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NFκB component p50 in blood mononuclear cells regulates endothelial tissue factor expression in sickle transgenic mice: Implications for the coagulopathy of sickle cell disease

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Abstract

Sickle cell anemia is accompanied by activation of coagulation and thrombosis. We have studied the abnormal expression of tissue factor (TF) by the pulmonary vein endothelium of the mild-phenotype NY1DD sickle transgenic. As detected by immunofluorescence microscopy, this appears only after the NY1DD mouse is exposed to hypoxia/reoxygenation (H/R), which actually causes ischemia/reperfusion in the sickle—but not the normal—mouse. We tested the hypothesis that the NFκB-activating inflammation that develops in post-H/R NY1DD mice is responsible for this phenotype switch. Various NFκB inhibitors (including p50-specific andrographolide) demonstrated that endothelial TF positivity is NFκB dependent. Several systemic inflammatory stimulators (TNFα, lipopolysaccharide, thioglycollate, carageenan) given to control mice showed that inflammatory promotion of TF expression by only pulmonary vein endothelium is not specific to the sickle model. We bred the NFκB(p50)−/− state into the NY1DD mouse. Combined with marrow transplantation, this allowed creation of NY1DD mice that were NFκB(p50)−/− only in peripheral blood cells (and marrow) versus only in vessel walls (and tissues). This revealed that endothelial TF expression in the NY1DD mouse is highly dependent upon NFκB(p50) in peripheral blood mononuclear cells—but not in the vessel wall. In confirmation, infusion of post-H/R sickle mouse blood mononuclear cells into naïve NY1DD mice stimulated endothelial TF expression; infusion of such cells from unstimulated sickle mice at ambient air did not stimulate TF expression. We conclude that peripheral blood mononuclear cells indirectly promote endothelial TF expression *via* a NFκB(p50)-dependent mechanism. This may be relevant to the role of coagulopathy in clinical sickle disease.

Keywords

sickle; endothelial; tissue factor; NFκB(p50); monocyte; inflammation

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AUTHOR CONTRIBUTIONS

RK performed the actual mouse experiments. AS performed all immuno-fluorescence measurements. LCM carried out molecular biology aspects of the project, and supervised breeding strategies which were carried out by FA who bred and genotyped the mice. RJK Jr created, characterized and provided the anti-murine TF antibody. And RPH provided overall supervision, analysis of raw data, data interpretation, creation of all experimental strategies, and wrote the manuscript.

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INTRODUCTION

A biochemical coagulopathy is part of the clinical spectrum of sickle cell anemia (1,2). These abnormalities persist in "steady state" between acute events, and they are accentuated during acute vasooclusive crisis (1). Further, this is accompanied by –although is not yet proven to be causally related to-- clinical thrombosis, in the form of ischemic stroke, pulmonary thrombosis, and possibly thromboembolism.

We previously examined transgenic sickle mice (referred to henceforth as "sickle mice" for simplicity) for expression of tissue factor (TF) by the vascular endothelium. We found this to be substantially elevated in the more severe-phenotype sickle mice (BERK, $S+S^{Antilles}$, hBERK1) examined at ambient air, but endothelial TF expression was restricted to the pulmonary veins (3). Remarkably, this endothelial TF expression pattern was re-created by exposure of the mild-phenotype NY1DD sickle mouse to hypoxia/reoxygenation (H/R), which converted the low-TF NYIDD mouse to a high-TF mouse (3). The present studies take advantage of this useful phenotype switch model to study regulation of endothelial TF expression in the NY1DD sickle mouse.

Notably, H/R exposure of the normal mouse only exposes it to hypoxic stress and, importantly, does not induce endothelial TF expression (3). On the other hand, H/R in the NY1DD mouse actually creates ischemia/reperfusion. This is undoubtedly due to the unique presence, in the sickle mouse of a ~10-fold increase in sickled red cells that is evident during the hypoxia period, but which resolves upon reoxygenation (4). This is accompanied by several key events that occur only in the first couple of hours of the reoxygenation period: observable vascular occlusion (5,6); marked increases in leukocyte count and leukocyte/endothelial interaction, including emigration across endothelium (a hallmark of inflammation) (7,8); development of whole body peroxidation (4); occurrence of oxidant generation within the vascular endothelium (7); greater conversion of xanthine dehydrogenase to xanthine oxidase in the liver (4); and further activation of NFκB (8). This composite picture is characteristic of an induced state of inflammation caused by ischemia/reperfusion (9), and none of these events develop in the normal control mouse exposed to H/R (4–8).

However, a limitation of our prior work is that, although inflammation is indirectly implicated, the actual mechanism of endothelial TF induction in sickle mice was not established. We did previously demonstrate that lovastatin inhibits endothelial TF expression (3), but in addition to their anti-inflammatory properties, statins are reported to inhibit Egr-1 (10) which is a key, non-inflammatory regulator of TF expression (11). Consequently, an inhibitory response to lovastatin by no means constitutes direct proof of an inflammatory etiology. Therefore, our goal here was to examine the specific hypothesis that it is the NFκB-activating inflammatory state that underlies the abnormal endothelial TF expression observed in the post-H/R NY1DD sickle mouse.

Note that this study focused upon the specific NFκB p50 component for three reasons. Our previous studies have repeatedly implicated activation of two NFκB components, p50 and p65, in the inflamed state characteristic of all sickle mice (12,13). Yet, this lies in direct contrast with the fact that the TF promoter (see Discussion) is designed so that p50 should *not* play a role in TF expression (14). Our study of this contrast was enabled because NFκB(p50) −/− mice are available, but NFκB(p65) −/− mice are not because the latter state is lethal.

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METHODS

Reagents

Curcumin, sulfasalazine, hydroxyurea, carageenan, salsalate, and LPS (cat. #L6529) were obtained from Sigma Chemical Company, St. Louis, MO; andrographolide and 4H-andrographolide from Dr. Jian-Guo Geng, University of Minnesota; didox and trimidox from Dr. Howard Elford, Molecules for Health, Richmond VA; isohelenin from EMD Biosciences, Inc., LaJolla, CA; recombinant murine TNFα from R&D System, Inc., Minneapolis, MN; thioglycollate from Fisher Scientific, Inc., Pittsburg PA.

Mice

All mice were raised and housed in the same specific pathogen free room at the University of Minnesota. The mice in our colonies had previously undergone in-house sterile re-derivization. The present studies were done with our IACUC approval and monitoring.

The sickle mouse model used for this study was the mild-phenotype NY1DD mouse, which has naturally-occurring murine β thalassemia and presence of linked transgenes for human alpha and human beta^S globins, on a C57BL6 background (3). Wild-type normal C57BL6 were used as the same-strain control animals. In addition, we used mice exhibiting the homozygous knock out (−/−) state for the NFκB(p50) gene, obtained from Jackson Laboratories, Bar Harbor, ME. As originally reported, such mice appear to be phenotypically normal (including marrow, spleen, lymph nodes, and lymphocyte T and B cell ratios), but they do have low levels of serum immunoglobulins and an impaired ability to mount inflammatory responses to laboratory infections (15). The mice used here had been backcrossed against C57BL6 at least 9 times.

We bred these non-sickle NFκB(p50)−/− mice into the NY1DD mouse so that we had NY1DD mice that were NFκB(p50)−/−. For this, we utilized the strategy of first breeding the NFκB (p50)−/− state into thalssemic C57BL6 mice (littermates of the NY1DD mice) and then proceeding into NY1DD mice until the NFκB(p50)−/− state was achieved. The NFκB(p50)−/− state did not appear to phenotypically alter the mice from the NY1DD NFκB(p50)+/+ state. However, we did verify that the NFκB(p50)−/− NY1DD mice had low levels of serum immunoglobulin's (~16% of wild-type IgA level, and ~12% of wild-type IgG level)), and an occasional knockout sickle mouse had conjunctivitis (<5% of them); the latter animals were not used for experimentation. We are currently attempting to breed the NFκB(p50)−/− state into a different mouse model that is more suitable than is the NY1DD mouse for studying occurrence of inflammatory, sickle-derived histopathological lesions at ambient air.

Each mouse used for experimentation was molecularly verified to have the knockout or over-expression status it should have. This evaluation used the following primers: sense, 5′-GCA AAC CTG GGA ATA CTT CAT GTG ACT AAG-3′; wild-type anti-sense, 5′-ATA GGC AAG GTC AGA ATG CAC CAG AAG TCC-3′; knockout anti-sense, 5′-AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG-3′. Upon analysis of the PCR product, wildtype showed a single 100 bp band, NFκB+/− showed 100 bp and 190 bp bands, and NFκB−/− showed a 190 bp band.

TF expression

We measured expression of TF by the pulmonary vein endothelium exactly as previously described (3). We used gentle hydrostatic pressure to inflate lungs with PBS. Then they were placed into OCT compound (Sakura Rinetechnical, Tokyo, Japan) and snap frozen in liquid nitrogen. Five micron frozen sections were prepared, fixed, and blocked with bovine albumin. Tissue sections were triple stained for nuclei (DAPI, 4,6 diamidino-2-phenylindole), and for an endothelial marker (rat anti-murine CD31, BD Bioscience Pharmingen, Palo Alto, CA), and

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for murine TF, using a partially purified (IgG enriched) rabbit polyclonal antibody preparation that was originally characterized by Voigtlander et al. (16). We then used FITC (fluorescein isothiocyanate) anti-rabbit and TRITC (tetramethylrhodamine-5(and6)-isothiocyanate) anti-rat labeled secondary antibodies. Images were acquired using an Olympus IX70 inverted fluorescence microscope. On each animal tested, we evaluated 50 pulmonary veins, as these were the only endothelia that expressed TF; this yielded one data point for each animal. TF was expressed as percentage of pulmonary veins that were positive for TF expression; in virtually all cases, if one endothelial cell in a given vessel is positive, then all of them are (3). Non-specific staining was tested using pre-immune rabbit sera.

Hypoxia/reoxygenation (H/R) model

Animals were studied at ambient air and after H/R which was imposed using a previously standardized model (3), by placing mice into an environmental chamber with 8% O_2 for 3 hours, followed by a return to ambient air for 18 hours.

Inhibitors and Stimulators

These were given on drug-individualized dose schedules, as reported by others in the prior literature. All experiments utilized control animals receiving parallel injections of vehicle only. When saline is indicated as vehicle, it was mouse saline (330 mOsmol/L). All injections were given intraperitoneally.

Andrographolide and 4H-andrographolide (its inactive control) were given in DMSO/saline at 5 μg/mouse per injection, once daily for 3 days prior to H/R (17). Curcumin was given in ethanol/mouse-serum at 3 μmol per injection, once daily for 5 days prior to H/R (18). Didox and trimidox were given in saline at 8 mg and 4mg, respectively, per injection per 30g mouse weight once before and once just after hypoxia (19). Isohelenin in Tween-80/saline was given at 2 mg/kg, one hour before H/R (20). Sulfasalazine was given in saline as 0.5 ml of 0.01% per injection for 6 injections (for 3 days before, 2 on day of, one just after hypoxia (21). Salsalate was given in saline at 15 mg/kg by same schedule as sulfasalazine (21). Hydroxyurea, control for the two hydroxamic acid derivatives, didox and trimadox, was given in saline by same dose and schedule as didox.

To induce systemic inflammatory states in the C57BL6 control mice, we gave 1 ml of 4% thioglycollate broth (22) or 1 mg/kg carageenan (23) in saline two days before animal sacrifice. As additional models based on our previous experience, bacterial lipopolysaccharide (LPS) was given at 50 μg/mouse in saline 4 hours before sacrifice, or TNFα was given at 1 μg/mouse/day for three days before sacrifice.

BMT

To differentially place the NFkB(p50)−/− state, we utilized bone marrow transplantation (3). The animals previously obtained by crossbreeding then underwent NY1DD NFκB(p50)−/− to NY1DD NFκB(p50)+/+ transplantation, yielding animals in which the NFκB(p50)−/− state was expressed in the marrow-derived peripheral blood cells but not in vessel walls. Conversely, other animals underwent NY1DD NFκB(p50)+/+ to NY1DD NFκB(p50)−/− transplantation, yielding animals in which the NFκB(p50)−/− state was expressed in vessel walls but not in peripheral blood cells. For this, recipient animals were prepared by exposure to 137Cs (5.2 Gy [520 rad]), which was repeated 3 hours later. Donor animals were euthanized between the 2 radiation doses with CO2 gas, after which both tips of both femurs were removed. Marrow cells were flushed out of bone with PBS and, after washing, were counted and diluted to 40 million cells per mL in PBS. Eight million cells were injected via tail vein for each transplantation.

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In every animal, confirmation of the expected blood and tail (as a surrogate for vessel wall) NFkB(p50)−/− or NFkB(p50)+/+ phenotype was obtained three months after BMT by specific evaluation using polymerase chain reaction. The primers used for these tests are indicated above.

As a technical note, we previously demonstrated that intentional induction of graft-versus-host-disease via our BMT protocol does not induce endothelial TF expression (3). Nor does the transplantation procedure itself (3).

Mononuclear Cell Infusion

Donor mice included NY1DD mice at ambient air and NY1DD mice post-H/R, in which case sampling time was one hour after end of hypoxia. Recipient mice, naïve NY1DD at air, were infused with one or the other type of donor peripheral blood mononuclear cells, and then lungs were harvested after 18 hours to assess TF expression. Ratio of donor to recipient mice was 2:1. Donor blood was collected by cardiac puncture, and placed on Histopaque-1077 (Sigma). The resulting mononuclear cell preparation (monocytes 20–25%, lymphocytes 74–78%, granulocytes 1–2%). was diluted to 4×10^7 cells/ml in mouse saline and each mouse received 0.1 ml by intravenous injection.

Statistics

Testing for significance utilized t testing or ANOVA, as required. Number of independent observations (number of mice) is shown for all data. We used small groups of animals (since data were sufficiently robust) so that we could conserve on animal subject use.

RESULTS

NFκB Inhibitors

We tested several agents that have been reported to inhibit NFκB, since this provides a partial test of our hypothesis. All but one of the NFκB inhibitors did significantly decrease endothelial TF expression in the post-H/R NY1DD sickle mouse (Table I). This Table shows actual raw data expressed as percent TF positivity; the average degree of inhibition is stated in the following text.

Effective agents included: andrographolide, a p50-specific inhibitor (48%); curcumin, which additionally is an Egr-1 inhibitor (33%); two iron-chelating hydroxamic acids, didox (59%) and trimidox (50%); isohelenin (69%); and sulfasalazine (27%). Salsalate failed to inhibit TF expression (see Discussion). 4H-andrographolide, an inactive form of andrographolide, was used as a control agent, and it had no effect. Neither did hydroxyurea, a drug class control for the hydroxamic acids and a drug of high interest in the sickle context. These results for didox/trimidox were published previously (19) but are here reproduced to allow direct comparison with present results.

Stimulation of Inflammation

A significant question is whether or not the limitation of endothelial TF expression to the pulmonary veins seen in this model is a unique, sickle-specific phenomenon. Therefore, we compared the results of four other established models of inflammation in normal wild-type mice (which without stimulation had 10.1±4.6% TF expression). Intraperitoneal injection of LPS, TNFα, thioglycollate, or carageenan all resulted in conversion, to some degree, to a state of elevated endothelial TF expression: an increase to 40.0±5.8% ($P<.001$), 18.9±4.8% ($P<.01$), 20.5±2.6% ($P<.05$), and 17.0±2.1% ($P<.01$), respectively. Also, this was again restricted to the pulmonary veins (other organs checked: spleen, liver, muscle, heart, kidney, brain).

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Studies of the NFκB(p50)−/− Sickle Mouse

To more rigorously test the role of NFκB(p50) in endothelial TF expression, we bred the NFκB(p50)−/− state into the NY1DD sickle mouse. Figure 1 compares the lower TF expression seen at ambient air and the elevated TF expression seen post-H/R for the wild-type NY1DD (p50)+/+ sickle animal *versus* the presence of this knockout state in the NY1DD NFκB(p50) −/− sickle mouse. Presence of the knockout state is associated with a great lowering of TF expression, both at ambient air and after H/R stress. In this case, of course, the whole mouse is in the p50 knockout state. The heterozygous state, NY1DD(p50)+/−, showed an intermediate phenotype with a lesser degree of TF suppression (not shown in Figure 1).

We next determined whether the apparent effect of the NFκBp50 knockout state on endothelial TF expression was exerted in the vessel wall (e.g., its endothelial cells) or in peripheral blood cells. As presented in Methods, we created two additional kinds of NY1DD sickle mice: those having NFκB(p50)−/− in vessel walls and tissues, but NFκB(p50)+/+ peripheral blood cells; *versus* those having NFκB(p50)−/− peripheral blood cells, but NFκB(p50)+/+ vessel walls and tissues.

As shown in Figure 1, specific knockout of vessel wall/tissue NFκB(p50) did not diminish TF expression or its response to H/R. In striking contrast, knockout of peripheral blood cell NFκB(p50) did greatly reduce TF expression by endothelial cells, at both air and after H/R (Figure 1). P values are shown in the legend to Figure 1.

Mononuclear Cell Infusion

In corroboration of the above data implicating blood cells, we infused 4×10^6 peripheral blood mononuclear cells obtained from post-H/R NY1DD sickle mice (one hour after start of the reoxygenation period) into naïve NY1DD mice at ambient air. These significantly stimulated increased endothelial TF expression in the naïve mice, to 19.0±7.0% (P=.0278). The control infusion of mononuclear cells from naïve mice into naïve mice did not do so, leaving TF expression at 6.5±2.1%.

DISCUSSION

The present studies examined the phenotype switch (increased endothelial TF expression) exhibited by the post-H/R NY1DD sickle mouse to address the hypothesis that it is their known inflammatory state (caused by actual ischemia-reperfusion physiology, as presented in Introduction) that is responsible for this abnormal TF expression. The present results, in aggregate, support this hypothesis and identify a regulatory role for NFκB component p50 in activated peripheral blood mononuclear cells in this event. We believe that this model is highly relevant to human sickle disease since, in our opinion, it very probably comprises a paradigmatic state of ischemia/reperfusion (13).

The simple hypothesis test of examining the effect of known NFκB inhibitors successfully demonstrated their efficacy in this *in vivo* model. The single exception was salsalate, even though it is metabolized to salicylic acid; however, this may be because it actually is a fairly weak NFκB inhibitor, for example having three logs lower activity that of sulfasalazine (24). Although the present study does not add to existing information about the mechanism of these agents, the data obtained do provide provisional support for our hypothesis; whereas, it would not be supported had these agents not inhibited TF expression. In particular, the fact that the p50-specific inhibitor, andrographolide (17), was effective provides further support for our pre-existing suspicion that endothelial TF expression would somehow involve p50, as described in Introduction.

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Consequently, we proceeded to examine NY1DD sickle mice that were NFkB(p50) deficient and found that this state greatly impaired endothelial TF expression. However, it is important to note that there remains a basal level of apparently NFkB(p50)–independent TF expression that still increases somewhat in response to H/R (Figure 1). This would reflect the effect of other NFkB components and the other transcription factors important in TF regulation, AP-1, Sp1, and Egr-1 (11).

To determine the whether the NFκB(p50) that was influencing the expression of TF in endothelium was located in peripheral blood cells *versus* endothelial cells themselves, we utilized a marrow transplantation strategy to create NY1DD sickle mice that had the NFκB (p50) knockout either in peripheral blood cells (and other marrow-derived tissues) but not endothelial cells (or vessel walls in general), or alternatively, NY1DD mice that had the knockout in endothelial cells but not peripheral blood cells. Results (Figure 1) clearly demonstrated that endothelial NFκB(p50) is not involved in endothelial TF expression, as expected (see below). Rather, endothelial TF expression was inhibited only when NFκB(p50) was absent from the peripheral blood cells.

Since this observation implicates blood cells as activators of endothelium, we demonstrated that infusion of a preparation of peripheral blood mononuclear cells obtained from post-H/R NY1DD mice, but not from unstressed NY1DD mice, caused significant subsequent enhancement of endothelial TF expression in the recipient naïve NY1DD mice. Since the mononuclear cells were harvested exactly one hour after the end of hypoxia, we know that the implicated mononuclear-activating event had occurred very shortly into the reoxygenation period, consistent with previous observations on other biomarkers of the expected inflammatory response to ischemia/reperfusion (4–9). Although the present results cannot exclude some contribution from the many lymphocytes or the few granulocytes contained within the mononuclear cell preparation, this seems unlikely because it is the monocytes and not the lymphocytes that typically generate the known endothelial-activating substances of interest.

This observation of an in *vivo* activating effect of mononuclear cells in the sickle mouse complements prior observations of activated monocytes being present sickle disease blood. For example, our group demonstrated that peripheral blood mononuclear cells from sickle patients exhibited increased levels of IL1β and TNFα and, importantly, that they stimulated activation of NFκB within co-cultured endothelial cells (25). Consistent with this, Perelman and colleagues demonstrated increased expression of multiple inflammatory mediators by sickle blood mononuclear cells (IL1β, IL8, monocyte chemoattractant protein-1, and VEGF) and have supported a monocyte-stimulating role for placenta growth factor in the sickle context (26), although it seems likely that multiple factors are involved *in vivo*. In turn, the mechanism of endothelial activation by blood mononuclear cells could involve multiple factors. We know that the stimulating effect of sickle human monocytes on cultured endothelial cells is blockable with antibodies to IL1β and TNFα (25). However, both monocytes and granulocytes additionally are abnormally productive of superoxide in sickle patients (27). Definitive identification of the specific activating mediators affecting endothelium *in vivo* has not yet been reported.

We had *not* expected the specific NFκB p50 component in endothelial cells *per se* to participate in the stimulation of TF expression in this model. The NFκB family of transcription factors includes several well-known components: p50, p52, p65(RelA), RelB, and c-Rel (14). The mechanism of NFκB regulation, e.g. of the inflammatory response, involves the binding of distinct heterodimeric or homodimeric combinations of these factors to the promoter region of many genes. Notably, different genes can have somewhat different genetic sequence of the at

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gene's regulatory NFκB site *per se*. It is this sequence variability that confers distinct selectivity at a given site, as to whether a given NFκB component can bind.

For example, the sequence of the NFκB site of the TF gene specifically promotes regulatory binding of c-Rel/p65 heterodimers, but it specifically prevents binding of p50/p50 or of p50/p65 dimers (14). For this basic reason, it should be expected that the specific p50 component in endothelium is *not* involved in direct endothelial TF regulation, although the overall regulation of TF expression definitely depends on other NFκB components in addition to AP1, Sp-1, and Egr-1 (11). It should be noted that data on TF transcriptional regulation were largely obtained from monocytes, so the possibility that regulation within endothelium differs in this respect cannot be completely excluded. However, because NFκB binding specificity is based on genetic sequence, it would presumably be true for all cells, e.g., both endothelial cells and peripheral blood monocytes. In any case, our results rule out a role for endothelial NFκB(p50) itself.

An important question has been whether this peculiar type of endothelial TF expression pattern (limited to pulmonary veins) is unique to the sickle mouse model of inflammation. We conclude that it is not, as our results demonstrate that it also develops in normal control mice that are exposed to systemic inflammatory stimuli (LPS, TNF∝) or chemical peritonitis (carageenan, thioglycollate). Interestingly, it thus appears that the endothelium of this particular geographic location is peculiarly susceptible (in terms of TF expression) to inflammatory stress. So this phenomenon may be of relevance to inflammation states in general.

It will have been noted that the control, non-stressed value for TF expression by the NY1DD sickle mouse was higher (~12%) in Table I than in Figure 1 (~7%). This was *not* due to assay insensitivity or variability. Reproducibility in this assay is high (for example, results are the same for different blinded microscopists [3]), and we have taken advantage of this to conserve on animal subject use. Rather, the change in baseline is explained by the fact that these two experiment types were done in different time frames (three years apart). In between, our animal facility changed its standard mouse chow from a higher-fat/lower-protein mixture to a lower-fat/higher-protein mixture. This resulted in the shift of basal TF expression, as noted. It was not accounted for by technical variability, reagent differences, and so on. We point this out as a caution to other investigators who may be studying TF expression in *in vivo* models. Regarding the present study, this shift over time does not affect data interpretation or results, since every experiment used its own internal controls.

Our results using the *in vivo* sickle mouse model of inflammation are potentially discrepant with the previously reported observations that administration of endotoxin to rats and rabbits did not result in lung endothelial TF expression (28,29). Many experimental differences could account for this, including: species differences, different avidities of the reagents for TF, different doses or schedules, different bioavailability of different LPS preparations, or technical differences. And it should be recalled that the endothelial TF expression we have studied is limited to a small sub-set of pulmonary vessels, something that easily could be overlooked absent meticulous examination. Yet, we find that the sickle transgenic mouse model of pulmonary venous TF expression is a robust, tractable model.

Whether or not endothelial TF is functionally active in this model is not known yet. Presence or absence of actual TF activity, of course, depends on several factors in addition to simple TF expression. An example is whether or not platelet activating factor is elevated, which is the case in sickle humans but not sickle mice. TF positive endothelial cells, particularly in the pulmonary veins, could play an important role in sickle vacular biology. First, they could be a source of TF-positive microparticles, at least in sickle human biology (30). In addition, this

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geographic location is immediately upstream of the vascular bed that most typically develops thrombosis in human sickle disease (Circle of Willis).

In conclusion, the present results further support the concept that the activated peripheral blood monocnuclear cells (which also are elevated in number) in sickle disease are critical players in perturbation of the endothelium in a vascular bed that is immediately upstream of the Circle of Willis. Insofar as tissue factor expression contributes to sickle disease pathobiology, our results argue for an increased investigative focus upon monocyte biology in this disease context.

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ABBREVIATIONS

H/R	hypoxia-reoxygenation
IL	interleukin
LPS	lipopolysaccharide
NFκB	nuclear factor – kappa B
TF	tissue factor
TNF	tumor necrosis factor

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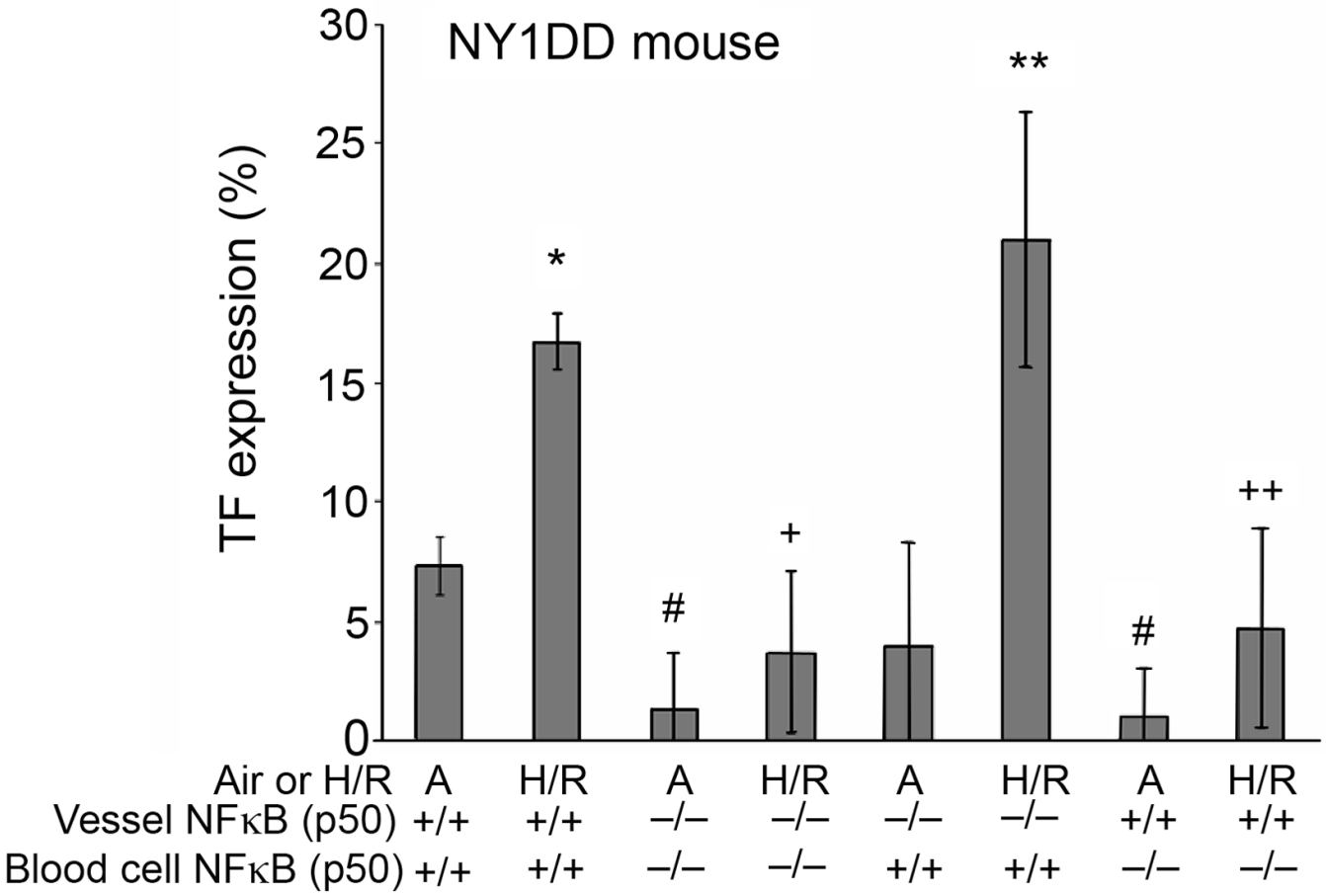

Figure 1.

Effect of NFκB(p50) knockout state. TF expression is indicated as percentage of pulmonary veins with TF positive endothelium, and is shown as mean ± SD for wild-type (p50+/+) NY1DD sickle mice in ambient air ("air") or after exposure to H/R ("H/R"). Compared to the TF response of wild-type NY1DD sickle mice to H/R (two bars on left), NY1DD NFκB(p50) −/− mice have lower TF at ambient air (third bar) and abrogated TF response to H/R (fourth bar). Remaining bars are from cross-transplantation experiments, NY1DD NFκB(p50)−/− to NY1DD NFκB(p50)+/+ and vice versa, and demonstrate that the TF response to H/R requires blood cell NFκB(p50) but not vessel wall/tissue NFκB(p50). * = P<.001, and # = P<.01, compared to air control (bar on far left). ** = P<.001 compared to respective air control (the fifth bar from left). And + = P<.01 and ++ = P<.001, compared to +/+ blood cells post H/R (the second bar from left). From left to right, number of animals used = 3,3,6,6,4,4,4,3.

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Table 1

Effect of NFκB inhibitors (and control drugs) on endothelial TF expression in the NYIDD sickle mouse[*]

Condition or Agent	n	TF expression (%)	P[#]
NY1DD at ambient air	10	12.8 ± 2.9	
NY1DD post-HR	27	34.7 ± 6.3	<0.001
+ andrographolide	6	18.0 ± 2.1	<0.001
+ curcumin	10	23.2 ± 7.3	<0.001
+ didox	6	14.3 ± 2.3	<0.001
+ trimidox	12	17.5 ±6.9	<0.001
+ isohelenin	5	10.8 ± 3.0	<0.001
+ sulfasalazine	5	25.2 ± 3.0	<0.05
+ salsalate	6	29.3 ± 5.7	ns
+ 4H-andrographolide[§]	6	31.3 ±2.1	ns
+ hydroxyurea[§]	6	35.6 ± 4.8	ns

[*]TF is expressed as percentage of pulmonary veins being TF positive, and data are shown as mean ± SD.

[#]Post-H/R is compared to ambient air; all others are compared to post-HR with vehicle but without the active drug.

[§]These agents are control drugs; all others are known NFκB inhibitors.