Annual Sickle Cell Disease Clinical Research Meetings

August 29 - September 2, 2005

The Natcher Conference Center
NIH Campus - Building 45
45 Center Drive
Bethesda, Maryland

Sponsored by
Department of Health and Human Services
National Heart, Lung, and Blood Institutes
Division of Blood Diseases and Resources
Blood Diseases Program
Hemoglobinopathy & Genetics Scientific Research Group

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SCHEDULE OF MEETINGS AND ROOM ASSIGNMENTS

DATE/TIME	MEETINGS	CONFERENCE ROOM
Monday, August 29		
7:30 am - 9:00 am	***REGISTRATION***	**Downstairs Lobby**
8:30 am - 5:00 pm	Renal and Urology Workshop	**E 1 & 2**
12 NOON - 1:00 pm	***LUNCH***	
6:00 pm - 10:00 pm	CSCC Steering Committee	**Regency Room (Double Tree Hotel)**
Tuesday, August 30		
7:30 am - 9:00 am	***REGISTRATION***	**Downstairs Lobby**
8:30 am - 5:00 pm	CSCC Steering Committee	**E 1 & 2**
8:30 am - 12:00 pm	MSH Patients Follow-up Steering Committee Meeting	**F 1 & 2**
12 Noon - 1:00 pm	***LUNCH***	
1:00 pm - 5:00 pm	Adult Sickle Cell Providers Network Meeting	**F 1 & 2**
1:00 pm - 5:00 pm	IASCNAPA	**G 1 & 2**
6:00 pm - 10:00 pm	Transfusion Alternatives Preoperatively in Sickle Cell Disease (TAPS) Randomized Controlled Trial	**Regency Room (Double Tree Hotel)**
Wednesday, August 31		
7:30 am - 9:00 am	***REGISTRATION***	**Downstairs Lobby**
8:00 am – 4:00 pm	CSCC Neuropsych Study – Psychologist Training	**G 1 & 2**
8:30 am - 5:00 pm	SWiTCH Steering Committee Meeting **(Closed Meeting)**	**E 1 & 2**
8:30 am - 5:00 pm	RFA PI Meeting: Genetic Modifiers of SCD and Thalassemia	**F 1 & 2**
12 NOON - 1:00 pm	***LUNCH***	

Thursday, September 1

7:30 am – 9:00 am	***REGISTRATION***	
8:30 am - 5:00 pm	BABY HUG Steering Committee and Training Session **(Closed Meeting)**	**E 1 & 2**
8:30 am - 5:00 pm	RFA PI Meeting: Pulmonary Complications in SCD	**F 1 & 2**
12 NOON - 1:00 pm	***LUNCH***	

Friday, September 2

7:00 am - 8:30am	***REGISTRATION***	**Downstairs Lobby**
8:30am - 3:00 pm	BABY HUG Steering Committee Meeting **(Closed Meeting)**	**E 1 & 2**

Renal and Urology Complications in Sickle Cell Disease Workshop
Monday, August 29, 2005

AGENDA

8:30 A.M. Introductions, Opening Remarks,
Meeting Charge, ***Dr. Duane R. Bonds***
Meeting Chair, ***Dr. Cage S. Johnson***

8:45- 9:15 Overview of Renal Problems in Sickle Cell Disease- Dr. Eugene Orringer

9:15 - 9:45 Baseline Renal Studies from BABY HUG - Dr. Russell Ware

9:45-10:15 Hydroxyurea and Renal Failure in Adults - Dr. Kenneth Ataga

10:15-10:30 Break

10:30-11:00 Treatment Interventions for Renal Problems - Dr. Antonio Guasch

11:00 - 11:30 Morphine Potentiates Sickle Cell Nephropathy - Dr. Kalpna Gupta

11:30-12:00 Genetic Modulation of Sickle Cell Renal Disease - Dr. Martin Steinberg

12:00 - 1:00 Lunch

1:00-1:30 Regional and Systemic Complications of Renal Ischemia in Transgenic Sickle Mice - Dr. Karl Nath

1:30 -2:00 Priapism in Children - Dr. Zora Rogers

2:00-2:30 Priapism in Adults - Dr. Arthur Burnett

2:30 - 2:45 - Break

2:45 - 3:00 Urine Concentration in Infants - Baseline Data from BABY HUG - Dr. Scott Miller

3:00 - 3:15 Summary Discussion - Dr. Cage Johnson

3:15 - 4:00 Discussion of Unanswered Questions and Areas of Research Opportunities

CSCC Steering Committee

MSH Patients' Follow Up - Extension I
Steering Committee and Coordinators Meeting
Tuesday, August 30, 2005
8:30 – 12:00 PM

Agenda

Continental Breakfast: 8:00 AM

1. **Welcome and Introductions - Dr. Bonds**

2. **Data Collection and Procedures – Dr. Barton and Ms. Brandon**
    a. **Certification**
    b. **Payments**
    c. **Recruitment and Data Collection Status**
    d. **Annual Visit Windows**
    e. **Data Collection Schedule**
    f. **MSH Web Site**

3. **Scientific Affairs – Dr. Barton**
    a. **Ancillary Studies**
        i. **Status of Pulmonary Hypertension Study**
        ii. **Status of Armstrong Ancillary Study**
    b. **Review of Status of Analyses and Manuscripts**

4. **Scientific Presentation – BNP Analyses – Mark Gladwin, MD**

5. **Long Term Follow-up Success - Louise Dorn, RN & Susan Jones, RN**

6. **Executive Committee Membership - Dr. Barton**

7. **Discussion**

8. **Other Business**

9. **Date of next meeting**

10. **Writing Group Meetings**
    a. **BNP Group**
    b. **Longitudinal Laboratory Determinations Group**
    c. **QOL Group**
    d. **Long-Term Follow-up Group**

Lunch: 12:00 PM

Adults Sickle Cell Providers Network Meeting

Transfusion Alternatives Preoperatively in Sickle Cell Disease Randomized Controlled Trial
(TAPS RCT)

Doubletree Hotel, Rockville, Maryland, USA

Tuesday 30 August 2005 6 – 10 pm

AGENDA

1 Welcome and introduction to the trial

2 Presentation on trial design (see Trial Summary attached)

3 Discussion of Protocol (see Draft Trial Protocol*)
- inclusion/exclusion criteria
- end points
- laboratory testing

4 Logistics (see Data Flow Chart*)
- randomisation
- trial flow chart
- data collection and analysis
- laboratory testing

5 Regulatory Aspects
- sponsorship/insurance
- regulatory approval
- trial monitoring

6 Trial Budget

7 Other Issues

8 Next Steps

* The draft protocol and data flow chart will be sent to all those centres who have expressed an interest in enrolling patients into this trial. For other centres, please e-mail: moira.malfroy@nbs.nhs.uk, to request an electronic copy.

Transfusion Alternatives Preoperatively in Sickle Cell Disease Randomized Controlled Trial - (TAPS RCT)

SUMMARY OF TRIAL DESIGN AND METHODOLOGY

Preparation of patients with sickle cell disease for elective surgery traditionally involved partial exchange transfusion to raise the haemoglobin (Hb) A level in the belief that this would reduce the frequency of postoperative sickle related complications. However, a large randomised study has shown that a conservative (top-up) transfusion regime is equally safe (Vichinsky et al 1995). This approach halved exposure to allogeneic blood, with a concomitant reduction in red cell alloimmunisation. A further development is that some clinicians now perform elective surgery successfully without any preoperative transfusion (Buck et al 2005). The benefits of routine preoperative transfusion as against no transfusion have not been rigorously proven in the era of modern anaesthesia and meticulous supportive care, a question in which we believe there is equipoise.

Consequently a new trial, the TAPS RCT, is being set up to investigate whether the administration of blood transfusion preoperatively to patients with sickle cell disease increases or decreases the overall rate of perioperative complications. Answering this question will provide valuable evidence to improve the care of sickle cell patients having surgery.

Type of design

A Phase III, multicentre, pragmatic, parallel group, sequential (Whitehead 1999), randomised controlled trial. Because of the nature of the intervention, the trial will be unblinded. Approximately 400 patients will be needed to detect a 10% difference in complication rates between the two groups (transfused and untransfused) with 90% power. The sequential design allows for interim analysis at pre-determined points as the outcomes accumulate, allowing for stopping should a significant or null effect be established. Patients will be randomly allocated to the two groups using block randomisation stratified by centre, surgical risk and history of sickle cell complications.

Patients to be studied

Patients with Sickle Cell Anaemia type Hb SS or Sβ^0thal undergoing low or medium risk elective surgery as defined by Koshy et al (1995), requiring general or regional anaesthesia.

Exclusion criteria:

- **High risk surgery (intracranial, cardio-thoracic)**
- **Haemoglobin level at randomisation <6.0 g/dL**
- **On a regular transfusion regime**
- **Children at high risk of stroke**

Trial interventions

Patients will be randomised to two arms:

Arm A will receive a preoperative transfusion. Patients who present with an admission Hb of <9g/dL will receive a simple transfusion (top-up). Patients presenting with a Hb of ≥9g/dL will undergo a partial exchange transfusion. The aim will be to achieve a preoperative post transfusion target Hb of 10 g/dL in both groups.

Arm B will receive no preoperative transfusion.

Clinicians will be free to follow local policies and procedures for other aspects of perioperative care, but a suggested care protocol is available for consultation if required. Intra and postoperative transfusions can be given as clinically indicated.

Outcome measures

The primary outcome measure is the frequency of all significant complications (sickle related, transfusion related and infections) between randomisation and 30 days post surgery. These are currently being defined. Secondary outcome measures include: total days in hospital (pre, intra and post-operative) up to 30 days post surgery, number of red cell units received intra and postoperatively, readmission, or failure to discharge within 30 days of surgery.

Duration

Patient participation will be from randomisation up to 30 days post surgery. Duration of the trial may be up to 4-5 years from the start of randomisation, dependent on recruitment rates and the results of sequential analysis.

Add-on study

We are considering doing a concurrent observational study (non-funded) of current practice, to collect data on patients with genotypes other than Hb SS or S ^{0}thal who would not be eligible for the trial. Basic and follow-up data only would be returned on a single form.

Health Economics

Health economic evaluation will be based on quality-adjusted life-years over the period of the trial using a one page EQ-5D questionnaire at baseline and follow-up at 30 days post surgery.

Flow-chart of trial entry, randomisation and treatment

References

Buck J, Casbard A, Llewelyn C, Johnson T, Davies SC, Williamson LM. Preoperative Transfusion in Sickle Cell Disease: a survey of practice in England. *Eu J Haem*, 2005;75:14-21.

Koshy M, Weiner SJ, Miller ST, Sleeper LA, Vichinsky E, Brown AK, Khakoo Y, Kinney TR. Surgery and anesthesia in sickle cell disease. Cooperative Study of Sickle Cell Diseases. *Blood*, 1995;86:3676-3684.

Whitehead J. A unified theory for sequential clinical trials. *Stat Med*. 1999 ;18(17-18):2271-86.

Vichinsky EP, Haberken CM, Neumayr L, Earles AN, Black D, Koshy M, Pegelow C, Abboud M, Ohene-Frempong K, Iyer RV and the Preoperative Transfusion in Sickle Cell Disease Study Group. A comparison of conservative and aggressive transfusion regimens in the perioperative management of sickle cell disease. *New England Journal of Medicine*, 1995;333:206-213

Contact for the trial: Moira Malfroy, Research Nurse and Trial Coordinator, National Blood Service
E-mail: moira.malfroy@nbs.nhs.uk Tel: +44 (0) 1223 548169

TAPS/Version 0.2

JOINT NHLBI RFA GRANTEES' MEETINGS:

RFA HL-01-001: GENETIC MODIFIERS OF SINGLE GENE-DEFECT DISEASES

RFA HL-04-015: PULMONARY COMPLICATIONS OF SICKLE CELL DISEASE

RFA HL-01-003: CARDIOVASCULAR, LUNG, AND BLOOD IMMUNOBIOLOGY IN HEALTH AND DISEASE

August 31- September 1, 2005

AGENDA

Wednesday, August 31, 2005

8:30 am Greg Evans, NHLBI
Welcome and Introduction

RFA HL-01-001: GENETIC MODIFIERS OF SINGLE GENE-DEFECT DISEASES

8:40 am Martin Steinberg, Boston University, Boston, MA
Genetic modulation of sickle cell anemia

9:40 am Ron Nagel, Albert Einstein College of Medicine, Bronx, NY
Pleiotropic and epistatic effects in sickle cell disease

10:40 am BREAK

10:55 am Tim Townes, University of Alabama, Birmingham, AL
Genetic modifiers of sickle cell disease

Noon LUNCH

1:00 pm Orah Platt, Children's Hospital, Boston, MA
Genetic modifiers of severity in sickle cell disease

2:00 pm Marilyn Telen, Duke University, Durham, NC
Outcome-modifying genes in sickle cell disease

3:00 pm BREAK

3:15 pm Andreas Braun, Sequenom, Inc, San Diego, CA
Genome-wide search for modifiers of severity in beta-thalassemia

RFA HL-01-003: CARDIOVASCULAR, LUNG, AND BLOOD IMMUNOBIOLOGY IN HEALTH AND DISEASE

4:15 pm Robert Means, Medical University of South Carolina, Charleston, SC
Inflammatory control of erythropoiesis in SCD

4:45 pm Paul Frenette, Mount Sinai School of Medicine, New York, NY
Adhesion mechansims in sickle cell vaso-occlusion

5:15 pm ADJOURN

Thursday, September 1, 2005

RFA HL-04-015: PULMONARY COMPLICATIONS OF SICKLE CELL DISEASE

9:00 am Greg Evans, NHLBI
Welcome and Introduction

9:10 am Punam Malik, Children's Hospital, Los Angeles, CA
Role of Placenta Growth Factor in Acute Chest Syndrome

10:10 am Marilyn Telen, Duke University, Durham, NC
Pulmonary Hypertension

11:10 am BREAK

11:25 am Kwaku Ohene-Frempong, Children's Hospital of Philadelphia
Oxyhemoglobin Desaturation and Vasculopathy

12:25 pm LUNCH

1:25 pm Michael Debaun, Washington University, St. Louis, MO
Asthma and Nocturnal Hypoxemia

2:25 pm Victor Gordeuk, Howard University, Washington, DC
Pulmonary Hypertension and the Hypoxic Response

3:25 pm Group Discussion- Collaborations

4:00 pm ADJOURN

BABY HUG STEERING COMMITTEE MEETING
September 1-2, 2005
Natcher Conference Center, NIH

September 1, 2005 (Thursday)

8:00 AM	Welcome and introductions	Wang
8:10	Report from NHLBI	Bonds
8:20	Comments from NICHD; BPCA Site Visits	Gerber
8:30	Clinical Center Quarterly Performance Report	Thompson
8:45	SAE start/stop dates and Morbidity Review Committee Files	Rees, Thompson,
9:00	Update on patient recruitment and log data	Wang, Wynn
9:15	Protocol Streamlining ▪ Review of ballot results ▪ Accepted changes to protocol ▪ Controversial proposed changes to protocol	Wang
10:00	BREAK	
10:20	TCD studies	Adams
10:40	Bayley studies	Armstrong
11:00	DTPA issues	Rana
11:15	Renal data update	Ware
11:30	Discussion with NHLBI Director	Nabel
12:00 PM	LUNCH	

1:00	Drug distribution		Perry Point
	Steering Committee Meeting		Coordinators'
1:30	Urine penicillin	Rogers	BREAKOUT SESSION
1:45	Urine concentrating studies	Miller	
2:00	Pitted red cell counts	Rogers	
2:15	Howell-Jolly Bodies	Ware	
2:30	DNA Mutations	Ware	

2:45	Immunology	Lederman, Casella
3:00	Discussion of studies	Wang
3:30	Coordinators' Report	Wynn, Debenham
4:00	Discussion of streamlining protocol	Steering Committee
4:45	Final recommendations for streamlining protocol	Steering Committee
5:00	Adjournment	

September 2, 2005 (Friday)

8:30	HU Pharmacokinetics	Rogers, Bonds, Gerber
9:30	Exit Strategy	Thompson, Bonds, Rogers, Gerber
10:30	BREAK	
10:45	Difficult Cases - problems during enrollment and follow-up	Wang, Ware
11:00	Publications Committee Report	Miller
11:15	Discussions for new centers	Rees, Thompson
12:00 PM	LUNCH	
1:00	Discussions for new centers	Rees, Thompson
2:30	ADJOURNMENT	

RENAL COMPLICATIONS OF SICKLE CELL DISEASE

Eugene P. Orringer, MD, University of North Carolina at Chapel Hill

Sickle cell anemia and the related hemoglobinopathies are associated with a broad spectrum of renal abnormalities. Most sickle cell patients acquire an impaired urinary concentrating ability. They also have defects in urinary acidification and potassium excretion, and they often have supra-normal proximal tubular function, evidence for which includes increased secretion of creatinine and increased reabsorption of phosphorus and β_2-microglobulin. Many young patients with sickle cell disease have supra-normal renal hemodynamics with elevations in both effective renal plasma flow and glomerular filtration rate, parameters that tend to decrease with age.

Proteinuria is a common finding in adults with sickle cell disease, with levels of protein excretion often reaching the nephrotic range. Proteinuria is often the first sign of sickle cell nephropathy, the pathologic features of which include enlargement of the glomeruli and focal glomerular sclerosis. The progression of sickle cell nephropathy to end-stage renal disease may be slowed by adequate control of both hypertension and proteinuria. Inhibitors of the angiotensin converting enzyme or an antagonist of the receptor may be particularly useful in this setting. Both proteinuria and hypertension appear to predict the progression to end-stage renal disease. While renal failure can be effectively managed by dialysis and/or renal transplantation, there is some suggestion that renal transplant recipients with sickle cell disease have an increased rate of complications.

Hematuria is seen in individuals with all of the sickle cell diseases as well as with sickle cell trait. In most cases, the etiology of the hematuria turns out to be benign with perhaps the most common cause being papillary necrosis resulting from medullary ischemia and infarction. However, there does appear to be an increased association between sickle cell disease and renal medullary carcinoma. Therefore, all sickle cell patients who present with hematuria should undergo a thorough evaluation in order to exclude the presence of this aggressive neoplasm.

Finally, erythropoietin levels in patients with sickle cell disease are usually lower than one would expect for the degree of anemia present, and the levels tend to decrease further as renal function deteriorates. In addition, acute renal failure is a common component of the acute multiorgan failure syndrome.

ASSESSMENT OF RENAL FUNCTION IN THE PHASE III MULTICENTER HYDROXYUREA (BABY HUG) TRIAL FOR INFANTS WITH SICKLE CELL ANEMIA

***Russell E. Ware*, Scott T. Miller, Renee C. Rees, Rathi V. Iyer, Zora R. Rogers, Sherri A. Zimmerman, Caterina Minitti, Julio Barredo, Sohail Rana, James F. Casella, Stuart Toledano, Daner Li, Bruce Thompson, Win C. Wang. St. Jude, Memphis TN; SUNY, Brooklyn NY; C-TASC, Baltimore, MD; U. Miss., Jackson, MS; UT Southwestern, Dallas, TX; Duke, Durham, NC; CNMC, Wash, DC; MUSC, Charleston, SC; Howard, Wash, DC; Johns Hopkins, Baltimore, MD; U. Miami, FL.**

Hydroxyurea therapy leads to induction of fetal hemoglobin and has both laboratory and clinical efficacy for persons with sickle cell anemia (SCA). To date, Phase I/II trials have been completed in adults, children, and even infants with SCA, and demonstrate minimal short-term toxicities other than transient reversible myelosuppression. In adults with clinically severe disease, the Phase III MSH trial demonstrated that hydroxyurea can reduce pain, acute chest syndrome, transfusions, and hospitalizations. Although hydroxyurea can help prevent acute vaso-occlusive events, its ability to prevent chronic organ damage is unknown. To address this critical issue, BABY HUG was designed as a multicenter, randomized, double-blinded, placebo-controlled Phase III trial with the primary aim of assessing the efficacy of hydroxyurea in preventing damage to the spleen and kidney in infants with SCA. The kidney and the spleen were chosen as the primary endpoints since both organs are damaged early in life and their function can be quantitatively assessed. BABY HUG infants are enrolled without selection for clinical severity; initial eligibility in the Feasibility and Safety Pilot Study was limited to age 12-17 months, but more recently infants as young as 9 months can be enrolled. Assessment of renal function in BABY HUG primarily focuses on the glomerular filtration rate (GFR), since the GFR increases early in life in SCA and may be an early marker of renal damage. GFR is calculated using a baseline radionuclide study with Tc-99m DTPA injection and plasma clearance at 1, 2, and 4 hours. In addition, GFR is estimated using the Schwartz equation: GFR (mL/min/1.73 m^2) = [(weight in kg) x (k)] / creatinine, where k is an age-dependent constant of 0.55 for toddlers and the creatinine is measured accurately by HPLC methodology. As of late June 2005, a total of 46 infants from 10 centers have had baseline GFR measurements. For the DTPA measurements, the average value (mean ± 1SD) = 122.7 ± 41.7 mL/min/1.73m^2, median 114.6, range 54.0 – 300.9 mL/min/1.73m^2. The DTPA GFR was elevated above 120 mL/min/1.73m^2 in 37% of infants age 12-15 months, but in 53% of infants above 15 months. There was a negative trend between DTPA GFR and creatinine (p=.07) but no correlation with age as a continuous variable (p=.52). The estimated GFR using the Schwartz equation had overall higher values: mean value of 159.0 ± 51.3 mL/min/1.73m^2, median 154.2, range 65.8 – 261.3 mL/min/1.73m^2. There was a positive correlation between estimated GFR and age (p=.08). The DTPA GFR measurement was significantly correlated with the Schwartz GFR estimate, r = .35, p= .03. These limited baseline BABY HUG data indicate that (1) GFR can be measured in infants by both DTPA clearance and Schwartz estimate; (2) GFR is elevated early in life in infants with SCA and may increase with age; and (3) GFR appears to be a good primary endpoint for assessing the efficacy of hydroxyurea to prevent organ damage in SCA.

THE INFLUENCE OF RENAL FUNCTION ON HYDROXYUREA PHARMACOKINETICS IN ADULTS WITH SICKLE CELL DISEASE

Jing-He Yan[1], PhD, *Kenneth Ataga[2], MD*, Sanjeev Kaul[1], PhD, Jeffery S Olson[3], PharmD, Dennis M Grasela[1], PharmD, PhD, Samantha Gothelf[3], PharmD, Abdulah Kutlar[4], MD, and Eugene Orringer[2], MD [1] Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, [2] University of North Carolina, Chapel Hill, NC, [3] Bristol-Myers Squibb Pharmaceutical Research Institute, Plainsboro, NJ, [4] Medical College of Georgia, Augusta, GA

Hydroxyurea is approved for the treatment of patients with severe sickle cell anemia. In addition, it is recognized that renal abnormalities are common in patients with sickle cell disease (SCD) as they get older. However, there is a paucity of pharmacokinetic data with the use of hydroxyurea in these patients, especially in those patients with varying degrees of renal function. This was an open-label, non-randomized, 2-center study conducted to assess the influence of renal function on the pharmacokinetics of hydroxyurea in adults with sickle cell disease (SCD). Seventeen patients were divided into 5 groups: normal renal function (n = 7), mild renal impairment (n = 2), moderate renal impairment (n = 3), severe renal impairment (n = 2), and end-stage renal disease (ESRD, n = 3). Except for patients with ESRD, all the patients received a 15-mg/kg single oral dose of hydroxyurea. Patients with ESRD received a 15-mg/kg oral dose of hydroxyurea on 2 occasions. Blood and urine samples were collected for the assessment of hydroxyurea pharmacokinetics. The results indicate that the systemic exposure increases and the urinary recovery decreases as the degree of renal insufficiency worsens. On the basis of the exposure and the apparent clearance from the current and 2 historical studies, we have proposed an initial dosing regimen of hydroxyurea (7.5 mg/kg/day) for SCD patients with CL(cr) <60 mL/min. This dosing strategy is anticipated to provide a safe dose for SCD patients with renal impairment.

Antonio Guasch, M.D.

Treatment Intervention for Renal Problems

MORPHINE POTENTIATES SICKLE CELL NEPHROPATHY

***Kalpna Gupta*,** Ramya Arerangaiah, Marc L Weber, Nagamala Chalasani, *Karl A Nath, Robert P Hebbel. Hematology Oncology and Transplantation, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN. *Division of Nephrology and Hypertension, Mayo Clinic College of Medicine, Rochester, MN

Like vascular endothelial growth factor (VEGF), opioids induce angiogenesis and accelerate wound repair in ischemic wounds via nitric oxide-dependent opioid receptor (OR) mediated mechanisms (Cancer Res 62; 4491, 2002; Wound Rep Regen 13;165, 2005). It is noteworthy that both chronic human use of heroin a precursor of morphine or modulation of VEGF in mouse kidney leads to renal pathology. We hypothesized that chronic therapeutic use of opioids in sickle cell anemia promotes renal disease at a multicellular level in the kidney via ORs and distinct signaling pathways. We examined the effect of morphine treatment on renal pathology of transgenic sickle mice with different severity (mild, NY1DD, medium, hBERK and severe, BERK). We injected mice subcutaneously with morphine doses used in human patients (1.4 – 2.8 mg/Kg, with increments of 0.1 mg/Kg/week). Three weeks of morphine treatment induced a 25% and 40.5% increase in the kidney weights of NY1DD and hBERK mice, respectively ($p<0.01$ & 0.003 vs. PBS), while 6 weeks of treatment of BERK stimulated 43.8% increase ($p<0.001$ vs. PBS). Histological responses to morphine were also concordant with each other in all 3 types of sickle mice. However, the intensity of pathological changes increased markedly after 12 weeks of treatment as compared to 3 weeks. Morphine induced an increase in, glomerular volume, glomerular hypercellularity, prominence of juxtaglomerular apparatus, peritubular congestion, tubular dilatation with proteinaceous casts, and vascular dilatation. Quantitatively, morphine increased glomerular volume by 30% and cellularity by 50% ($p<0.001$ and 0.05, respectively vs PBS), recapitulating the glomerular alteration observed in heroin associated nephropathy (HAN). These pathological changes were associated with an eight-fold increase in protein:creatinine ratio, suggestive of an alteration in renal function. Morphine-induced hyperplasia in the kidney is further corroborated by upregulation of growth-promoting and cytoprotective nitric oxide (NO) and hemoxygenase-1 (HO1) pathways. Morphine treatment increased kidney NO by 15% and HO1 activity by 60-70% ($p<0.05$ and 0.0001 vs. PBS, respectively), and eNOS as well as HO-1 protein and RNA. Increased eNOS co-localized with the endothelium, whereas, increased HO1 colocalized with the non-endothelial compartments in the kidney. Morphine stimulates NO via mu opioid receptor (MOR) and promotes vasodilation. We observed that morphine mediates mesangial cell activity via kappa opioid receptor (KOR). Morphine stimulated the proliferation of mesangial cells derived from both wt and MOR KO mice, but not of those derived from KOR KO mice. Moreover, KOR agonist U50,488H stimulated mesangial cell proliferation *in vitro* by 101% ($p<0.01$ vs. PBS) and stimulated phospho-STAT3 in a time dependent fashion, but MOR agonist DAMGO did not, suggesting that KOR mediates mesangial cell proliferation via STAT3 signaling. STAT3 inhibitor peptide PpYLKTK completely abrogated morphine as well as U50,488H induced mesangial proliferation. In aggregate, these data suggest that morphine promotes hypercellular, cytoprotective and vasodilatory renal alteration via MOR and KOR in a cell specific manner by activating NO, HO1 and STAT3 signaling in sickle mice. Therefore, prolonged treatment with morphine may lead to an exacerbation of sickle nephropathy.

GENETIC MODULATION OF SICKLE CELL RENAL DISEASE

***Martin H Steinberg (1)*, Vikki G. Nolan (1), Ann C. Rybicki (2), Clinton Baldwin (1), Diego F. Wyszynski (1), Mary E. Fabry (2), Lindsay A. Farrer (1), Ronald L. Nagel (2); Boston University School of Medicine, Boston, MA (1), Albert Einstein College of Medicine, NY, NY (2)**

Renal damage, commonly seen in patients with sickle cell anemia, as with other complications of this disease, is likely to be genetically modulated. In a sickle transgenic mouse with renal defects similar to those seen in sickle cell anemia, differential gene expression in the kidney identified genes highly up-regulated. Among these genes were cytochrome P450 4a14 (*cyp411*), mitochondrial hydroxyl-methylglutaryl CoA synthase (reductase) (*hmgcr*), cytokine inducible SH-2 containing protein (*cish*), retinol dehydrogenase type III(*rdh3*), 2-hydroxy oxidase (h*ao2*), renin-1 (*ren*), alkaline phosphatase 2 (*alplp)*and arginase II (a*rg2*). These genes can be integrated into several different responses to organ damage: response to hypoxia cascade, a replacement cascade involving the loss of renal proteins in the urine, or a tissue damage-ameliorating cascade in which damage repair is the main goal. We hypothesized that polymorphisms in these pleiotropic genes might permit them to modulate the development of renal failure and other organ damage in patients with sickle cell anemia. Accordingly, we examined single nucleotide polymorphisms (SNPs) in the human homologs of these selected genes in patients with sickle cell anemia. The Cooperative Study for Sickle Cell Disease (CSSCD), a longitudinal study of the natural history of sickle cell disease, provided a database of 4,082 patients who were observed for about 5 years. Laboratory data included renal function estimated by serum creatinine. Blood samples were obtained for globin gene analysis and were used for SNP genotyping. We studied only patients with sickle cell anemia, with or without coincident _ thalassemia. DNA samples and sufficient clinical and laboratory data were available for 779 patients. An adjusted creatinine clearance, using the Cockcroft-Gault and Schwartz formulas was computed for adults and children, respectively. These formulas were used since glomerular hyperfiltration and increased secretion of creatinine reduces the utility of serum creatinine as a measure of renal function in sickle cell anemia. We analyzed the adjusted creatinine clearance as a continuous variable using multivariate linear regression and as a categorical variable by comparing the lower quartile (25th percentile) to the upper (75th percentile) using logistic regression. All analyses were adjusted for the presence of coincident _ thalassemia. We found an association of a single ht SNP (rs2295644) marking the *ARG2* gene with creatinine clearance. *ARG2*, expressed in the kidney, is believed to play a role in NO metabolism.

We then screened patient DNA for SNPs in other candidate genes that included inflammatory mediators, modulators of oxidant injury and NO biology, vasoregulatory molecules and cell adhesion factors and that might be associated with the renal failure phenotype. Genotyping was first done using the Sequenom mass spectrometry SNP genotyping system. For quality control purposes about 3% of the DNA samples were regenotyped and Hardy-Weinberg equilibrium was assessed for each SNP among controls. In this initial screen, we considered a SNP to be associated with renal function when the p-value was less than or equal to 0.01, or more than one SNPs in the same gene was significant at the 0.05 level. If a SNP met these criteria, a second phase of genotyping was done to study additional haplotype tagging (ht) SNPs. We previously reported the association of genes in the TGF-$/BMP pathway with selected sickle cell subphenotypes (Sebastiani et al, Nature Genet 37: 435, 2005; Baldwin et al, Blood 106: 372, 2005). Additional genes in this pathway were genotyped using the ABI SNPlex system. These genes included *BMP6*, bone morphogenetic protein (BMP) receptors, TGF- receptors, SMADs, MAP kinases and their associated co-factors such as SARA, CDH1 and SMURF1. *BMPR1B*, a BMP receptor gene, was

associated with creatinine clearance in both analyses. When analyzed as a continuous trait, 5 ht SNPs were associated with creatinine clearance (p values ranging from 0.0008 and 0.04) and when treated as a qualitative trait, there were 4 significant associations (p values ranging from 0.01 to 0.05) Less striking associations of creatinine clearance with other genes in the TGF-/BMP pathway were also found.

Priapism, rare in the general population, is one of the many serious complications associated with sickle cell disease and few studies have described the clinical and hematological characteristics of individuals with sickle cell-related priapism. Using data from the CSSCD, we found 273 cases and compared them with 979 controls. Cases, compared with controls, had significantly lower levels of hemoglobin, higher levels of lactate dehydrogenase, bilirubin, and aspartate aminotransferase, and higher reticulocyte, white blood cell and platelet counts. These findings suggest an association of priapism with increased hemolysis and suggest that priapism may be one vascular manifestation of reduced nitric oxide (NO) bioavailability. Hemolysis decreases the availability of circulating NO which plays an important role in erectile function. We examined the possible association of single nucleotide SNPs in candidate genes of different functional classes for an association with priapism. One-hundred forty-eight patients with sickle cell anemia and incident or a confirmed history of priapism were compared with 529 controls who had not developed priapism. Polymorphisms in the *KLOTHO* gene (*KL;* 13q12) showed an association with priapism by genotypic and haplotype analyses. These findings may have broader implications in sickle cell disease since *KL* encodes a membrane protein that regulates many vascular functions, including vascular endothelial growth factor expression and endothelial NO release. Inhaled NO, arginine or even sildenafil, a PDE5 inhibitor, may be worthy of study for the management of sickle cell priapism.

REGIONAL AND SYSTEMIC COMPLICATIONS OF RENAL ISCHEMIA IN TRANSGENIC SICKLE MICE

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Sickle cell disease (SCD) is characterized by recurrent cycles of vaso-occlusive disease, the latter occurring either as overt painful crises or in a clinically silent fashion. Such episodes of vaso-occlusion impair vascular perfusion and impose tissue ischemia. The present study examined the regional as well as systemic complications of renal ischemia in a transgenic murine model of SCD (*Am J Path* 166:963-972, 2005). The kidney was chosen as the organ subjected to ischemia since ischemic insults to the kidney in SCD are well recognized clinically, and the kidney is vulnerable to acute and chronic involvement in SCD often for unclear reasons.

The transgenic murine SCD model used in this study is on a C57Bl/6 background, and is homozygous for the murine β-globin deletion and carries two transgenes, $\alpha^{H}\beta^{S}$ and $\alpha^{H}\beta^{S\text{-Antilles}}$. Renal ischemia was induced in wildtype (WT) and transgenic sickle mice (SM) by bilateral occlusion of the renal arteries (15 or 22.5 minutes in duration depending on the protocol), and endpoints were assessed either at 6 hours or 24 hours after the ischemic insult.

After renal ischemia for 15 minutes, and at 24 hours, SM as compared to WT exhibited greater renal dysfunction as reflected by a significantly higher BUN (71 ± 12 vs 113 ± 11 mg/dl, means ± SEM) and plasma creatinine (0.8 ± 0.2 vs 1.7 ± 0.3 mg/dl). This was accompanied by much more extensive acute tubular necrosis on histological analysis. With renal ischemia for 22.5 minutes, and assessed after 6 hours, SM exhibited increased vascular congestion involving the glomerular, cortical, and medullary circulations, RBC sickling, and more marked tubular necrosis; glomerular endothelialitis and mesangiolysis were also observed in SM. With renal ischemia of either duration, expression of the cell death-related protein, caspase-3, was more prominent in SM as compared to WT mice. The injurious complications resulting from renal ischemia in SM was not confined to the kidney but was also observed in distant organs, as reflected by more prominent capillary congestion in the heart and lungs. In an attempt to determine a mechanism whereby renal ischemia in SM elicits an adverse response in distant organs, serum amyloid P-component, the murine homologue of C-reactive protein, and a marker of inflammation and vascular injury, was measured in SM and WT, at 6 hours after ischemia. Following ischemia, SM, as compared to WT, exhibited a marked increase in circulating levels of serum amyloid P-component (49 ± 7 vs 311 ± 72 μg/ml). Twenty-four hours after renal ischemia for 22.5 minutes, mortality appeared in SM, attaining 28%, whereas no mortality was observed in WT.

In summary, SM exhibit an amplified response to renal ischemia characterized by exacerbation in renal dysfunction and more severe histologic injury, an exaggeration in the systemic inflammatory response, increased vaso-occlusion in distant and vital organs such as the lungs and the heart, and increased mortality. We suggest that such amplification of localized injury induced by regional ischemia in SCD, in conjunction with the exaggerated systemic inflammatory response and increased vaso-occlusion in distant organs may contribute to the morbidity and increased mortality that occur in this disease.

PRIAPISM IN CHILDREN AND ADOLESCENTS WITH SICKLE CELL DISEASE

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Priapism, a painful, persistent, unwanted erection of the penis has been associated with sickle cell anemia (SCA) since 1934. Reports initially suggested that 30-45% of adults and 2-5% of pediatric patients (pts) with sickle cell disease (SCD) had a history of this complication. However, it was our clinical impression that this complication was far more common than reported in pediatric pts. Subsequently our center has systematically investigated the occurrence, natural history, and management of priapism in children and adolescents with SCD by a variety of approaches.

Initially we conducted a survey of male pts with SCA 5 to 20 years (yrs) of age to determine the prevalence and characteristics of priapism episodes. In this sample of 98 pts, 11 of whom were previously known to have had priapism, 16 of 87 (18%) of the remaining pts reported having had priapism on one or more occasion. The actuarial probability of experiencing priapism by 20 yrs of age was 89$\pm$ 9%. The mean age was 12 yrs at first episode and there was a median of 1 episode per pt. Episodes typically began at 4am and had a median duration of 125 minutes.

Once we began to discuss this complication with pts and their families it became apparent that a protocol for management was needed. For pts with recurrent episodes we advise a bedtime dose of pseudoephedrine, an approach which has not been formally studied. We instruct pts to try simple maneuvers to relieve the priapism at home – voiding, warm bath or shower, gentle exercise, opioid analgesia – but if an episode lasts 2 hours (hrs) or more to present to the emergency department (ED). Since 1993 our protocol driven response to prolonged priapism (lasting 4 hrs or more) that fails to respond to hydration and opioid analgesia in the ED has been aspiration and irrigation (A&I) of the corpora cavernosa with a dilute solution (1:1,000,000) of epinephrine. The initial experience with this protocol was reported in 2000. At that time 39 A&I had been performed on 15 pts, 1-15 times per pt, with a median age of 13.7 yrs (range 3.9-18.3 yrs) at first procedure. A&I resulted in detumescence on 37 of 39 occasions (95% efficacy, CI 81-99%). The two failures were pts who presented after 27 and 36 hrs of priapism. Through 2004, A&I has been attempted during 74 episodes in 20 pts, resulting in detumescence in all but the 2 above. Repeat A&I was required within 24 hours in 3 episodes. The only side effects have been a hematoma in 2 pts and vomiting related to conscious sedation in one. Pts are now discharged home from the ED after A&I. For pts who require A&I on more than one occasion or who have frequently recurrent stuttering priapism (self resolving episodes lasting 0.5-4 hrs), we have administered leuprolide at decreasing doses monthly for 6 months or more which successfully prevented reoccurrence.

The natural history of priapism in pediatric pts was evaluated by review of the medical records of all 726 males with SCD (464 SS, 193 SC, and 69 S-thalassemia) seen between 1977 and 2003. Priapism was reported in 57 (7%) of all males; 49 (10.6%) with SS, 2 (1%) SC, 1 (4.8%) with S-beta zero thalassemia and 0 with S-beta plus. The youngest age at first episode was 3.2 yrs. The mean was 10.1 yrs, well before the onset of puberty.

There was no difference in age at onset by type of episode: stuttering 9.7 yrs and prolonged 8.9 yrs. Omitting 5 pts whose 40 - 100 events would skew the group statistics, the remaining 47 pts had 192 episodes: 16 pts with 1 episode only, median 3 per pt (range 1-15) occurring over 0.1-10 yrs. Episodes were longer than 2 hrs on 102 (53%) occasions and less than 0.5 hrs in 16 (8%). Most pts had both stuttering and prolonged episodes, 17 having stuttering episodes only. Stuttering priapism occurred before, between, and after prolonged episodes. Priapism occurred separate from other SCD related events in all but 6 cases: 1 stroke 24 hours after priapism treated with transfusion and morphine, 1 acute chest syndrome after prolonged priapism unresponsive to A&I, and 4 during generalized painful crises.

Priapism is a significant complication of SCD even in pediatric pts. Pt and family education can improve dialog about the complication and perhaps time of presentation for A&I with prolonged episodes. New approaches are urgently needed to prevent frequently recurrent episodes.

PRIAPISM ASSOCIATED WITH SICKLE CELL DISEASE: MECHANISMS OF DISEASE AND THERAPEUTIC IMPLICATIONS

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Priapism, defined as persistent penile erection in the absence of sexual excitation, is readily identifiable as a physically conspicuous, often painful clinical presentation. Despite its known clinical complications including erectile tissue damage and loss of erectile function and sexual ability, the entity is under-recognized and poorly managed. Hematologic dyscrasis, including sickle cell disease, are understood to have a major etiologic role, although the exact pathophysiologic basis for the association is not fully understood.

The classic paradigm of veno-occlusion in the penis has long been held to be the pathophysiologic basis for priapism associated with a host of disease states and conditions in which priapism occurs. Several investigators have supported venous outflow obstruction of the corporal bodies and increased intracavernous blood viscosity as likely mechanisms for priapism associated with hematologic dyscrasias. Predisposing conditions and susceptibility factors are also recognized to exert roles in the pathogenesis of priapism. Erections sustained for prolonged durations of sexual activity and sleep-related erections have been associated with recurrent priapism episodes.

New science in the field has supported the notion that dysregulatory mechanisms of penile erection contribute to priapism, beyond simply the veno-occlusive/venous congestion hypothesis. A major dysregulatory mechanism appears to involve aberrations in the nitric oxide signaling pathway, the main erection mediatory system in the penis. In penes of genetically altered mouse models lacking endothelial nitric oxide synthase (eNOS) and in transgenic sickle cell mice, both displaying priapism phenotypes, phosphodiesterase-5 (PDE5) expression and activity are downregulated. Restoration of eNOS by gene transfer to penes of eNOS mutant mice restores normal PDE5 levels and corrects priapism. These findings offer a molecular explanation for idiopathic, stuttering, and other forms of priapism, including those previously attributed solely to hematologic abnormalities.

While the management of priapism is based on sound principles derived from consensus body recommendations, it remains reactive for the most part. As a result, adverse consequences occur amid available clinical management practices for priapism. Preventative action taken early in the course of recurrent presentations would seem advantageous. As the disorder is increasingly understood from a pathophysiologic standpoint, effective preventative treatments having a mechanism-specific basis are welcome.

Based on the suggestion that PDE5 may serve as a molecular target for the treatment and prevention of priapism, we administered PDE5 inhibitors using a long-term therapeutic regimen to 3 men with sickle cell disease-associated priapism recurrences and one man with idiopathic priapism recurrences. The intervention is based on the rationale that the erection regulatory function of PDE5 in the penis would be reset by this treatment and protect against further episodes. Findings were that long-term PDE5 inhibitor treatment alleviates priapism recurrences. These preliminary data support the clinical use of PDE5 inhibitors to treat priapism, including that associated with sickle cell disease. However, various practical matters deserve attention if such therapy is being considered. Observations include the fact that the therapy occurs with some latency such that sympathomimetic treatment or other interventions may still be required for acute management, particularly with the presentation of a major priapism episode. Further evaluation of PDE5 inhibitor therapy as long-term prevention for priapism awaits completion of a controlled clinical trial.

RENAL CONCENTRATING ABILITY IN INFANTS WITH SICKLE CELL ANEMIA; PRELIMINARY DATA FROM BABY HUG, A MULTICENTER TRIAL

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A defect in concentration of urine is well described in sickle cell disease (SCD) and trait. Present in most adults, its onset is early in childhood and may be reversible with transfusion therapy until age 10 years

BABY HUG is an NHLBI-NICHD sponsored double-blind, placebo-controlled Pediatric Phase III Clinical Trial designed to critically assess the efficacy of hydroxyurea in preventing organ damage in young children with SCD. Primary endpoints are spleen function and glomerular filtration rate, but an attempt to assess urine concentrating ability is also under study. To date, 81 infants with confirmed Hb SS or S-Beta^{O} thalassemia recruited without regard to disease severity have entered the screening process. As part of this assessment, parents were given materials and instructions to obtain a timed second-void urine specimen from infant subjects after four to 10 hours of fluid deprivation at home; more aggressive deprivation was avoided due to safety concerns. Infants were taken to a Clinical Center in the morning to submit urine specimens and have serum obtained; specimens were sent with frozen gel packs by overnight mail to a central laboratory where urine and serum osmolality were determined.
Urine was obtained from 63 infants and serum from 61. Mean age of infants was 13.7 mo (range 8.3 – 17.9) and mean duration of fluid deprivation reported was 7.5 hr (3-13 hr; one deprived 0 hr was excluded). Serum osmolality was 287.4 $\pm$ 5.7 mOsm/L and independent of age, height, weight, or duration of NPO.

Urine osmolality (mean 401.8 $\pm$ 166.3, median 433, range 83-794 mOsm/L) highly correlated with duration NPO (figure). Thirty-nine infants (68.4%) were able to concentrate their urine (urine > mean serum osmolality + 1 SD) with five infants (8.8%) having urine : serum osmolal ratio > 2 and 18 (30.0%) having urine > 500 mOsm/L. Four infants were isosthenuric (urine within mean serum osmolality $\pm$ 1 SD) and 18 had urine osmolality values more dilute than serum (> 1 SD below mean serum osmolality) despite the attempt to withhold fluid. There was no significant correlation between urine osmolality and serum osmolality, age, height, weight or creatinine-based glomerular filtration rate (GFR); there was correlation with DTPA GFR and serum urea nitrogen.

Even with our suboptimal fluid deprivation challenge, urine concentrating capacity was evaluable, with more than two-thirds of our screened young infants with SCD concentrating urine. BABY HUG may determine whether this concentrating ability can be preserved/restored with

hydroxyurea therapy.

RENAL COMPLICATIONS OF THE SICKLE CELL DISORDERS - CHAIR'S SUMMARY

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The kidney is an organ of considerable impact on the clinical course of the sickling disorders. The kidney modulates both acid-base and water balance, and these aspects of physiology influence hemoglobin (Hb) S polymerization and contribute to the intra-renal vaso-occlusion that is responsible for the renal complications of the sickle cell syndromes. Consequently, the entire kidney is affected in ways that lead to alterations in glomerular, tubular and medullary structure and function. With advanced tissue destruction, hematuria, proteinuria and/or renal insufficiency occur.

Glomerulus: The glomeruli are significantly larger than those of age-matched controls; glomerular size increases with age and shows an inverse relationship to the degree of anemia, suggesting that glomerular hypertrophy and increased renal blood flow are contributing factors to glomerular hypertrophy. Increased nitric oxide synthease activity has been found in the transgenic mouse model of sickle cell disease; synthesis of nitric oxide by this enzyme could further contribute to vasodilatation and hyperfiltration. Ischemic hyperfiltration as a consequence of the increased renal blood flow and glomerular hypertrophy are thought to cause segmental glomerular sclerosis with eventual proteinuria and renal failure.

Tubules: Numerous abnormalities in both proximal and distal tubular function have been described. Most important is the tubular hypersecretion of creatinine, which contributes to the overestimation of GFR by creatinine clearance as compared to inulin or para-aminohippuric acid. Consequently, by age 2, if not sooner, serum creatinine is distinctly lower than in normals. Uric acid production is increased due to expanded hematopoiesis, but serum uric acid levels are normal because of increased tubular secretion; urate concentrations rise with renal failure, and gout or tophi can occur. There is an incomplete distal renal tubular acidosis. Systemic acidosis is unusual except in situations where there is another source of hydrogen ion; the degree of systemic acidosis may be greater in sickle cell disease than in non-Hb S patients. Potassium excretion is also impaired; hyperkalemia is rare, but spurious hyperkalemia may be seen with the release of potassium from erythrocytes or platelets on standing.

Medulla: A nearly universal feature of the nephropathy is the loss of urine concentrating ability. The hyperfiltration and glomerular hypertrophy are accompanied by mesangial proliferation, reduplication of the basement membrane, effacement of foot processes and progressive focal segmental sclerosis. Surveys show that macroalbuminemia is detectable in 20 to 30% of those with Hb SS and to a lesser extent in Hb SC and Hb S thal. The prevalence of proteinuria increases with age and is a predictor for the development of renal insufficiency. As in other complications of sickle cell disease, proteinuria has been reported in a wide variety of diseases other than the hemoglobinopathy. Thus, the diagnostic evaluation must consider these alternative. Treatment with ACE inhibiting agents, based upon the hypothesis that they reduce intra-glomerular pressure by decreasing efferent arteriolar constriction, were successful in sickle cell disease, as well as other conditions with proteinuria such as diabetes.

Hematuria: Hematuria as a result of micro- or macro-papillary necrosis or rupture of dilated neo-vascularization occurs. Papillary necrosis may be seen in as many as 25% of patients with Hb SS on urography, not all of whom have a history of hematuria. Hematuria is often presumed to be secondary to sickling, but there are sufficient reports of other disorders causing hematuria in sickle cell patients to justify an aggressive approach to differential diagnosis. Reports of a rare medullary cell carcinoma in patients, all of whom had evidence for Hb S as well as a 17-fold excess frequency of renal cell carcinoma in Hb SS further support the need for careful diagnostic evaluation of hematuria. In the absence of a specific etiology other than Hb S, treatment is empiric. Supportive care with bedrest and a fluid intake sufficient to produce a dilute urinary flow

of approximately 2 ml/kg/hr have been the usual approach. Individual case reports indicate responses to DDAVP infusions of 0.3 Φg/kg q 12 h for two doses, then q 24 h for three doses or to epsilon aminocaproic acid in doses of 6 to 8 grams per day. Additional responses have been reported with balloon tamponade.

Renal Failure: Acute renal failure is rarely reported, but surveys suggest that acute doubling of the serum creatinine is more common than generally supposed. Chronic renal failure (CRF) occurs in 4 to 20% of patients in the reported surveys. The prevalence increases with advancing age. Proteinuria is an important predictor of risk for the development of CRF, as patients with proteinuria but with normal serum creatinines show a loss of GFR of 13% and of renal plasma flow of 16% compared to Hb SS controls without proteinuria. The presence of CRF predicts a considerably shortened survival.

Treatment of CRF includes management of the biochemical abnormalities of renal failure as well as treatment of the anemia and its cardiovascular complications. Treatment with recombinant human erythropoietin (rHu-EPO) is successful in raising the hematocrit in some but not all patients. The evidence that hydroxyurea prolongs erythrocyte survival indicates that a combined rHu-EPO/ hydroxyurea approach could ameliorate the transfusion requirements and its consequences. During treatment of CRF, careful adjustment of drug dosing for those agents excreted by the kidney is important. Normeperidine, a metabolite of the commonly used analgesic, meperidine, is excreted by the kidney; in renal failure, this metabolite accumulates and can cause seizures. Diuretic therapy can produce hypovolemia, which may impair renal blood flow and GFR.

As renal insufficiency progresses, dialysis and transplantation are used in its management. More recent data from national registries in both children and adults indicate that the 5-year survival after transplant of 18% is at least as good as the reported 17% on dialysis and argue for broader use of transplant in these patients. In registry data, the one year allograft survival was identical to that of age-matched Afro-American transplant recipients, although the three year graft survival of 48% was significantly less than the 60% in the control population. Mortality and morbidity were related to bacterial infection in this asplenic population on immunosuppression and to hepatic failure related to iron overload and chronic viral hepatitis. Graft failure often accompanied patient death from other causes. A similar survey in children showed allograft survival at one and two years was similar to that of controls, and patient survival was 89%. Improvements in graft outcome were attributed to more effective immunosuppression of graft rejection following the introduction of cyclosporine as well as progressive improvements in general supportive care. These data indicate that the benefit of transplantation is similar to that in the general transplant population.

Hypertension: The prevalence of hypertension is surprisingly low in sickle cell disease as compared to matched controls. Blood pressures in the 90th percentile (but still within the normal range) are associated with shortened survival. These data raise the question of whether patients with blood pressures at the upper end of the normal range should be treated. Treatment should be initiated for persistent blood pressures >130/80. Beta blockers, calcium channel blockers, and ACE inhibitors have all been used successfully. ACE inhibitors are particularly useful when there is concomitant proteinuria. Diuretic therapy should be restricted to low dose because of its potential for inducing hypovolemia and its associated negative effect on GFR and renal blood flow.

GENETIC MODULATION OF SICKLE CELL ANEMIA

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The complications of sickle cell disease are likely to be influenced by genetic heterogeneity in genes that modulate inflammation, oxidant injury, nitric oxide (NO) biology, vasoregulation, cell-cell interaction, blood coagulation and hemostasis. We previously focused on our studies on stroke, osteonecrosis and priapism.(Sebastiani et al, Nature Genet 37: 435, 2005; Baldwin et al, Blood 106: 372, 2005; Nolan et al, Br. J Haematol 128: 266, 2004). In the past year: additional phenotypes were examined for their association with SNPs in potential modifier genes; laboratory data in selected disease phenotypes was re-analyzed, and, in the context of this data and work by other investigators, we reformulated our view of the pathophysiology of disease; genetic admixture in sickle cell disease patients has been studied; a global estimation of disease severity, integrating clinical, laboratory and genotyping information, was developed.

Estimating sickle cell disease severity has been difficult making the integration of clinical and laboratory abnormalities of into a predictive model of disease complications and death, an unrealized goal. We first integrated clinical and laboratory data from nearly 3500 individuals from the Cooperative Study of Sickle Cell Disease and applied to this data advanced statistical machine learning techniques to identify the significant associations between selected complications of disease and laboratory variables. We looked for predictors of the risk for early death in three separate age groups. The resulting model revealed that complex networks of interactions between clinical and laboratory variables underlie common disease complications and ultimately death. Predictors of risk in this model were the *HBA1, HBA2* genotype, stroke, sepsis and acute chest syndrome. While this model can predict the risk for early death given the presence of other disease complications and variations among common laboratory variables, it did not provide an understanding of the genetic basis for our observations. Accordingly, in more than 1000 patients with sickle cell disease we genotyped SNPs in genes chosen because of their possible link to the pathophysiology of disease. We then used our estimate of global disease severity to find associations of genotypes with severity. In the initial screening studies we identified several genes in the TGF-∃/BMP pathway that were associated with selected disease subphenotypes. To expand our initial findings, we examined haplotype tagging SNPS in a more comprehensive fashion in 21 genes that are key members of this pathway. The genes included *BMP6*, BMP receptors, TGF-∃ receptors, SMADs, MAP kinases and their associated co-factors such as SARA, CDH1 and SMURF1. A Bayesian significance test was used to model the associations between the score of disease severity and individual SNPs and also a traditional association test with p-values computed using permutation tests was employed. To reduce the problem of multiple comparisons, we selected as significantly associated with disease severity, only those SNPs that passed both tests with strong evidence of association. The most striking associations were found for *BMP6*, its receptors *BMPR1* and *BMPR2*, several *SMAD* proteins and *SARA1*.

Sickle cell disease patients have a high risk of developing leg ulcers. In 378 patients with a confirmed history of leg ulcers and 920 controls we found that ∀ thalassemia was more frequent among controls than cases. Also, leg ulcer patients had lower hemoglobin levels, higher LDH, bilirubin, AST, reticulocyte and white blood cell count than controls. When analysis was restricted

to the 243 cases vs. 516 controls that were also genotyped for SNPs in candidate genes, the results were similar. One-hundred-thirty-two SNPs in 47 candidate genes were examined. Candidate genes having multiple SNPs associated with leg ulcers were *KL, TEK* , *SMAD1*, and *SARA1*. The *TEK* receptor tyrosine kinase (TIE2) is expressed almost exclusively in endothelial cells, is involved in angiogenesis and is the ligand for angiopoietin-1 (*ANG1*). *SMAD1* and *SARA1* are members of the TGF-∃/BMP pathway. Patients with sickle cell disease have an increased risk of bacteremia. Among 201 subjects with bacteremia and 1238 controls, there was no significant difference in age, sex, HbF concentration, distribution of ∃-globin gene cluster haplotypes or the presence of coincident ∀ thalassemia. Four SNPs in *BMP6,* 2 SNPs in *TGFBR3* and 2 SNPs in *SMAD3* were associated with bacteremia. Renal failure is also common in sickle cell anemia. We analyzed adjusted creatinine clearance as a continuous variable using multivariate linear regression and as a categorical variable by comparing the lower quartile (25th percentile) to the upper (75th percentile) using logistic regression. All analyses were adjusted for the presence of coincident ∀ thalassemia. *BMPR1B*, a BMP receptor gene, was associated with creatinine clearance in both analyses. Our results confirm the importance of the TGF-∃ signaling pathway in sickle cell disease pathophysiology and suggest that subtle variation in this cell signaling network can be associated with the severity of disease. The TGF-∃/BMP pathway regulates a wide range of biological functions including cell proliferation, cellular differentiation, extracellular matrix production, cell death, tissue repair and immune regulation.

Admixture mapping examines allele frequencies across historically separated populations. Disease-causing variants are likely to differ in frequency due to either drift or selection and will be more frequent in patient populations descended from recently mixed ethnic groups. These groups should have an increased probability of inheriting the alleles derived from the ethnic group that carries the disease-susceptibility alleles. Recently, several high-density SNP maps have become available. There are also several maps for regions of Africa including Beni Nigeria, Ghana, Cameroon, Senegal and Botswana. These maps focus on 100 SNPs that have been identified as having the most potential for admixture mapping. We compared allele frequencies among patients with sickle cell disease, healthy African Americans, European Americans and the specified African regions. While still preliminary, our results suggest that for some genomic regions it appears that the allele frequencies are ordered such that the sickle cell population more closely resembles the African samples than does the non-sickle cell disease African American population. Both are very different from the European American frequencies.

Hemolysis is likely to be an antecedent of certain vascular complications of sickle cell disease. Prompted by the work of Gladwin and associates, we studied the relationship of hemolysis to the subphenotypes of sickle cell disease. Our studies suggest that there is a hemolysis-driven phenotype in sickle cell disease that now includes priapism (Nolan, et al, Blood ePub, 2005) leg ulcers (ASH 2005 abstract), stroke and pulmonary hypertension. Priapism patients and patients with leg ulcers, when compared with controls, have evidence of increased hemolysis. Less likely to have coincident ∀ thalassemia, they have significantly lower levels of hemoglobin, higher levels of LDH and AST, and higher reticulocyte, white blood cell and platelet counts. High HbF levels reduce the incidence of some subphenotypes of sickle cell disease, like osteonecrosis, acute chest syndrome and acute painful episodes. HbF level was not associated with protection from pulmonary hypertension, stroke or priapism. Conversely, ∀ thalassemia increases the risk of osteonecrosis, acute chest syndrome and acute painful episodes, so-called vasocclusive complications, and high HbF reduces their incidence. Hemolytic and viscosity-vasoocclusive phenotypes must have substantial areas of overlap. Nevertheless, this dichotomization helps place subphenotypes of sickle cell disease into a new context. Hemolytic anemia and increased NO scavenging play a major role in the propensity to acquire the subphenotypes of stroke, pulmonary hypertension, leg ulcer and priapism. All are ameliorated by ∀ thalassemia that

reduces hemolysis and improves anemia. HbF, while it should not be disregarded as a modulator of these subphenotypes, has little direct protective effect. Distinct from these phenotypes are ones associated with increased blood viscosity, like osteonecrosis, acute chest syndrome and painful episodes. Adversely affected by ∀ thalassemia, their prevalence is directly associated with hemoglobin concentration; HbF has a protective effect. Hemolytic anemia-induced phenotypes are likely to be improved by transfusion and agents that increase NO bioavailability or reduce hemolysis but helped to a lesser extent by drugs that induce HbF expression unless the response is very robust. The PDE5 inhibitor sildenafil may be effective in pulmonary hypertension. While hydroxyurea may help prevent stroke in susceptible children it appears to have little effect in sickle cell pulmonary hypertension. Hydroxyurea is associated with a reduction in the incidence of acute chest syndrome and acute painful episodes and this is related to its ability to increase HbF production.

RONALD NAGEL, M.D.

PLEIOTROPIC AND EPISTATIC EFFECTS IN SICKLE CELL DISEASE

GENETIC MODIFIERS OF SICKLE CELL DISEASE

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In our progress report last year we described blood cell gene expression profiles of 50 human sickle cell patients with different disease severities. We defined severe disease as 3 or more ER/Hospital visits in the past year. Moderate disease was 1-2 ER/Hospital visits per year. Mild disease was 0 ER/Hospital visits per year. The initial results, which are illustrated in Figure 1, were encouraging. The gene expression profiles of all 5 patients with severe disease clustered tightly in the hierarchical array. The Stanford cDNA spotted arrays (40,000 dots) were used for this analysis.

During this year's funding period, we analyzed blood RNA of 50 additional patients. Duplicate blood samples or duplicate microarray slides were analyzed for many of these patients. Unfortunately, severe patients were not significantly clustered in this expanded group (data not shown). We are confident that the expression data is accurate because duplicate samples, which are analyzed on separate slides and on separate days, are adjacent (tightly clustered) in the hierarchical array. The reproducibility and sensitivity of the assay is also confirmed by the adjacent location of expression profiles from identical sickle twins.

Therefore, we changed the criteria for categorizing the patients into severe, moderate or mild phenotypes. The patients were assigned to one of two categories: severe or non-severe. Severe disease was defined as one or more hospitalizations for sickle related illness during the lifetime of the individual. Non-severe disease was defined as no hospitalizations for sickle related illness. The data was recently analyzed by a new statistical program which compares expression from all genes of two groups. The analysis identified 100 genes that are up-regulated or down-regulated with high significance in the severe group. When these 100 genes were used to construct hierarchical arrays, severe and non-severe patient samples were clearly separated. During the next funding period, we plan to construct a "sickle chip" composed of these 100 genes and to use this DNA array to analyze blood RNA of new-born sickle patients. The goal of these studies is to identify patients who will develop severe disease so that therapeutic interventions can be initiated before significant tissue and organ damage has occurred. During this year, we also plan to initiate mechanistic studies of the top 20 genes on this list.

Another goal of our grant was to clone severely and non-severely affected SCD mice and to define QTL that modify SCD disease. We and many other groups have had difficulty producing ES cell lines from outbred animals. Our initial results were promising; however, after establishing 15 new ES cell lines during the previous funding period, we were unable to produce any additional lines. Our failure to produce new lines is most likely due to genetic drift in our outbred sickle animal colony. To overcome this problem, we have produced a new mouse model of sickle cell disease by replacing the mouse beta-globin genes with human gamma- and betaS-globin genes and replacing the mouse

alpha-globin genes with human alpha-globin genes. These mice synthesize human hemoglobin exclusively in adult red blood cells and develop the entire pathology of the disease. These new animals are important for Aims 2 and 3 of the grant because they enable efficient breeding of the human alpha and gamma/betaS knockins onto multiple, defined genetic backgrounds. We are presently breeding the knockin alleles onto C57Bl/6, BalbC, DBA and 129 backgrounds and we expect different disease severities on the different backgrounds. SNP and microsatellite analysis will then be utilized to map loci that segregate with severe disease.

GENETIC MODIFIERS OF SEVERITY IN SICKLE CELL ANEMIA (SS)

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Strong epidemiologic evidence suggests that the non-specific inflammatory marker, the baseline white blood cell count (WBC), is a substantial predictor of clinical severity in SS. This marker, with its own set of inherited and environmental influences, is also notably a risk factor for mortality in the general population. Our study is designed to identify the genes that influence baseline WBC in SS. Our strategy is to detect, characterize, and localize the genes that influence baseline WBC in SS patients and their extended families, mice, and baboons. We report here on our preliminary findings in our animal populations.

1. The mouse

To identify QTL for baseline WBC count, we established F2 intercrosses between (NZW/LacJ x SM/J)F1 hybrids (NZSM cross) and (C57BLKS/J x SM/J)F1 hybrids (KSSM cross). F2 mice from both crosses were phenotyped at 8 weeks of age. Genome-wide scans for significant single QTL are shown below.

The allele effects of the WBC QTL identified are shown in Fig. 2. In the KSSM cross, a dominant KS allele linked to the peak marker for Wbcq1 increases WBC count (Fig. 2A). In the case of Wbcq2 and Wbcq3, recessive and dominant SM alleles, respectively, increase WBC count (Fig. 2B, C). Hence, a QTL inherited from the parental strain with the low WBC count, SM, increases WBC count compared with the allele inherited from the high WBC count strain, KS, at these loci. This phenomenon, known as transgressive segregation, is common in QTL crosses and indicates that both parental strains carry alleles that influence the trait in both directions.9,10 Hence, examination of allele effects suggests that additional WBC QTL await identification. Clearly, a complex regulatory gene network influences baseline WBC count. No sex-specific QTL were noted for any of the peripheral blood traits studied,

Several candidate genes that make "biological sense" are under investigation. All candidates show appropriate expression patterns according to publicly available gene array databases (http://symatlas.gnf.org/SymAtlas/)(e. g., expression in bone marrow, hematopoietic precursors cells, lymphoid cell lines, myeloid cells).

2. The baboon

We previously reported the detection of significant (P<0.05) genetic correlations indicative of polygenotypic and QTL pleiotropy between a number of hematological traits; and, in particular, between WBC and MPV. Similarly, we reported observations of significant pleiotropy between WBC and circulating levels of biomarkers of inflammation and oxidative stress, as an example, between WBC and p-selectin.

Using whole genome linkage screens, we conducted bivariate linkage analyses to search for QTLs influencing both WBC and p-selectin. These analyses returned suggestive evidence (LOD = 1.49) for a pleiotropic QTL influencing both traits on baboon chromosome (PHA) 1p in a region that is orthologous to human chromosome (HSA) 1p35-34. Several key genes lie in this region including:

- **1p35.3 ;tRNA selenocysteine associated protein**
- **1p35.3; sestrin 2**
- ***1p35-p34.3; platelet-activating factor receptor**
- **1p35-p34.1; syntaxin 12**
- **1p35.3; tRNA selenocysteine associated protein**
- ***1p35-p34.3; colony stimulating factor 3 receptor (granulocyte)**
- ***1p34.3; cell division cycle associated 8**
- **1p34.3; ischemia/reperfusion inducible protein*1p32-p36; poly(A) binding protein, cytoplasmic 4 (inducible form)**

Genetic Polymorphisms Associated with Aging and Priapism in Sickle Cell Disease

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Despite the constancy of the genetic abnormality responsible for sickle cell disease (SCD), the clinical course of patients with SCD is remarkably variable. Our study is therefore designed to determine whether additional genetic polymorphisms affecting cell adhesion, inflammation and coagulation contribute to the clinical course of SCD and help determine the frequency of occurrence of specific end-organ complications. In that regard, we have most recently focused our efforts on two clinical areas: aging and priapism.

Little is known about the biological factors that protect certain SCD patients from early demise while others never reach mid-adulthood. Understanding this variability in the aging process in SCD is becoming more important as some patients are now living into their 5th and 6th decade of life. Recently, McKerrell and colleagues (2004) compared the clinical and laboratory profiles of SCD patients aged 40 years and over with SCD patients who were between 21 and 30 years of age. Similarly, we have compared clinical and genetic correlates of older SCD patients (50 years and over) with those of younger patients (18-30 years). Among 514 patients in our total study population, 49 (10%) were categorized as "older" and 194 (38%) were categorized as "younger." Older SCD patients had lower hemoglobin (older: 7.8 ± 1.1 vs. younger: 8.5 ± 1.2, p=0.004), platelet count (older: 372 ± 126 vs. younger: 460 ± 225, p=0.02), MCV (older: 92 ± 12 vs. younger: 89 ± 9, p=0.08), MCHC (older: 33.6 ± 1.4 vs. younger: 34.3 ± 1.8, p=0.05), and WBC (older: 10.2 ± 2.7 vs. younger: 13.1 ± 4.1, p<0.001). Older patients also had lower total bilirubin (p=0.01), and increased alkaline phosphatase (p=0.0002) and creatinine (p=0.0002), which was associated with poorer creatinine clearance (p<0.0001). The older SCD patients also had increased systolic (p<0.0001) and diastolic (p=0.008) blood pressure, decreased O2 saturation (p=0.03), and a history of fewer pain episodes per year requiring medical treatment (p<0.0001). Many of our findings are consistent with those of McKerrell et al. (2004). In order to identify genetic factors associated with longevity in SCD, we examined 155 SNPs in a total of 41 genes, primarily involved in red blood cell adhesion and inflammation pathways. Chi Square tests of association were constructed for the genotypes of each SNP with the two clinical categories: "older" and "younger." When the number of rare homozygotes was less than 5 individuals, we combined those individuals with the heterozygote individuals for analysis. All p-values are uncorrected for multiple testing. We found putative associations with 5 SNPs in 3 genes. Three non-coding SNPs in Klotho, not in linkage disequilibrium, exhibited different genotype frequencies in the older versus younger SCD patients (p=0.007, p=0.01 and p=0.01). Similarly, a single non-coding SNP in NOS2A (p=0.02) and TGFBR2 (p=0.02) also exhibited significantly different

genotype frequencies in the older versus younger patients. These data support the clinical findings in aging SCD patients reported by McKerrell and colleagues (2004), and they also suggest that genetic factors contribute to variability in longevity in SCD. Interestingly, multiple SNPs in Klotho exhibited differing genotype frequencies in older versus younger patients. Mutations in Klotho have been previously associated with aging-related phenotypes in mice. A better understanding of the biological mechanisms associated with longevity in SCD may help identify those at risk for early demise and in need of more specialized medical care.

In addition, we have also examined priapism in relation to several clinical and genetic factors in 249 of our adult male patients, 92 (37%) of whom reported a positive history of priapism. The mean age of male patients without a history of priapism was 35.2 years (± 10.8 years) compared with a mean age of 36.4 years (± 11.3 years) in male patients with a positive history of priapism. Because of the possible relationship with nitric oxide biology, we examined the co-occurrence of priapism with proteinuria, leg ulcers and stroke. Of the males with a positive history of priapism, 20% also had a history of 2+ or greater proteinuria, compared with a presence of 2+ or greater proteinuria in only 10% of males without a history of priapism (p=0.03). Similarly, 34% of males with a positive history of priapism also had a history of leg ulcers, compared with the presence of leg ulcers in 22% of males without priapism (p=0.03). No statistically significant association between the occurrence of priapism and stroke was observed. In an effort to identify genetic risk factors for priapism, we examined 262 single nucleotide polymorphisms (SNPs) in a total of 56 genes, primarily involved in red blood cell adhesion and inflammation pathways. Chi Square tests of association were constructed for the genotypes of each SNP with two clinical categories: patients with a positive history of priapism and patients without a history of priapism. As in the aging analysis, when the frequency of rare homozygotes was less than five individuals, we combined these rare homozygote individuals with heterozygote individuals for analysis. All p-values were uncorrected for multiple testing. We found associations with 12 SNPs in 8 genes: SLC4A2 (p=0.003); ITGAV (p=0.004 and p=0.02 for two different SNPs); F13A1 (p=0.004 and p=0.02 for two different SNPs); AQP1 (p=0.01 and p=0.04 for two different SNPs); TGFBR2 (p=0.01 and p=0.02 for two different SNPs); ADRB2 (p=0.03); MGC (p=0.04); and ARG2 (p<0.05). These genes are involved in a variety of functions, including adhesion, coagulation, signal transduction, NO biology and immune response. We examined 21 non-coding SNPs in the Klotho gene, but we did not find an association between priapism and Klotho, as was recently reported by Nolan and colleagues (2005). The only possible trend for association we observed in Klotho was at marker rs1888057 (p=0.07); we did not observe association with the SNP (rs2249358; p=0.82) Nolan and colleagues found associated with priapism.

These data support our over-arching hypothesis that genetic factors mediate the variability in clinical course of patients who have SCD. A better understanding of the genetic factors that contribute to the occurrence of complications such as early demise and priapism should ultimately lead to a better understanding of SCD pathophysiology as well as to improved treatment for patients with SCD.

GENOME-WIDE SEARCH FOR MODIFIERS OF SEVERITY IN β^0-THALASSEMIA/ HbE DISEASE

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β^0-thalassemia/HbE disease is one of the most common thalassemias in Southeast Asia, representing the main cause of childhood chronic disease. Patients compound heterozygous for HbE and β^0 mutant alleles display remarkable variability in clinical expression, ranging from nearly asymptomatic to severe, transfusion-dependent disease. It is believed that genetic factors may account for at least part of the observed variability in disease severity.

A universal platform technology (MassARRAY®) based on automated mass spectrometry has been developed for analyzing nucleic acids at a high level of precision and accuracy. We have used this technology to evaluate the contribution of polymorphisms within the β-globin gene cluster to variation in disease severity. More than 70 SNPs in this region were genotyped in unrelated patients from Thailand representing the mildest (n=209) and most severe (n=327) forms of the disease, and having normal α-globin genes. Eleven SNPs uninformative in this sample were excluded from further analysis. Adjusting all statistical analyses for age, gender, and geographic region, logistic regression was used to investigate the association between each SNP and disease severity. Thirty-nine SNPs spanning the LCR region and Aγ-globin gene showed strong association with disease severity. The strongest association was observed with the *XmnI* polymorphism located 158 bp upstream of the Gγ-globin gene (global p=1.4E-12). Patients with the *XmnI* CC genotype were 4.1 times more likely than CT patients (p=2.4E-07) and 34.5 times more likely than TT patients (p=1.7E-12) to have severe disease. Haplotype analysis of five tagging SNPs (*rs4601817; HincIIE; XmnI; HindIII-Gγ; HincII-5′ψβ*) showed that β^0/HbE disease was milder among patients with the haplotype T-TTA (freq=0.4;p=6.3E-16), and more severe among patients with haplotypes CACGG (freq=0.48;p=5.8E-07) and TACGG (freq=0.09;p=5.2E-04). Haplotype analysis of the *XmnI* site and β-globin mutations among 536 patients and 50 HbE homozygote individuals revealed that the T allele of *XmnI* was nearly always in *cis* with the HbE allele (haplotype frequencies: T-HbE=0.39; T-β^0=0.04; C-β^0=0.43; C-HbE=0.14). Linear regression analysis showed the *XmnI* genotypes TT and CT were also associated with increased expression of fetal hemoglobin (HbF) in both the mild (p=0.004) and severe (p=9.3E-09) patient groups.

The MassARRAY platform can also be used to rapidly estimate and compare allele frequencies in pooled DNAs from different subject groups. To identify additional genetic modifiers influencing severity among β^0-thalassemia/HbE patients, we utilized this approach to conduct a genome-wide association study involving approximately 110,000 gene-based single nucleotide polymorphisms (SNPs). The assay panel corresponds to SNPs with a median spacing of 10.4 kilobases, in approximately 99% of all known and predicted human genes. As a first-pass filtering step, comparing estimated allele frequencies for all SNPs between DNA pools offers advantages in both time and cost over individual genotyping. To begin, DNAs from approximately 200 regionally matched patients representing the extremes of disease severity (mildest vs. severe) were included in each DNA pool. Allele frequencies for all SNPs were then estimated in both pools, and those showing suggestive significant differences (p values <0.02) were selected for verification by repeated pooled DNA analysis. From among the approximately one-fourth of these that showed reproducible allelic differences at p<0.05 by pooling, about 700 SNPs were selected for genotyping individual patient DNAs to determine precise allele and genotype frequencies. Prior to correction for multiple testing, 440 SNPs at the level of allele frequencies and of which 260 SNPs at the level of genotype frequencies showed evidence for association with disease severity at p<0.05. These included SNPs in several reported quantitative trait loci (QTLs) associated with fetal hemoglobin HbF levels.

However the most strongly associated SNPs were within a region centromeric to the beta globin gene cluster containing many olfactory receptor genes. Logistic regression was used to investigate association between SNP genotypes and disease severity (mild vs. severe). The logisitic models included two SNP genotype predictor variables (one homozygote class randomly selected as the referent) and terms for age, gender, and geographic region. Three of the 19 SNPs examined from this region were found to be strongly associated with disease severity (global p values: 1.8E-7, 3.2E-10, 9.2E-5). Haplotype analysis of SNPs in the region using the Haplo.Stats program revealed associations between certain haplotypes and either severity of disease or %HbF (of total Hb).

Analysis of data from the genome-wide association study also identified many candidate modifier genes unlinked to the beta globin gene region. These candidates are currently being evaluated for their potential roles in the biology of this disease. Additional support for these findings must come from replication in independent patient collections and functional analyses.

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INFLAMMATORY CONTROL OF ERYTHROPOIESIS IN SICKLE DISEASE

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Although sickle cell anemia is the result of an abnormality in the beta globin chain of hemoglobin, its clinical manifestations cannot be explained solely on that basis. It has become clear over the last several years that inflammation and inflammatory mechanisms contribute to the clinical syndromes associated with sickle cell disease even in steady-state, whether as a mediator of erythroid suppression or as a facilitator of thrombotic events.

Determinations of the concentrations of the cytokine mediators of inflammation in serum or plasma of sickle cell patients have shown disparate results, particularly in patients not experiencing an acute crisis at the time. In studies of anemic patients with either AIDS or rheumatoid arthritis, determinations of bone marrow cytokine concentrations have provided unique correlations with clinical and laboratory parameters. We determined concentrations of IL-1, IL-6, TNF, and placental growth factor (PlGF) in bone marrow aspirates from 6 homozygous sickle cell (SS) patients who were not acutely ill and who were not receiving hydroxyurea, erythropoietin, or chronic transfusion, and compared them to specimens from 7 healthy controls. Concurrent plasma measurements were made when available. We also measured concentrations of soluble transferrin receptor (sTfR) and of marrow erythroid colony-forming units (CFU-E) as markers of erythropoietic activity. As expected, sTfR concentration, whether measured in marrow or plasma, was significantly higher in SS patients (p = 0.024). CFU-E concentration was not significantly different between the two groups. Although plasma IL-6 was significantly higher in SS patients, and plasma IL-1 significantly higher in controls, values measured were in the normal range. Bone marrow concentrations of IL-6 and IL-1 did not differ between the study groups. TNF was undetectable in all specimens, plasma or marrow.

Placental growth factor (PlGF) is a member of the vascular endothelial growth factor family and is associated with inflammation and with pathologic angiogenesis. Perelman and colleagues have reported that PlGF is released from marrow erythroid cells and that its circulating concentration is 50% higher in patients with severe sickle cell disease than it is in healthy controls, and also that PlGF induces the expression of inflammatory cytokines like interleukin-1 (IL-1) and tumor necrosis factor (TNF) (*Blood* 2003;102:1506-13, and 1514-24). In our studies, plasma PlGF did not differ between SS patients and controls, but bone marrow PlGF concentrations were significantly higher in SS patients (p = 0.004). Since PlGF is a product of erythroid cells, the ratios of marrow PlGF to marrow CFU-E and sTfR were determined, and also found to be significantly greater in SS patients (p = 0.016 and p = 0.024, respectively). None of the cytokines measured in SS patients correlated with clinical parameters. These studies demonstrate that the bone marrow of stable SS patients exhibits an increased PlGF concentration not seen in circulating blood. This does not represent a phenomenon general to cytokines, and does not reflect increased erythroid activity but rather an increase per erythropoietic unit.

During studies of the regulation of erythropoiesis by inflammation in sickle disease, we observed that CFU-E from SS patients were less sensitive to inhibition by recombinant human (rh) γ interferon (IFN) than those from healthy controls, with this effect being most pronounced at lower rhγIFN concentrations. The potential contribution of PlGF to this process was studied. At PlGF concentrations 10 – 1000 pg/mL, no inhibition or enhancement of CFU-E colony formation was observed, and there was no difference between the responses of progenitors from SS patients or from normal volunteers. However, PlGF 100 pg/mL reversed the inhibitory effects of rhγIFN on

CFU-E colony formation (p = 0.04). This effect was most apparent at lower rhγIFN concentrations. Studies using the HCD57 cell line demonstrated that PlGF decreased apoptosis induced by 24 hour exposure to rhγIFN.

In conclusion, PlGF concentrations are increased in the marrow of stable SS patients. This increased level of PlGF exposure may contribute to protecting erythroid progenitors from cytokine-induced apoptosis and inhibition of colony formation, and may be a mechanism by which erythropoiesis in sickle cell disease is preserved despite concurrent inflammation.

ADHESION MECHANISMS MEDIATING SICKLE CELL VASO-OCCLUSION

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Vascular occlusion is a major cause of morbidity and mortality in sickle cell disease but its mechanisms are poorly understood. Previous studies demonstrated that sickle RBCs are more adherent to vascular endothelium than normal RBCs, and that the endothelium in sickle cell patients and mice is inflamed. Clinical studies have suggested a link between elevations in WBC counts and poor outcome in sickle cell disease and *in vitro* data indicate that sickle cells can bind neutrophils in a static adhesion assay. We initiated studies to evaluate the molecular mechanisms mediating sickle RBC and leukocyte (WBC) adhesion on activated endothelium *in vivo*. We chose to use mice that expressed human sickle hemoglobin exclusively because these animals display the most severe phenotype among sickle animal models. We have generated age-matched male sickle cell animals using transplantation of sickle cell bone marrow into lethally irradiated C57BL/6 recipients. Using this approach, we can generate from a single donor several genetically identical sickle mice that express >97% donor sickle hemoglobin. The interactions of sickle blood cells in the microcirculation of the cremaster muscle was evaluated before and after stimulation with TNF-α, a cytokine that is frequently elevated in sickle patients. We have found that sickle RBCs interacted with WBCs that were adherent in post-capillary and collecting venules. These interactions were enhanced following TNF-α administration, leading to the death of sickle animals. Adherent leukocytes likely play an important role in the vascular occlusions and subsequent death since sickle mice lacking both endothelial selectins—which are required for leukocyte recruitment on the vessel wall—were protected from venular occlusions and death. To evaluate the molecular mechanisms responsible for these interactions, we treated sickle mice with monoclonal antibodies directly against leukocyte adhesion molecules. In the course of experiments, we noticed that control antibodies inhibited RBC-WBC interactions. We have thus evaluated commercial preparations of intravenous gammaglobulins (IVIG) in sickle mice using our intravital microscopy protocol. We observed that IVIG reduced both the number of interactions between RBCs and WBCs and the number of adherent WBCs attached to the endothelium in a dose-dependent manner. Sickle mice treated with ≥ 200mg/kg of IVIG were significantly protected from blood flow reductions and also death following TNF-α administration. Only 1/14 sickle mice was alive at the end of the experiment in groups treated with control PBS and low-dose (<10mg/kg) IVIG *vs* 8/10 alive in groups treated with ≥ 200mg/kg IVIG (p=0.0004, Fisher's Exact Test). The effect of high dose IVIG was not due to non-specific protein coating since albumin administration (400mg/kg) did not protect sickle mice. Since IVIG was administered before the inflammatory challenge, the protocol does not necessarily reflect the clinical situation in which patients seek medical attention after a crisis is established. To address this issue, we modified the protocol and administered IVIG after the initiation of RBC-WBC interactions, a surrogate marker on ongoing disease activity. We observed that IVIG (400mg/kg) dramatically inhibited the number of interactions between RBCs and WBCs, improved blood flow, and more than doubled the survival time of sickle cell mice. These studies suggest that IVIG may have activity in acute sickle cell crises. We have designed a randomized double-blind placebo-controlled dose-escalation pilot clinical study to investigate IVIG's activity clinically. This grant has also supported studies to identify selectin ligands that mediate the recruitment of neutrophils in postcapillary and collecting venules. We have recently identified CD44 as a bona fide E-selectin ligand that mediate slow rolling of neutrophils. To dissect the cellular components that contribute to vaso-occlusion in vivo, we have begun analyses of the subset of adherent WBCs recruited in the cremasteric microvasculature using real time multichannel fluorescence digital

videomicroscopy. Our preliminary studies indicate that we can identify precisely the various subsets of adherent WBCs recruited in venules of normal and sickle mice using three-color fluorescence intravital microscopy with AlexaFluor-labeled lineage-specific antibodies. These results revealed that while neutrophils account for about 50% of all adherent WBCs, they capture the vast majority (~75%) of circulating sickle RBCs. We have also begun to identify the subsets of adherent WBCs recruited in the bone marrow microcirculation. Our preliminary results suggest that many WBCs are constitutively recruited in post-sinusoidal venules in wild-type mice. In contrast to the systemic microcirculation, the majority of these adherent WBCs are lymphocytes. Studies of sickle cell mice are ongoing. Taken together, our studies suggest that sickle cell vaso-occlusion involves multicellular interactions among RBCs, WBCs, and the venular endothelium. Targeting leukocyte adhesion and/or the interactions between sickle RBCs and WBCs may be useful for the treatment or prevention of sickle cell vaso-occlusion.

ROLE OF PLACENTA GROWTH FACTOR IN ACUTE CHEST SYNDROME

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Acute chest syndrome (ACS), a devastating complication of sickle cell disease (SCD), is the most common cause of disease-related mortality. Nonetheless, little is known about its pathophysiology. It is diagnosed and treated only after the disease process is well underway. Work from our laboratory suggests a novel insight into the underlying pathophysiology of ACS. We show that an erythroid-cell derived angiogenic growth factor, placental growth factor (PlGF) promotes a strong inflammatory response in SCD: it increases expression of VEGF, IL-1β, IL-8, MCP-1, TNFα and tissue factor (TF) from monocytes via binding to VEGFR1/Flt-1. PlGF levels are increased in SCD plasma and correlate with disease severity. PlGF is inducible by hypoxia and erythropoietin (Epo), factors elevated in SCD. We show that PlGF initiates downstream signaling events resulting in activation of early growth response-1 (Egr-1). Downstream targets of Egr-1 include VEGF, IL-1β, MCP-1, TNFα, TF and 5-lipoxygenase (5LO), all of which increase leukocyte chemotaxis and inflammation; 5LO initiates the cascade that produces leukotrienes (LT). We show that SCD patients have evidence of reactive airway disease at baseline; PlGF increases expression of 5LO and 5LO activator protein from human pulmonary endothelial cells; and that PlGF-/- mice have a reduced inflammatory response to acute lung injury. ACS often follows an acute event, associated with a drop in hemoglobin. The latter would increase hypoxia, Epo and erythropoiesis, all of which increase PlGF production. *We hypothesize that elevated PlGF, via its effect on inflammatory cytochemokines and leukotrienes results in increased inflammation and reactive airway disease at baseline in patients with SCD. Further elevations in PlGF levels during acute sickle events amplify the inflammation and reactive airway disease and contribute significantly to the cascade of events that results in ACS. Aim 1:* Knock out the PlGF gene in transgenic sickle mice and study their disease severity and response to acute lung injury. *Aim 2:* Determine whether elevated PlGF levels will predict ACS in SCD patients hospitalized for an acute event and that PLGF levels and LT levels will predict the magnitude of airway reactivity and obstructive lung disease in patients with SCD. *Together, these aims are a focused to elucidate a mechanism that likely contributes to ACS, in order to enable early intervention in patients at high risk for ACS and target therapy at the underlying disease process.*

PULMONARY HYPERTENSION IN SICKLE CELL DISEASE

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Pulmonary complications of sickle cell disease (SCD), such as pulmonary hypertension (pHTN), constitute a major source of both morbidity and mortality. The Duke-UNC Comprehensive Sickle Cell Center offers a large and well-characterized patient population with which to study many aspects of pHTN in SCD. Moreover, our ongoing research in the areas of genetics, cell adhesion, nitric oxide (NO) biology, and oxidative tissue injury in the lungs and pulmonary vasculature enable us to ask fundamental questions regarding pHTN in SCD. Normally, bioactive NO downregulates ET-1 production, decreases NADPH oxidase activity, inactivates superoxide, and preserves the ability of the pulmonary vasculature to vasodilate. We hypothesize that the defective ability of red cells containing primarily hemoglobin S (SS RBCs) to transport and deliver bioactive NO is a critical mechanism causing pHTN in SCD. This defect results in upregulation of endothelin-1 (ET-1), which then results in increased superoxide (O_2^-), most likely as a result of increased NADPH oxidase activity, as shown below. In contrast, normal delivery of bioactive NO downregulates ET-1 production, leads to decreased NADPH oxidase activity, inactivates O_2^- in the presence of thiols, has an antiproliferative effect, and causes pulmonary vasodilation.

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The long-term goals of our proposed work are (1) to increase our understanding of both the natural history of pHTN in SCD as well as the pathophysiologic mechanisms leading to its development, as hypothesized above; and (2) to identify specific therapeutic interventions that can ameliorate the course of pHTN by intervening in the pathway outlined above. The overall objective of this proposal is to improve both diagnosis and treatment of pHTN in SCD. We will accomplish this by pursuing the following three Specific Aims:

AIM 1: We will study the natural history and disease progression of pHTN, as well as identify biological disease markers of SCD and disease-modifying genes associated with pHTN.
AIM 2: We will determine the effects of two therapeutic maneuvers—bosentan and transfusion—on pulmonary hypertension and other parameters of pulmonary and cardiovascular function.
AIM 3: We will investigate basic pathophysiologic mechanisms, which contribute to pHTN, including red cell biology, NO and superoxide. We will characterize the association between RBC nitric oxide (NO) processing and export and the pulmonary complications of SCD. We will use mouse models of SCD to characterize the individual contributions of the sickle RBC and the

endothelium to pulmonary vasculopathy using an isolated-perfused murine lung model under normoxic and hypoxic conditions, and in an acute lung injury model designed to mimic the acute chest syndrome. Finally, we will use a murine model of chronic hypoxia (CH)-induced pHTN to study the role of superoxide (O_2^-) and extracellular superoxide dismutase in pHTN in mice with SCD.

At the conclusion of the proposed work, we will have characterized the natural progression of pHTN in SCD and identified clinical characteristics, disease markers, and disease-modifying genes that correlate with slower or more rapid disease progression. In addition, we will have determined whether bosentan and transfusion each has a salutory effect on pHTN in patients with SCD. Finally, we will have studied several mechanisms that we hypothesize contribute to development of pHTN in SCD and thereby will have identified new therapeutic avenues for treatment of this pulmonary complication of SCD.

OXYHEMOGLOBIN DESATURATION AND VASCULOPATHY IN SICKLE CELL DISEASE

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The overall goal of this proposal is to investigate the significance of oxyhemoglobin desaturation in pulmonary complications and vasculopathy in sickle cell disease (SCD). A multidisciplinary approach will involve collaborations among hematology, radiology, neuroradiology, pulmonary science, and biochemistry and utilize state-of-the-art techniques within each discipline. Studied will be the SCD (SS type) patients between the ages of 2 and 18 years. Nearly 500 patients with SCD (SS type) receive treatment at the Children's Hospital of Philadelphia. The proposal has 4 specific aims. The first is directed toward answering the question of whether children with SCD have a higher prevalence of oxyhemoglobin desaturation than the normal population. This will be a cross-sectional study with a prospective component. Three clinical categories are expected to emerge: persistent desaturation (OHD), intermittent sleep-related desaturation, and normal oxyhemoglobin desaturation. Specific Aim 2 will investigate mechanisms leading to OHD and intermittent oxyhemoglobin desaturation in SCD. Pulmonary consequences will be addressed, as will the hypothesis that sleep-related intermittent desaturation results from obstructive sleep apnea due to anatomical and/or functional mechanisms. Anatomical factors may include overgrowth of adenoid and tonsils as a result of functional asplenia known to occur in this population. The third aim concerns determination of anatomical or functional evidence for vascular pathology, including that in the lung and brain, in children with SCD and oxyhemoglobin desaturation. In addition to imaging studies, assays for biomarkers for vasculopathy, including but not limited to markers of oxidative stress, endothelial injury, RBC flexibility and morphology, will be performed. Aim 4 will examine the effects of intervention for persistent and intermittent OHD on health, vascular manifestations and peripheral biomarkers. Interventions will include clinically-indicated adenotonsillectomy, positive pressure oxygen administration, and nocturnal oxygen supplementation.

ASTHMA AND NOCTURNAL HYPOXEMIA IN SICKLE CELL ANEMIA

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Our overall goal is to elucidate the physiologic, genetic, and molecular aspects of two common co-morbid conditions, asthma and nocturnal oxygen desaturation, that increase the incidence rate of pain in sickle cell anemia (SCA). In separate studies, we have shown children with SCA and either asthma or nocturnal desaturation have an increased pain rate when compared to children without the condition. Also, our group demonstrated that in a murine model of SCA there was significant susceptibility to hypoxia-induced pulmonary vasocongestion when compared to mice without SCA. At present, we do not know the interrelationships between asthma, nocturnal desaturation and lung disease in SCA. We propose three interrelated projects to explore these relationships. The first and second clinical projects will delineate the physiological basis for the association of asthma and nocturnal desaturation with SCA related morbidity. We will perform a case-control study evaluating whether genes associated with asthma increase the risk of pain and ACS episodes. The basic science project will have experiments aimed at defining the molecular mechanisms by which asthma and nocturnal hypoxemia, separately and together, increase lung injury in a transgenic SCA murine model. We will test three global hypotheses: 1) phenotypic and genotypic features of asthma are risk factors for pain and ACS episodes in children with SCA; 2) intermittent nocturnal oxygen desaturation is associated with morbidity in SCA; and 3) chronic airway inflammation and intermittent nocturnal hypoxia, separately and together, increase lung injury in a murine model of SCA when compared to controls.Taken together, the results of this highly interactive collaboration of clinical and basic scientists will permit new insights into the mechanisms of lung disease, thus providing a strong foundation for targeted therapy for this vulnerable group.

PULMONARY HYPERTENSION AND THE HYPOXIC RESPONSE

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Our research initiative is designed to determine the prevalence and risk factors of pulmonary hypertension (PHT) in children and adolescents with sickle cell disease (SCD), and to determine the role of the hypoxic response in its pathogenesis. Published preliminary studies indicate that PHT occurs in nearly one-third of adults with SCD, that it is associated with increased mortality although pulmonary artery pressures are lower than in patients with primary PHT, and that chronic hemolysis with nitric oxide scavenging may be a part of the pathogenesis (Castro et al, 2002; Gladwin et al, 2004; Reiter et al, 2002).

The planned research is based on three postulates.

- **First, the problem of SCD-associated PHT may begin during childhood and adolescence. A retrospective analysis (Morris et al, 2004) and our own preliminary data suggest PTHN prevalence of up to 25% in adolescents.**
- **Second, the pathogenesis of SCD-associated PHT may not only include the effects of chronic hemolysis, but also the consequences of chronic hypoxia related to severe anemia and repeated vasoocclusive episodes. PHT is a recognized complication of conditions marked by chronic hypoxia (Hultgren and Grover, 1968; Moraes and Loscalzo, 1997; Naeije, 1997), and we have recently found evidence that PHT complicates Chuvash polycythemia (CP), a congenital disorder of oxygen sensing in which the hypoxic response is constitutively up regulated in the absence of hypoxia and in which high hemoglobin concentrations would promote nitric oxide scavenging.**
- **Third, the pathophysiology of SCD-associated PHT may be elucidated by comparing components of the hypoxic response in patients with SCD and CP according to the presence or absence of PHT.**

CP, common in a specific region of Russia, is caused by a homozygous mutation in the von Hippel-Lindau gene (*VHL)* and characterized by up regulation of hypoxia inducible factor (HIF) under normoxic conditions (Ang et al, 2002a&b). HIF is the principal transcriptional regulator of the response to hypoxia in mammalian cells, and many of its target genes are up regulated in CP, such as those coding for erythropoietin, vascular endothelial growth factor (*VEGF*) and plasminogen activator inhibitor 1 (*PAI-1*) (Ang et al, 2002b; Gordeuk et al, 2004), and most likely others such as endothelin 1, angiopoietin 1, and inducible and endothelial nitric oxide synthase (iNOS and eNOS). Based on a comparison of the clinical and pathophysiologic features of SCD and CP, we hypothesize that, in addition to increased nitric oxide-scavenging effects of hemoglobin, altered expression of a HIF-regulated gene or genes is central to the pathophysiology of PHT in both SCD and CP. A coordinated study of PHT in both conditions may therefore help to make a substantial advance in our understanding of molecular basis for PHT in SCD, and lay the groundwork for the rational development of preventive and therapeutic strategies.

Based on these considerations, our research has the following specific aims.

1. Determine the prevalence, risk factors and clinical consequences of pulmonary hypertension (PHTN) in children and adolescents with sickle cell disease (SCD).

2. Elucidate the pathophysiology of PHTN in SCD by comparing proliferative vascular responses mediated by i) HIF-regulated pathways and ii) nitric oxide-scavenging in patients with SCD and patients with Chuvash polycythemia (CP). We will also examine genes implicated in primary PHTN.

3. Utilize high throughput microarray and genotyping technologies to identify candidate gene polymorphisms involved in pathologic responses to hypoxia in SCD and CP patients with and without PHTN.

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