

# blood

2001 97: 1937-1941  
doi:10.1182/blood.V97.7.1937

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

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## Modulation of endothelial cell activation in sickle cell disease: a pilot study

Alex A. Solovey, Anna N. Solovey, Jeanne Harkness, and Robert P. Hebbel

The vessel wall endothelium undoubtedly plays a role in the vascular pathobiology of sickle cell disease. This pilot study tested the feasibility of using an inhibitor of nuclear factor (NF)- $\kappa$ B, a transcription factor, to modify the endothelial activation state of patients with this vascular disease. For a total of 7 separate drug exposure tests, 3 subjects with sickle cell disease took sulfasalazine (given orally at 1 g every 8 hours), and the activation state of their circulating endothelial cells (CECs) was assessed using immunofluo-

rescence microscopy. Companion studies were also performed using sulfasalazine in sickle transgenic mice to verify its effect simultaneously on both CECs and vessel wall endothelium. Both CECs and tissue vessel wall endothelium in sickle mice have an activated phenotype. In these mice sulfasalazine significantly reduced CEC expression of vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM), and E-selectin, and it correspondingly reduced expression of these molecules in some tis-

sue vessels. In humans with sickle cell disease, sulfasalazine significantly reduced CEC expression of VCAM, ICAM, and E-selectin, but it did not reduce expression of tissue factor. Addition of a second transcription factor inhibitor, salsalate, did not change this result. This pilot study suggests that endothelial cell activation state can be modified and down-regulated in vivo by sulfasalazine. (Blood. 2001;97:1937-1941)

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### Introduction

The complex pathophysiology of sickle cell disease is undoubtedly influenced by the many physiologic functions of the vascular wall endothelium.<sup>1</sup> Even the characteristic development of acute vascular occlusion due to red cell sickling may be triggered by proximate adhesion of red cells to endothelial cells.<sup>2,3</sup> This abnormal cell-cell interaction uses various mechanisms, many of which involve adhesion receptors that can be expressed on vessel wall endothelial cells. Similarly, other processes that involve the endothelium, such as thrombosis or white cell adhesion, may play a role in vascular occlusion. Through these mechanisms, it is likely that function or dysfunction of the vascular endothelium contributes to the overall vascular pathobiology of this disease, which includes recurrent vaso-occlusions, stroke, chronic organ damage, and neovascularizing retinopathy.

Indirect evidence that the vascular endothelium is abnormally activated in sickle cell disease comes from the study of circulating endothelial cells (CECs). We earlier observed that the CECs from patients with sickle cell disease have abnormally increased expression of adhesion molecules—vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and E-selectin—as well as tissue factor.<sup>4,5</sup> Therefore, we tentatively concluded that sickle cell disease involves an abnormally activated, pro-adhesive, and procoagulant endothelial cell state. This could derive from the stimulating action of various biological modifiers such as hypoxia, thrombin, and cytokines on the normally quiescent endothelium<sup>6</sup> as well as from the pro-inflammatory effects of reperfusion-injury physiology.<sup>7,8</sup> This view of sickle cell disease as a state of abnormal endothelial activation presents a potential opportunity for novel therapeutic approaches in that pharmacologic inhibition of endothelial cell activation might be clinically beneficial.

Recognizing that the clinical efficacy of this approach would

need to be tested and established by appropriate long-term clinical studies, we conducted the present short-term pilot study to test the basic notion that inhibition of endothelial activation state is feasible in sickle cell disease. For this experiment we administered sulfasalazine to sickle mice and humans with sickle cell disease because it is a powerful inhibitor of activation of nuclear factor (NF)- $\kappa$ B,<sup>9</sup> the transcription factor that promotes expression of genes for a number of pro-adhesive and procoagulant molecules on endothelium.<sup>10,11</sup>

### Materials and methods

#### Human subjects

In designing this proof-of-concept pilot study, we assumed that both safety and clarity of interpretation were of paramount importance. Therefore, we studied 3 carefully selected subjects with sickle cell disease (Table 1). Each volunteer gave informed, signed consent for this study, which was approved by the human subjects review board of the University of Minnesota, Minneapolis. In addition to agreeing to have multiple blood samples taken, each subject met 4 strict inclusion criteria: (1) Meeting our safety criterion, each of the patients had normal levels of serum creatinine and hepatocellular enzymes, and none of the patients had any history that would predict risk for exacerbation of hemolytic rate due to deficiency of glucose-6-phosphate dehydrogenase. (2) Meeting our requirement for absolute reliability, each patient had an unblemished record of compliance and reliability during their period of attendance in our clinic. (3) Meeting our requirement for interpretability, each patient was taking folic acid as their only medication, allowing us to avoid potentially confusing effects of the multiple medications typically taken by patients with sickle cell disease. Each patient agreed to take no analgesics during the observation periods described here. (4) Finally, we only reported observations made when these patients were in

From the Department of Medicine, University of Minnesota Medical School, Minneapolis.

Submitted November 24, 1999; accepted November 24, 2000.

Supported by grant PO1 HL55552 from the National Institutes of Health, Bethesda, MD.

**Reprints:** Robert P. Hebbel, Box 480 UMHC, 420 Delaware St SE, Minneapolis, MN 55455; e-mail: hebbe001@tc.umn.edu.

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**Table 1. Study subject characteristics**

Patient (phenotype)	Subject		Hemogram			Disease manifestations
	Sex	Age, y	Hb, g/L	WBCs, $\times 10^9/L$	Platelets, $\times 10^9/L$	
A (HbSS)	F	24	9	12 000	340 000	3 pain crises per year 3 acute chest syndrome events
B (Hb $\beta^{+thai}$ )	M	28	8	13 500	290 000	<1 pain crisis per year 1 acute chest syndrome event 1 splenic sequestration crisis
C (HbSC)	M	39	13	13 000	375 000	1 pain crisis per year splenic infarction retinopathy

The indicated hemogram is typical for each subject in their steady state. All subjects were taking folate as their only medication.

their steady state, which we defined operationally as being at least 6 weeks remote from a preceding or following acute clinical event.

For this study the subjects took enteric-coated tablets of sulfasalazine (Azulfidine; Pharmacia & Upjohn AB, Stockholm, Sweden) and/or sal-salate. Both drugs were given at the dose of 1 g orally every 8 hours. Patients A and C tolerated the medications without difficulty and reported taking all scheduled doses; Patient B interrupted his second trial exposure for 3 days due to mild gastric distress and then resumed his regimen. Duration of therapy was from 1-4 weeks. We obtained peripheral blood samples (10-15 mL each) at multiple time points before, during, and after this protocol for testing of CECs. Each patient was studied on more than one occasion, separated by periods of documented return to their baseline for the study end-point (activated CEC phenotype).

### CEC phenotype

We fixed fresh whole blood with 0.25% paraformaldehyde and used immunomagnetic beads (Dynal, Oslo, Norway) coated with an anti-endothelial monoclonal antibody (mAb P1H12) to obtain cell preparations somewhat enriched for CECs, exactly as previously described.<sup>4,5</sup> We studied these using immunofluorescence microscopy and a combination of direct and indirect immunostaining.<sup>4,5</sup>

We confirmed that the CECs were endothelial by using fluorescein isothiocyanate (FITC)-labeled mAb P1H12, and we examined them for expression of selected adhesion molecules by double-staining with murine mAb to human ICAM-1 or VCAM-1 (Southern Biotechnology Associates, Birmingham, AL) or E-selectin (Novocastra Laboratories, Burlingame, CA). We detected tissue factor expression using a polyclonal rabbit antibody (gift of Dr Ron Bach, University of Minnesota, Minneapolis). The unlabeled primary antibodies were detected using secondary antibodies: lissamine-rhodamine-labeled mAb to murine immunoglobulin (Ig) and TRITC-labeled mAb to rabbit Ig (Jackson Immunoresearch Laboratories, West Grove, PA). We used 2 different negative controls: (1) same-species, same-isotype irrelevant primary antibodies and (2) omission of the primary antibodies. For positive controls we used cultured human umbilical vein endothelial cells that were stimulated for 6 hours with 1  $\mu$ g/mL bacterial lipopolysaccharide or 10 ng/mL tumor necrosis factor (TNF)- $\alpha$ . For each blood sample we recorded the percentage of CECs that were positive for expression of the molecule of interest. CECs were scored as negative if they had no more staining than the negative control samples. Because blood from patients with sickle cell disease averages 13 CEC/mL in the steady state,<sup>4</sup> the volume of blood used for these studies allowed us to score at least 20 CECs for each antigen in virtually all blood samples.

### Animal studies

We used 6- to 8-month-old animals drawn from our colony of sickle transgenic mice, which we established from acquired mice (gift of Dr Mary Fabry, Albert Einstein College of Medicine, Bronx, NY). These mice have a C57Bl/6 genetic background, they are homozygous for a deletion of murine  $\beta^{major}$  globin gene, and they carry linked transgenes for human alpha and  $\beta^S$  globins, as thoroughly characterized previously.<sup>12</sup> This study was conducted under supervision of our institution's animal use committee. The sickle mice were treated with sulfasalazine, given intraperitoneally as 0.5 mL of a 0.1% solution of drug dissolved in normal mouse saline (1.024%

sodium chloride [NaCl]) adjusted to pH 8.0. Control mice with sickle cell disease were given only vehicle in identical fashion. These injections were given 3 times daily for 10 days. On a per-weight basis, this amount of drug approximates a dose of 4 g/d given to a 70-kg human. Actual drug levels were not measured.

Animals were euthanized by carbon dioxide (CO<sub>2</sub>) asphyxiation, and blood was collected by cardiac puncture, followed by rapid collection and freezing of tissue samples. For assessment of activation molecule expression, CECs from mice with sickle cell disease were prepared in the very same manner described above because mAb P1H12 also recognizes murine endothelial cells. These CECs were studied in the same manner as human CECs, but now using rat mAb to murine ICAM-1 or VCAM-1 (Southern Biotechnology) or E-selectin (PharMingen, San Diego, CA). These were detected using secondary antibodies, Cy3-labeled antirat Ig (Jackson Immunoresearch). Because sickle mouse blood contained 70-160 CEC/mL, we also were able to evaluate at least 20 CECs for each antigen in the mouse in virtually all samples. For tissue assessment, frozen tissues were cut to a thickness of 5  $\mu$ m and fixed in 4% paraformaldehyde for 30 minutes. Sections were stained for the same antigens using the same primary antibodies as used for CECs, except that secondary antibodies were tagged with alkaline phosphatase (Jackson Immunoresearch). Tissues were counterstained with hematoxylin (Sigma Chemical Co, St Louis, MO). As control, we used same-species, same-isotype irrelevant primary antibodies and omission of the primary antibodies. Adjacent sections of tissue were stained with test versus control antibodies to ensure accurate reading of any background staining. For each tissue we evaluated 20-50 high-power fields in at least 5 nonadjacent sections, so that each data point for tissue endothelial antigen expression was derived from analysis of more than 200 vessel segments for capillaries or more than 50 vessel segments for large vessels.

We used a quantitative method to score the degree of tissue positivity for adhesion molecules. This was based on histochemical staining (as above) and a 0 to 4+ scale of expression. Values were assigned as follows: 0 indicated no detectable expression; 1+, minimal expression, defined as occasional positive endothelial cells, but overall less than 5% of the vessels being positive; 2+, mild expression, defined as less than 33% of vessels being positive; 3+, moderate expression, defined as the range between 2+ and 4+; and 4+, high degree of expression, defined as more than 80% of blood vessels expressing the test molecule. Tissues were evaluated independently and in blinded fashion by 2 microscopists. They concurred in most (more than 85%) cases; when they did not concur, the microscopists reviewed material together (still blinded) to reach consensus.

### Statistical analysis

Statistical comparison of off-drug versus on-drug murine CECs and endothelium was done using the Student *t* test. Evaluation of the human trial data employed 3 methods. (1) First, simple inspection of the data was used. In our extensive prior experience with evaluating CECs in patients with sickle cell disease (in the absence of any intervention such as that used here), we examined more than 100 samples each for ICAM, VCAM, E-selectin, and tissue factor. With the exception of one single sample in which VCAM positivity was lower, every one of these samples exhibited 50% or greater CEC positivity. Thus, the expected typical baseline range for sickle samples (50% to 100% positive) is shown by the broken-line box in

each panel of Figure 2. (2) For each subject we compared the on-drug values to the 95% CL for the off-drug values. (3) We used the Student *t* test to generate a nominal *P* value so we could compare on-drug with off-drug values for each patient. This can be done here for the following reason: Because of the nature of our data set, the value of the covariance term in the calculation of the standard *t* statistic is unknown. Therefore, the magnitude of *t* calculated here necessarily is lower than it would be if covariance were known. Therefore, for data comparisons having the nominal *P* values reported here that are in the significant range (*P* < .05), the actual *P* value would be smaller (ie, more significant) than the value shown here.

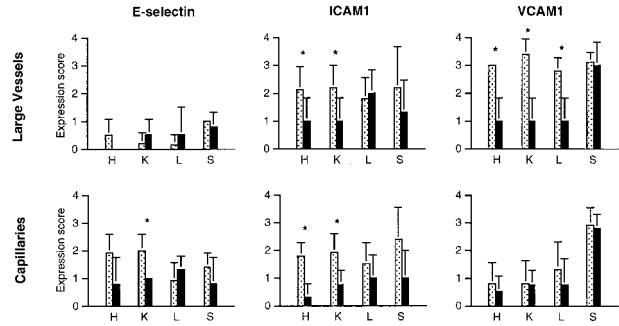
## Results

### Sickle mice

We examined both capillaries and large vessels in 4 tissues (heart, kidney, liver, and spleen) for expression of 3 molecules that appear on activated endothelium. Of these, VCAM-1 and E-selectin are expressed only upon endothelial activation, while ICAM-1 is constitutively expressed at low levels but increases upon activation.<sup>13</sup> In humans with sickle cell disease, all 3 are relevant adhesion molecules for white cells, and VCAM additionally is an adhesion molecule for sickle red blood cells.<sup>2,3</sup>

Using these sickle transgenic mice, we assessed the activation state of CECs obtained from live animals and then the tissues obtained immediately thereafter. CECs in the sickle mice were in an activated state, with a high percentage exhibiting expression of VCAM-1, ICAM-1, and E-selectin (Table 2, left column), just as we found previously for humans with sickle cell anemia.<sup>4</sup> Correspondingly, these molecules were expressed in murine tissue vessels, but the expression pattern was complex. Details are provided in Figure 1, but results are briefly summarized here. E-selectin was expressed moderately in the capillaries of most tissues, but was expressed only weakly in large vessels. ICAM-1 was expressed moderately strongly in all large and small vessels of all tissues examined. VCAM-1, on the other hand, was expressed strongly in large vessels of all tissues, but not in the capillaries (except for the spleen, which was uniformly strongly positive). Thus, tissue vessel endothelial activation was geographically variable, but nevertheless present, in sickle mice at baseline. Whether or not this activation state in sickle mice is different from healthy mice cannot be discerned from this study because our goal was limited to examination of treated and untreated sickle mice. Our examination of these murine tissues revealed no overt endothelial damage or denudation.

Compared to animals that received vehicle only, the animals given sulfasalazine for 10 days showed significant decreases in percentage of CECs that were positive for these activation markers (Table 2, right column). Correspondingly, tissue expression of these activation markers on vascular endothelial cells also decreased, variably but significantly for some tissues (Figure 1). In these animal studies we noted that variability in results from animal-to-animal was rather limited (and is evident in error bars in Figure 1) and less

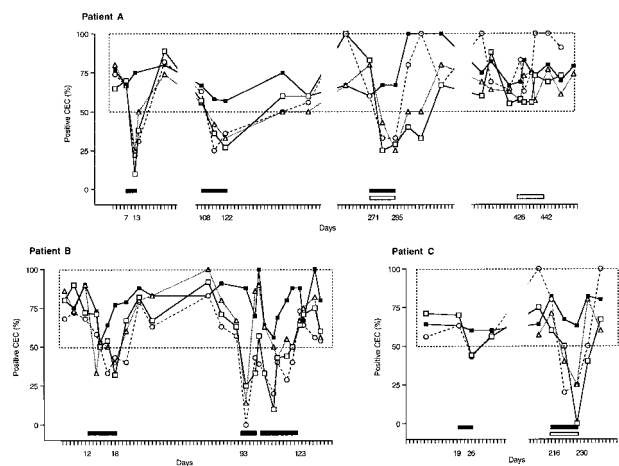


**Figure 1. Endothelial activation in murine tissues.** Tissues were scored (per "Materials and methods") for endothelial expression of E-selectin (left), ICAM-1 (middle), and VCAM-1 (right). Large vessels (top row) and capillaries (bottom row) were scored separately. Tissues examined were heart (H), kidney (K), liver (L), and spleen (S). Data are shown as the mean  $\pm$  SD (*n* = 4-8 different mice for each bar). Data from untreated mice with sickle cell disease are shown by stippled bars; data from sulfasalazine-treated mice with sickle cell disease are shown by filled bars. The asterisk indicates differences that are statistically significant. The apparent absence of a bar for E-selectin in the large vessels of the heart of treated animals (upper left panel) is because that value was  $0 \pm 0$ .

than the evident striking organ-to-organ variability in activation antigen expression or even the response to sulfasalazine.

### Humans with sickle cell disease

Our pilot study of endothelial-modulating intervention in humans with sickle cell disease necessarily was limited to analysis of CEC phenotype, and it was deliberately limited to 3 patients for the reasons outlined in "Materials and methods." The patients were given sulfasalazine, and we tested their CEC phenotype (VCAM-1, ICAM-1, E-selectin, and tissue factor) before, during, and after drug administration. Using these 3 subjects we conducted a total of 7 separate trial exposures to sulfasalazine. Results are shown in Figure 2, in which the days on sulfasalazine are indicated at the bottom of each graph by a solid bar, with start and stop days noted on the horizontal axis. Results were dramatic for the adhesion molecules.



**Figure 2. Trial of sulfasalazine in patients with sickle cell disease.** Data are shown as the percent of positive CECs for VCAM-1 (open squares), ICAM-1 (open triangles), E-selectin (open circles), and tissue factor (heavy solid line). Time scale is in days, with each mark indicating a 2-day interval. Drug administrations are indicated at the bottom of the graphs by bars (solid bar indicates sulfasalazine and open bar, salsalate), and the starting and ending days of the individual exposure trials are indicated on the horizontal axis. Patient A was given sulfasalazine 3 separate times (once with salsalate added) and was once given salsalate alone. Patient B was given sulfasalazine alone 2 separate times. The second drug exposure period was interrupted for 3 days when no drugs were taken. Patient C was given sulfasalazine 2 times (once with salsalate added).

**Table 2. Sulfasalazine given to sickle mice: expression of activation antigens on CECs**

Adhesion molecules	CECs with positive expression					<i>P</i>
	Off sulfasalazine		On sulfasalazine			
	Mean $\pm$ SD, %	<i>n</i>	Mean $\pm$ SD, %	<i>n</i>		
E-selectin	64 $\pm$ 5	4	43 $\pm$ 9	4	.008	
ICAM-1	82 $\pm$ 17	3	42 $\pm$ 7	3	.018	
VCAM-1	70 $\pm$ 9	3	47 $\pm$ 6	4	.009	

**Analysis by inspection of Figure 2.** During periods of sulfasalazine administration, the degree of CEC activation was lower than during the preceding or following off-drug periods. Virtually all of the on-drug measurements fell below the typical sickle range (Figure 2). The latter is indicated by the dashed box in Figure 2, which identifies the range within which sickle measurements virtually always fall, as indicated in our prior experience. For each of the trial exposures, expression of these 3 CEC activation markers returned to their baseline state promptly after cessation of drug (Figure 2). The transience of this drug effect is perhaps most clearly indicated in the second sulfasalazine trial for patient B, which was interrupted for 3 days (the break in the bar on days 101-103), during which time, ICAM and VCAM positivity immediately returned to baseline range.

**Analysis of Figure 2 by CL.** In addition to the above analysis by inspection, for each patient we compared the on-drug values to the 95% CL calculated for that patient's own off-drug values. As shown in Table 3, there was very little overlap between values for patients while on drug and their own baseline data for the 3 adhesion molecules.

**Statistical analysis of Figure 2.** For each patient we calculated the apparent *P* value for the difference between off-drug and on-drug values. This reveals a significant treatment effect for sulfasalazine for the 3 adhesion molecules (Table 4). Because we entered this project without knowing how long any beneficial effect of sulfasalazine might last after a drug was stopped, we used 2 conventions to define whether data samplings were on-drug or off-drug values. In the above analyses, data obtained after the subject had stopped the study drug were considered to be off-drug values, even in the cases where there was some lag before return of values to baseline (eg, for the third sulfasalazine trial for patient A). However, the values obtained on patient B during a brief interruption in administration of study drug (in the second trial exposure) are still included in our analysis as being on-drug values. It should be noted that both of these conventions would tend to diminish, rather than strengthen, the significance of these results.

In striking contrast to the responsiveness of the 3 adhesion molecules, expression of tissue factor on CECs did not respond to administration of sulfasalazine (Figure 2 and Tables 3 and 4). Therefore, in 2 of the trial exposures (the third for patient A and the second for patient C), we added salsalate in the hope of recruiting tissue factor responsiveness ("Discussion"). This had no evident beneficial effect (Figure 2 and Table 4). To monitor for possible placebo effect on CEC phenotype, we also administered one trial of salsalate alone to patient A. This revealed no effect of salsalate whatsoever (Figure 2 and Table 4).

## Discussion

Clinical considerations have led us to propose that sickle cell disease is characterized by an abnormal state of endothelial cell

**Table 3. Evaluation of sulfasalazine response by CL**

Patient	No. of on-drug values that fall above the lower 95% CL for the off-drug data/ No. of on-drug observations			
	VCAM-1	ICAM-1	E-selectin	Tissue factor
A	0/6	0/6	0/6	3/6
B	1/12	2/12	0/12	5/11
C	0/3	0/2	0/3	2/3
A, placebo	5/5	3/5	5/5	4/5

For each patient, the 95% CL was calculated for the off-drug values.

**Table 4. Statistical comparison of on-drug versus off-drug values**

	CECs with positive expression, mean $\pm$ SD, %		<i>P</i>
	Off drug	On drug	
<b>Sulfasalazine trial</b>			
Patient A			
E-selection	73 $\pm$ 17	30 $\pm$ 5	<.001
ICAM-1	64 $\pm$ 13	36 $\pm$ 10	<.001
VCAM-1	66 $\pm$ 20	28 $\pm$ 10	.001
Tissue factor	78 $\pm$ 16	66 $\pm$ 9	NS
Patient B			
E-selection	64 $\pm$ 12	36 $\pm$ 15	<.001
ICAM-1	77 $\pm$ 12	52 $\pm$ 21	.001
VCAM-1	73 $\pm$ 10	42 $\pm$ 16	<.001
Tissue factor	84 $\pm$ 9	73 $\pm$ 15	NS
Patient C			
E-selection	71 $\pm$ 21	29 $\pm$ 12	.009
ICAM-1	66 $\pm$ 10	33 $\pm$ 11	.01
VCAM-1	63 $\pm$ 11	31 $\pm$ 27	.017
Tissue factor	69 $\pm$ 11	63 $\pm$ 4	NS
<b>Placebo trial</b>			
Patient A			
E-selection	85 $\pm$ 17	84 $\pm$ 16	NS
ICAM-1	66 $\pm$ 5	68 $\pm$ 10	NS
VCAM-1	69 $\pm$ 15	62 $\pm$ 8	NS
Tissue factor	75 $\pm$ 6	76 $\pm$ 6	NS

Data shown as mean  $\pm$  SD for data depicted in Figure 2. The nominal *P* value indicated is explained in "Materials and methods." NS indicates not significant.

activation<sup>1,2</sup>; that is, a state of inflammation. This perspective represents a paradigm shift in how the vascular pathobiology of sickle cell disease might be understood. Supporting this concept, we previously described an abnormal activation phenotype for the CECs found in sickle blood.<sup>4,5</sup> Insofar as CEC phenotype is an indicator of the phenotype of vessel wall endothelium, this would imply that an abnormally pro-adhesive and procoagulant vessel wall contributes to clinical sickle disease. This perspective on sickle disease, in turn, predicts that clinical benefit would derive from therapeutics designed to impair unwanted vascular wall participation in disease pathophysiology.

Therefore, the primary goal of this very focused pilot study was to examine the feasibility of pharmacologic modification of endothelial activation as a potential therapeutic approach to sickle cell disease. Our results imply that this is possible because we found that sulfasalazine causes a prompt and significant decrease in CEC positivity for VCAM, ICAM, and E-selectin. Therefore, to the extent that activated endothelium participates in the vascular pathobiology of sickle cell disease, we would hope that this interventional strategy would have the clinical benefit of preventing either acute or chronic aspects of sickle disease. It must be emphasized, however, that further data are needed to support this notion. In particular, the extent to which CEC phenotype actually reflects that of vessel wall endothelium needs to be further bolstered (see below), and the extent to what degree of down-regulation of endothelial activation molecules would actually impact on disease pathophysiology needs to be documented.

Thus, although this pilot study yielded encouraging results, it has not attempted to optimize the regimen or even drug selection for down-regulating endothelial cell activation. In this study we chose sulfasalazine because it is a powerful inhibitor of NF- $\kappa$ B activation and is effective at micromolar concentrations *in vitro*.<sup>7</sup> The results we observed are consistent with the pre-eminent role of NF- $\kappa$ B in regulating expression of these adhesion molecules.<sup>6,10,11</sup> However, sulfasalazine was unable to diminish CEC tissue factor expression in our study, presumably because the tissue factor gene

is controlled by additional transcription regulators including API.<sup>14,15</sup> Because API is not inhibited by sulfasalazine,<sup>9</sup> the failure of sulfasalazine to impair CEC tissue factor expression was not wholly unexpected. Therefore, because salicylate inhibits both NF- $\kappa$ B and API activities at millimolar concentrations,<sup>9,16</sup> we added salsalate (which is metabolized to salicylate) in 2 trial exposures with the hope of observing tissue factor responsiveness because of dual transcription factor inhibition. However, we still did not observe a beneficial effect on tissue factor expression. We cannot say whether this is because even additional transcription factors would have to be inhibited to accomplish this goal or simply because dose-response considerations were not optimized in this pilot study.

A number of agents, most notably glucocorticoids<sup>17</sup> and other anti-inflammatory agents,<sup>18</sup> have demonstrated benefit *in vitro* for inhibiting NF- $\kappa$ B activation. However, few data address corresponding efficacy of these drugs for inhibition of NF- $\kappa$ B *in vivo*. Reports of such beneficial effects in rodents are limited to observation of whole-organ benefits from administration of high-dose glucocorticoids or diethyldithiocarbamate<sup>19,20</sup> to intracerebroventricular injection of a proteasome inhibitor<sup>21</sup> or to gene therapy approaches.<sup>22</sup> Indeed, as far as we know, the present report is the first attempt to experimentally test for salutary effects of transcription inhibitors on endothelial cells *in vivo*. The reported efficacy of high-dose glucocorticoids for treatment of 2 acute events of sickle disease, painful crisis<sup>23</sup> and chest syndrome,<sup>24</sup> may derive from the ability of these drugs to inhibit NF- $\kappa$ B activation,<sup>17</sup> although it could just as well derive from the many other effects of steroids.

In patients receiving sulfasalazine, we have preliminarily observed an increase in CECs that do not have activated NF- $\kappa$ B, as assessed by absence of nuclear localization by immunohistochemistry.<sup>25</sup> However, the effect of sulfasalazine could be mediated by direct inhibition of endothelial cell NF- $\kappa$ B, or they could be

mediated indirectly via down-regulation of monocyte activation,<sup>26</sup> for example, by inhibiting monocyte elaboration of endothelial-activating TNF. The actual mechanism of drug effect must be defined by future studies.

A secondary goal of this study was to obtain simultaneous measurements of activation markers on circulating and tissue endothelial cells. We found that both CEC and vessel wall endothelium were activated in the mouse with sickle cell disease, and the degree of activation of both declined in parallel upon exposure to sulfasalazine. Thus, these data indirectly support the notion that human CEC phenotype reflects that of the vessel wall, at least in a very general sense. In practicality, however, usefulness of CEC analysis clearly is limited because of the highly variable state of endothelial activation we observed from tissue-to-tissue, from vessel-to-vessel, and from antigen-to-antigen. If this variability extends to the human vascular tree, assessment of CEC phenotype cannot provide information of this complexity unless validated tissue-specific endothelial markers become available. Insofar as endothelial activation state impacts on disease genesis, it will be important to develop such methods so that the role of endothelial activation heterogeneity in disease pathophysiology can be understood.

Thus, from the standpoint of eventual endothelial therapeutics, there is a great need for studies that bridge the very wide gaps between *in vitro* observation of transcriptional regulation, measurement of blood CEC phenotype, and potential clinical effects. However, the theoretical value of endothelial-directed therapeutics certainly argues that this strategy deserves further exploration in sickle cell disease. Likewise, there are other vascular diseases that involve endothelial activation and an inflammatory vascular wall phenotype<sup>13</sup> such as atherosclerosis,<sup>27</sup> so these results have implications beyond sickle cell disease.

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