidneys from C57 mice (**fig. 3A and 3C**) showed overall less staining for HO-1 than kidneys from NY1DD mice (**fig. 3B and 3D**). C57 mice with normal oxygenation showed little staining for HO-1 in tubules and glomeruli (**fig. 3A**) while kidneys of C57 mice subjected to 4 days of hypoxia showed HO-1 staining in the cytoplasm of a small percentage (~20%) of proximal convoluted tubules (**fig. 3B, insert**). Normally oxygenated NY1DD kidneys showed HO-1 staining in the proximal straight tubules in a granular cytoplasmic pattern (**fig. 3C, insert**).

DISCUSSION

Renal failure (RF) occurs in 4-10% of SCA patients (11, 12). Proteinuria, hypertension, severe anemia, and hematuria are predictors of RF and the Bantu haplotype

significantly increases the risk of chronic RF (12). According to Platt et al (13), RF in SCA adults has an 18% mortality with 40% of those (7.6%) having overt RF. Sklar et al (14) studied 368 patients and found an increased risk of multiorgan failure, including RF.

In this study, we focused on correlating gene expression with hypoxia inducible kidney pathology in the NY1DD mouse since it may be physiologically closer to the deoxy sickling/vaso-occlusive crisis events occurring in SCA patients. Due to the complex anatomy and physiology of the kidney, many genes are differentially regulated by sickling/vaso-occlusion. One approach to understanding the relationship of individual genes to the hypoxic stimuli is to identify and classify these genes into well-defined functional pathways as we did in this study. Another approach is to look for coordinated gene expression.

Cluster analysis allows determination of which genes are being expressed in a coordinated manner, and in our case, the graphical representation of which genes are expressed at 0, 2 and 4 days of hypoxia. We examined one node that contained HO-1 and found ceruloplasmin, a Cu2+ containing enzyme with ferroxidase activity that converts Fe2+ to Fe3+ in the presence of molecular oxygen, to be up-regulated in a similar fashion to HO-1. Ceruloplasmin is also under transcriptional activation of HIF-1α similar to HO-1 (15, 16). Although HIF is known to induce a number of genes involved in erythropoiesis (erythropoietin, erythropoietin receptor, ferrochelatase, transferrin receptor), angiogenesis (VEGF, HO-1) and glucose regulation (some glycolytic enzymes, glucose transporter) , HO-1 and ceruloplasmin were the only 2 genes in the kidneys that were up-regulated.

In addition to hypoxia inducible genes, pro-apoptotic genes are also up regulated in renal ischemia (17). Fas associated via death domain (FADD) is a member of the extrinsic cell death pathway that transduces an apoptotic signal from a cell surface receptor that activates caspase 8 (18). Previously observed to be up regulated in renal ischemia (19), FADD is also up-regulated >2 fold after 4 days of hypoxia in this study.

In addition to validating the hypoxic up-regulation of HO-1 determined by microarray analysis, HO-1 immunocytochemistry (**figure 3**) localizes the site of HO-1 inducible gene expression to the renal tubules, specifically in the S1 and S3 segments of the proximal straight and convoluted tubule. Cellular studies demonstrated that renal proximal tubular epithelial cells are more susceptible to oxidative stress and are more dependent on HO-1 for protection against noxious stimuli than human mesangial cells (20). Human HO-1 gene deficiency results in advanced tubulo-interstitial injury, while the glomeruli are minimally affected (21) and suggests that the cells in these segments may be susceptible to oxidative damage and that HO-1 up-regulation is in response to this oxidative stress. Overexpression of HO-1 also confers resistance to apoptosis (21) and may be a mechanism to allow cell to cycle and initiate turnover of damaged proteins e.g. transporters. Hence, HO-1 up-regulation in response to acute hypoxia/hemolysis could involve the protection of renal proximal tubules.

CONCLUSION

The genes that were expressed in the kidney of sickle transgenic mice exposed to hypoxia of 2 and 4 day duration include genes involved in protection against oxidative damage, regulating ion transport, regulating cell division, maintaining cell structure and tissue repair. Real-time quantitative PCR confirmed the differential expression of selected genes. These data suggest that changes in gene expression will help to identify effects of hypoxia on molecular pathways in kidney cells of sickle cell mouse, and will provide useful information about the molecular responses associated to cell damage. On the other hand, pharmacological approaches in sickle cell patients with polymorphisms of the genes reported here would be a productive avenue to explore.

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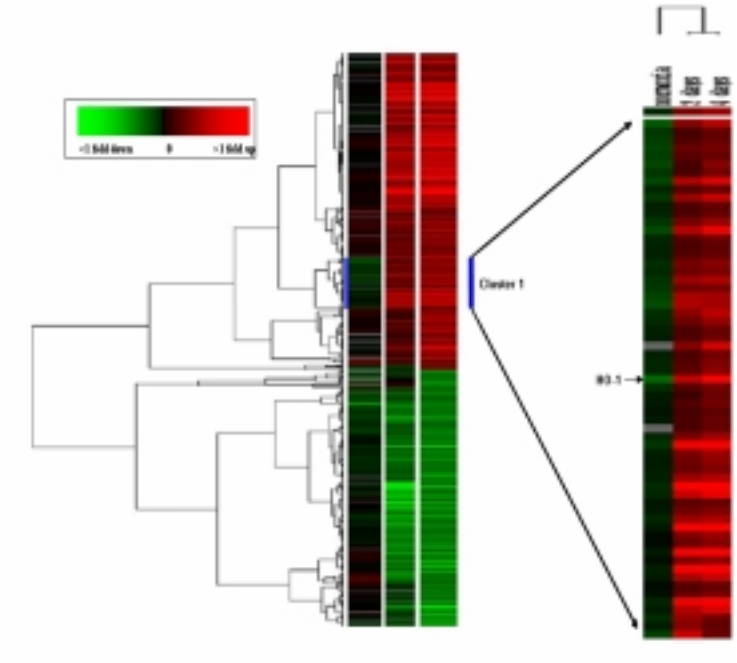

Figure 1. Hierarchical cluster analysis of normoxic, 2 and 4 day hypoxic mouse kidney genes. 723 genes that were found to be significantly up or down regulated (log_2 ratio > 0.5 and log_2 < -0.5) by SAM at day 4 hypoxia were linked to the same genes at day 0 and day 2 hypoxia and clustered using the Cluster program (http://rana.stanford.edu/software). One node (Cluster 1) was enlarged and the position of heme oxygenase-1 (HO-1) within the node was identified.

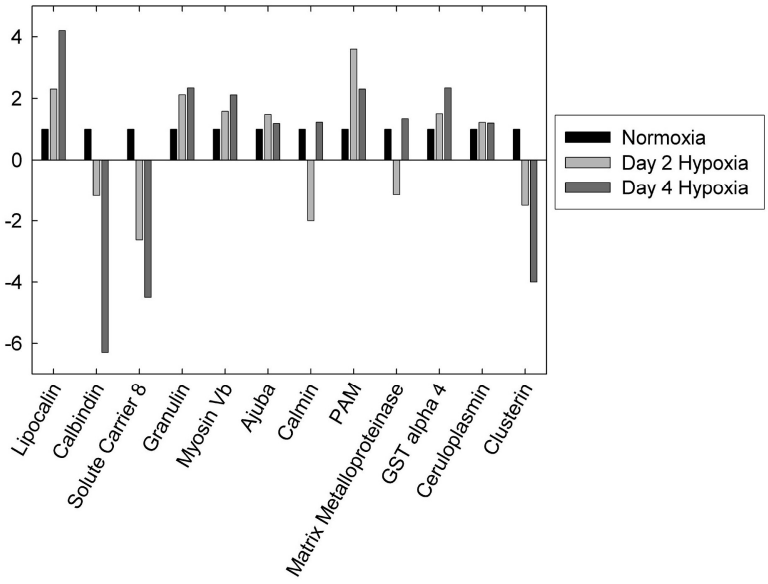

Figure 2. Confirmation of the changes in gene expression by real-time PCR. Real-time PCR (RT-PCR) was performed on RNA isolated from NY1DD mouse kidneys on 0 (normoxia), 2 and 4 days of hypoxia. The fold change of each gene is compared to the β2 microglobulin gene used as an internal standard and is represented by a histogram for each gene. All of the genes found to be up or down regulated by microarray analysis were also up and down regulated by RT-PCR although the fold changes were different in some cases.

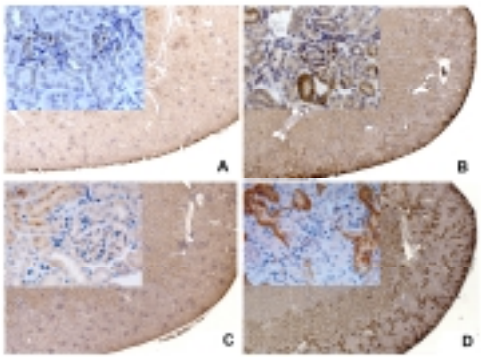

Figure 3. HO-1 immunohistochemistry in NY1DD and C57 mice kidneys exposed to 0 and 4 days of hypoxia. Original magnification 4x, high magnification inserts 40X. Panels A and B, C57 mice; panels C and D, NY1DD mice; panels A and C, 0 days of hypoxia; panes B and D, 4 days of hypoxia.