MEK1/2 inhibitors reverse acute vascular occlusion in mouse models of sickle cell disease

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ABSTRACT In sickle cell disease, treatment of recurrent vasoocclusive episodes, leading to pain crises and organ damage, is still a therapeutic challenge. Vasoocclusion is caused primarily by adherence of sickle erythrocytes (SSRBCs) and leukocytes to the endothelium. We tested the therapeutic benefits of MEK1/2 inhibitors in reversing vasoocclusion in nude and humanized sickle mouse models of acute vasoocclusive episodes using intravital microscopy. Administration of 0.2, 0.3, 1, or 2 mg/kg MEK1/2 inhibitor to TNF–α–pretreated nude mice before human SSRBC infusion inhibited SSRBC adhesion in inflamed vessels, prevented the progression of vasoocclusion, and reduced SSRBC organ sequestration. By use of a more clinically relevant protocol, 0.3 or 1 mg/kg MEK1/2 inhibitor given to TNF–α–pretreated nude mice after human SSRBC infusion and onset of vasoocclusion reversed SSRBC adhesion and vasoocclusion and restored blood flow. In sickle mice, 0.025, 0.05, or 0.1 mg/kg MEK1/2 inhibitor also reversed leukocyte and red blood cell adhesion and improved microcirculatory blood flow. Cell adhesion was reversed by shedding of endothelial E-selectin, P-selectin, and αvβ3 integrin, and leukocyte CD44 and β2 integrin. Thus, MEK1/2 inhibitors, by targeting the adhesive function of SSRBCs and leukocytes, could represent a valuable therapeutic intervention for acute sickle vasoocclusive crises.—Zhao, Y., Schwartz, E. A., Palmer, G. M., Zennadi, R. MEK1/2 inhibitors reverse acute vascular occlusion in mouse models of sickle cell disease. FASEB J. 30, 000–000 (2016). www.fasebj.org

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Recurrent vasoocclusive episodes contribute to morbidity and accelerated mortality in patients with sickle cell disease (SCD), primarily because of acute pain crises and ischemic irreversible end-organ damage (1). The pathophysiology of vasoocclusion is a multifactorial event involving sickle erythrocytes (SSRBCs), leukocytes, platelets, the endothelium, adhesion, and signaling molecules (2–4). Although therapies to treat sickle cell acute vasoocclusive episodes are being developed, none of these therapies targets the signaling mechanisms in SSRBCs and activated leukocytes to reverse adhesion of these cells once vasoocclusion is established.

The ERK1/2 is abnormally activated in SSRBCs, but not in normal red blood cells (RBCs) (5). Aberrant ERK1/2 signaling is known to occur in a wide range of pathologies, including cancer, diabetes, viral infection, and cardiovascular disease (6, 7). MEK1/2 (MEK)-dependent ERK1/2 signaling in SSRBCs mediates adhesion to TNF–α–activated microvascular endothelial cells (ECs) in vitro and in vivo, and vasoocclusion in vivo (8). MEK-dependent ERK1/2 signaling in SSRBCs is also involved in stimulation of neutrophil adhesion to nonactivated ECs (8). Studies have previously shown that stimulation of the ERK1/2 pathway in neutrophils activates neutrophil adhesion (9). ERK1/2 pathway inhibition in neutrophils down-regulates both adhesion molecule expression induced by endothelin-1 and cell-adhesive response (9).

Members of the ERK1/2 cascade have been identified as potential pharmacotherapeutic targets (10, 11). Inhibition of the ERK1/2 pathway with MEK inhibitors reduces the infarct volume together with down-regulation of inflammation and apoptosis in experimental models (12). MEK inhibitors also afford brain protection against forebrain ischemia and focal cerebral ischemia in animals (13). In SCD, reversal of ongoing vasoocclusion is still a therapeutic challenge. We have now evaluated whether the existing MEK inhibitors, RDEA119/BAY 869766 and trametinib, which have been developed for use in humans, can reverse adhesion of SSRBCs and leukocytes, and established vasoocclusion in both nude and sickle mouse models of vasoocclusion in vivo. Thus, these studies may lead to the establishment of existing MEK inhibitors as a potential therapeutic strategy to treat acute vasoocclusive episodes in SCD.

MATERIALS AND METHODS

ECs

Human dermal microvascular endothelial cells (HMVECs-d) (Lonza, Walkersville, MD, USA) were grown as monolayers in
EBM2 medium (Clonetics, Walkersville, MD, USA) supplemented with EGM2 (Clonetics) (14). Before in vitro adhesion assays, HMVECs-d were treated for 4 h with 10 ng/ml recombinant human TNF-α (Sigma-Aldrich, St. Louis, MO, USA).

**Blood collection and RBC preparation**

Obtaining blood samples from human participants has been approved by the institutional review board at Duke University, and written informed consent has been obtained from the participants. Blood samples were collected from adult patients with SCD homozygous for sickle hemoglobin (HbS) and from healthy donors. Patients with SCD had not been transfused for at least 3 mo and had not experienced acute vasoocclusive crises for 3 wk, and 30% of these patients were on hydroxyurea. Blood samples collected into citrate or heparin tubes were used within 24 h of collection. Packed RBCs were separated as previously described (4) and were analyzed for leukocyte and platelet contaminations using an Automated Hematology Analyzer K-1000 (Sysmex Corp., Kobe, Japan).

**Reticulocyte enrichment and depletion**

Reticulocytes (reticulocyte enriched) were separated from mature RBCs (reticulocyte depleted) based on reticulocyte transferrin receptor expression, using anti-human transferrin receptor mAb 5E9 (ascitic fluid diluted 1:100) and goat anti-mouse IgG-coated microbead affinity columns (Miltenyi Biotec, Auburn, CA, USA), following the manufacturer’s instructions.

**RBC and reticulocyte treatment**

For in vitro adhesion assays, packed SSRBCs, sickle (SS) reticulocytes, mature SSRBCs, and normal reticulocytes were treated at 37°C for 1 h with the MEK inhibitor RDEA119 (CGeneTech, Incorporated, Indianapolis, IN, USA) at 0.5–500 nM, then washed extensively. Sham-treated cells were incubated with the same buffer and vehicle but without the active agent. Treated cells were fluorescently labeled for in vitro and/or in vivo adhesion studies as described previously (4, 14).

For ERK1/2 phosphorylation, normal reticulocytes were sham treated, treated at 37°C with 20 nM epinephrine (Sigma-Aldrich) for 1 min, or pretreated for 1 h with the MEK inhibitor U0126 (Calbiochem, La Jolla, CA, USA) before epinephrine treatment. Cells were washed before cell lysis (5).

**Leukocyte separation**

Separation of peripheral blood mononuclear cells and neutrophils from blood from patients with SCD was performed as described previously (8, 15). Neutrophils and peripheral blood mononuclear cells were then mixed (SS leukocytes) before adhesion to washed TNF-α-activated HMVECs-d.

**Flow chamber in vitro adhesion assays**

Slides coated with activated HMVECs-d were washed then fit into a variable height flow chamber and tested for their ability to support adhesion of treated SSRBCs, SS reticulocytes, mature SSRBCs, normal reticulocytes, and nontreated SS leukocytes. The flow chamber was mounted on the stage of an inverted phase-contrast microscope (Diaphot; Nikon Inc., Melville, NY, USA) connected to a thermoplate (Tokai Hit Company, Ltd., Fujinomiya-shi Shizuoka-ken, Japan) set at 37°C. Cells suspended at 0.2% (vol/vol) in PBS with Ca²⁺, Mg²⁺ were infused into the flow chamber and allowed to adhere to the slide for 10 min without flow. Before exposure to flow, a minimum of 3 fields at each of 7 different locations along a line oriented normal to future flow was examined, and the total number of fluorescent cells was counted. Fluid flow (PBS with Ca²⁺, Mg²⁺) was then started using a calibrated syringe pump. After exposure to flow, the fields were again examined, and the number of fluorescent adherent cells was counted. The fraction of adherent cells was presented as the following:

\[
\text{Number of cells attached per field after exposure to flow} = \frac{\text{Total number of cells present per field before flow}}{\text{Number of cells attached per field after exposure to flow}}
\]

The wall shear stress was calculated as the following:

\[
\tau_w = \frac{6\mu Q}{wH(x)^2}
\]

where \(\tau_w\) is wall shear stress (dyne per square centimeter), \(Q\) is volumetric flow rate (cubic centimeter per second), \(\mu\) is medium viscosity, \(w\) is the width of the flow channel, and \(H(x)\) is the height of the flow chamber as a function of position along the microscope slide (16).

For adhesion molecule shedding, after exposure to fluid flow of activated HMVECs-d coincubated or not with SSRBCs or SS leukocytes, adherent cells to HMVECs-d were then vehicle treated or treated with 1 \(\mu\)M RDEA119 for 1 h. After exposure to fluid flow again, activated HMVECs-d, SSRBCs, and SS leukocytes were collected and analyzed for adhesion molecule expression by flow cytometry.

**Western blot**

Normal reticulocytes were lysed with hypotonic buffer [5 mM Na₂HPO₄, plus 1 mM EDTA plus 0.1% NaN₃ (pH 8)] containing 2 mM PMSF, phosphatase inhibitor cocktail, and protease inhibitor cocktail (all from Sigma-Aldrich). Protein separation by PAGE and Western blots using rabbit anti-human ERK1/2 (Upstate, Charlotteville, VA, USA) and rabbit anti-human phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) antibodies were performed as previously described (5). Bands were analyzed densitometrically using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Phospho-ERK1/2 data were normalized according to total ERK1/2 and are presented as fold change in ERK1/2 phosphorylation.

**Mice**

The Institutional Animal Care and Use Committee and the Committee on the Ethics of Animal Experiments at Duke University approved this animal work. All surgery was performed under anesthesia by injection of 100 mg/kg, i.p. ketamine (Abbott Laboratories, Chicago, IL, USA) and 10 mg/kg, i.p. xylazine (Bayer, Shawnee Mission, KS, USA), and all efforts were made to minimize suffering. Female athymic homozygous nude (nu/nu) mice, 8–12 wk of age, were bred at Duke University. Male transgenic Townes sickle (TS) mice, 8–10 wk of age, were obtained from colonies originally established by Dr. T. Townes from The University of Alabama (Birmingham, AL, USA) (17, 18). The murine TS model expresses exclusively human α- and sickle β-globin. Compared with wild-type controls, TS mice have decreased hematocrits (Hcts), elevated reticulocyte counts, lower Hb concentrations, splenomegaly, marked activation of coagulation, vascular inflammation, and EC injury, which are all
indications of the anemia and vasculopathy associated with human SCD (19, 20).

Window chamber surgery

Dorsal skin-fold window chamber surgery was performed on anesthetized mice as we have previously described in detail (4).

Animal treatments and fluorescence intravital microscopy

Animals were injected with 500 ng, i.p. murine recombinant TNF-α to activate ECs. In protocol 1 (Fig. 1), to reduce precipitation of vasoocclusion, 210 min following TNF-α administration, anesthetized nude mice were infused through the dorsal tail vein with vehicle (0.4% DMSO in saline), or the MEK inhibitor RDEA119 or U0126 at 0.2 or 2 mg/kg, or trametinib at 0.3 or 1 mg/kg. Thirty minutes later, fluorescence-labeled human RBCs (300 μl, Hct 50% in saline) were then infused (4, 8). Intravital microscopy of postcapillary venules and arterioles was performed immediately after RBC infusion and as described previously (4). At least 30 venules of each mouse were recorded for a period >60 min.

For more clinically relevant protocols, MEK inhibitor was administered intravenously (Fig. 1, protocol 2) or by oral gavage (Fig. 1, protocol 3) to nude mice or intravenously to sickle mice (Fig. 1, protocol 4) after onset of vasoocclusion. In protocols 2 and 3, 2 h following TNF-α administration, anesthetized nude mice were infused with human SSRBCs (300 μl, Hct 50%). In protocol 2, intravitral microscopy was performed immediately after SSRBC infusion for at least 15 min, a time during which vasoocclusion has already occurred. Vehicle, 1 or 2 mg/kg RDEA119, or 0.3 or 1 mg/kg trametinib was then infused into nude mice. Intravitral microscopy was resumed immediately after drug administration for a period >60 min. In protocol 3, vehicle or 0.3 or 1 mg/kg trametinib was administered by oral gavage to nude mice 15 min after human SSRBC infusion and onset of vasoocclusion. Intravitral microscopy was performed 30 min following drug administration for a period of 240 min. In protocol 4 (Fig. 1), 2 h following TNF-α injection and onset of an acute vasoocclusive crisis in TS mice, anesthetized animals were infused with vehicle (0.02% DMSO in saline), 0.025, 0.05, or 0.1 mg/kg RDEA119, or 0.05 mg/kg trametinib. Leukocytes and RBCs were monitored by labeling cells in vivo with 100 μl rhodamine 6G (Sigma-Aldrich) (0.02%) in sterile saline and 0.12 μg/g body weight phycoerythrin-conjugated anti-mouse TER119 (Ly-76) mAB (BioLegend, San Diego, CA, USA) specific for leukocytes and RBCs, respectively.

Figure 1. Schematic representation of intravitral microscopy protocols. In protocol 1, nude mice were injected intraperitoneally with murine TNF-α (time 0). After 210 min, animals were anesthetized then infused with 0.2, 0.3, 1, or 2 mg/kg MEK inhibitor or vehicle (0.4% DMSO in saline). At 240 min following TNF-α administration, human RBCs (50% Hct) were infused. Images of the subdermal vasculature under intravitral microscopy were recorded between the time points of 240 and 420 min (T 240 → T 420). Protocols 2 and 3 were designed to assess the effect of RDEA119 and trametinib on ongoing vasoocclusion in TNF-α-pretreated nude mice challenged with human SSRBCs. Nude mice with window chamber implants were injected intraperitoneally with murine TNF-α (time 0). After 120 min, animals were infused with human SSRBCs (50% Hct). In protocol 2, images of the subdermal vasculature were recorded immediately after SSRBC infusion between the time points of 120 and 165 min (T 120 → T 165) during which vasoocclusion has occurred. RDEA119 at 1 or 2 mg/kg, trametinib at 0.3 or 1 mg/kg, or vehicle was then infused at 165 min after TNF-α injection (time 0). Recording of images was resumed immediately after drug administration for a period of 90 min (T 165 → T 255). In protocol 3, trametinib at 0.3 or 1 mg/kg or vehicle was administered by oral gavage to nude mice 135 min after TNF-α injection (time 0). Images of the vasculature were recorded 30 min after drug administration between the time points of 165 and 405 min (T 165 → T 405) during which vasoocclusion has occurred. RDEA119 at 0.05 mg/kg trametinib, or vehicle (0.02% DMSO in saline) 120 min after TNF-α challenge (time 0). Images of the subdermal vasculature were recorded for 80 min between the time points of 130 and 210 min (T 130 → T 210). PE, phycoerythrin.
infused through a tail vein. Intravital microscopy was performed 10 min after drug administration, and at least 30 venules of each mouse were recorded for a period of 80 min.

Cell adherence was quantified on still images by measuring the fluorescence intensity (fluorescence unit) of adherent fluorescence-labeled cells using ImageJ software. The values were averaged among groups of animals (n = 5 for each treatment nude mouse group; n = 4 for each treatment sickle mouse group) to obtain mean fluorescence intensity and for statistical analysis. In some instances, data were normalized according to human SSRBC adhesion before drug infusion and plotted as a function of time.

**Histology**

The lungs, spleen, kidney, and liver were collected from euthanized vehicle-treated and 0.2 mg/kg RDEA119-treated nude mice immediately after intravital microscopy. Tissue section preparation (10 μm thickness) was performed as described previously (4). There were 3 random fields imaged for each section of each organ, and fluorescence intensity for each field was quantified using ImageJ software. The values were averaged among groups of animals (n = 3) for statistical analysis.

**Flow cytometry**

Vehicle or 0.2 mg/kg RDEA119 was infused into TNF-α–treated nude mice (n = 6 per treatment group). Thirty minutes later, fluorescence-labeled human SSRBCs (300 μL, Hct 50%) were then infused. Blood samples were collected from nude mice 15, 60, and 120 min following human SSRBC infusion and analyzed for the presence of SSRBCs using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The values were averaged among groups of animals for statistical analysis. Adhesion molecule expression on SSRBCs and SS leukocytes detached from activated HMVECs-d by RDEA119 and on activated HMVECs-d was tested by flow cytometry.

**Blood toxicity studies**

TS mice were treated daily for 14 d with vehicle or 0.1 or 0.3 mg/kg trametinib (n = 7 per treatment group). Blood samples were collected prior to treatment and 12 h following d 3, 7, and 14 treatments for hematologic analyses, including complete blood count with manual differential.

**Statistical analysis**

Data were compared using parametric analyses (Prism 5; GraphPad Software, San Diego, CA, USA), including repeated and nonrepeated measures of ANOVA. One-way and 2-way ANOVA analyses were followed by Bonferroni corrections for multiple comparisons (multiplying the P value by the number of comparisons). A value of P < 0.05 was considered significant.

**RESULTS**

**MEK inhibitor RDEA119 inhibits SSRBC adhesion to microvascular ECs at very low concentrations in vitro**

To evaluate the therapeutic utility of MEK inhibitors in SCD, we have determined the dose response of RDEA119 in inhibiting SSRBC adhesion to TNF-α–activated HMVECs-d in vitro. To attain the achievable biologic effect of the drug, we considered a drug concentration inducing 50% cell adhesion inhibition at a shear stress of 1 dyne/cm² as the effective biologic drug concentration. Sham-treated SSRCs adhered markedly to TNF-α–activated HMVECs-d under intermittent flow conditions at a shear stress of 1 dyne/cm² (Fig. 2A). However, RDEA119 at escalating concentrations ranging from 0.5 to 500 nM caused a dose-dependent inhibition of SSRBC adhesion (Fig. 2A). To inhibit adhesion of SSRCs by 50%, RDEA119 necessitated a 50% cell adhesion inhibition of 3.748 nM, suggesting that effective SSRBC adhesion inhibition in vivo may be achieved with very low doses of MEK inhibitor.

The effect of RDEA119 on adhesion of SS reticulocytes and mature SSRCs was also determined. Up to 13% of unseparated SSRCs expressed the transferrin receptor, a marker of reticulocytes. After separation, 93% of the reticulocyte-enriched cells expressed the transferrin receptor, whereas the mature SSRCs reacted with the anti-transferrin receptor antibody no more strongly than with the negative control Ig. SS reticulocytes and mature SSRCs adhered markedly to TNF-α–activated HMVECs-d, and RDEA119 inhibited adhesion of both cell populations (P < 0.001; Fig. 2B). However, no more than 10% of unseparated normal RBCs and normal reticulocytes adhered to TNF-α–activated HMVECs-d at a shear stress of 1 dyne/cm² (Fig. 2B). In normal reticulocytes, ERK1/2 was not phosphorylated, and we did not detect increased ERK1/2 phosphorylation by epinephrine, a stimulator of ERK1/2 activation (Fig. 2C, D). These data confirm that ERK1/2 activity is preserved in immature and mature SSRCs (5), but not in normal reticulocytes or normal RBCs (5), and suggest that RDEA119 can affect adhesion of both SS reticulocytes and mature SSRCs.

**MEK inhibitors prevent human SSRBC adhesion and precipitation of vasooclusion in nude mice in vivo**

Vasooclusion in response to inflammation is one of the major pathophysiologic processes in SCD. These experiments were designed to specifically study the effect of MEK inhibitors on human SSRBC adhesion in an intact vasculature, in the context of physiologic blood flow and shear stresses in vivo, and in the presence of an acute inflammation. In addition, our animal model allows studying the effect of these compounds on human SSRBC adhesion where vessels were noninstrumental and contralateral to the window chamber. Our human SSRBC preparations contained 1.05 ± 0.03 × 10⁶/μL RBCs, very low levels of contamination with leukocytes (0.3 ± 0.1 × 10⁵/μL) in some of the samples tested, and no contamination with platelets (0 cells/μL), making it unlikely that human platelets and the low numbers of leukocytes could affect SSRBC adhesion in our studies. In addition, the quantity of human RBCs infused into animals never exceeds 10% of the total circulating RBCs, assuming that the mouse blood volume is 1.5 ml, thereby minimizing any possible rheologic effects attributable to increased Hct (21). RBCs in these small concentrations should also not influence vascular regulatory mechanisms or O₂ delivery.
We first assessed whether the MEK inhibitors, U0126, RDEA119, and trametinib, prevent precipitation and/or progression of a vasoocclusive event in TNF-α-treated nude mice in vivo (Fig. 1, protocol 1). Continuous intravital microscopy observations of the microvasculature of nude mice immediately after human SSRBC infusion and for a period of 2 h showed extensive SSRBC adherence to inflamed vessels with a mean diameter of 43 ± 4.5 μm (Fig. 3A, C, D). Human SSRBC adhesion occurred within 5 min following RBC infusion, occluding microvessels with evident blood stasis, which was persistent for the whole period of the intravital microscopy (Fig. 3A, C, D). In contrast, normal RBCs showed almost no adhesion to TNF-α-activated ECs (Fig. 3B).

Treatment of nude mice with vehicle failed to inhibit human SSRBC adhesion, which was observed in 77 ± 4.6% of vessels with a mean diameter of 41 ± 2.9 μm (Fig. 3A, C, D), obstructing 55% of vessels recorded (Fig. 3E, F). In sharp contrast, U0126 at 0.2 mg/kg had marked anti-SSRBC adhesive activity (Fig. 3A, C, D). Human SSRBC adhesion occurred in 38 ± 7.5% of the total vessels recorded leading to obstruction of only 17% of the vessels (P < 0.001) (Fig. 3E, F). U0126 at 2 mg/kg had a stronger effect than that of 0.2 mg/kg U0126 on SSRBC adhesion, which was reduced by 90% (P < 0.0001) (Fig. 3A, C, D), and occurred in vessels with a mean diameter of 25 ± 3.3 μm. As a result, blood flow was restored in 83 ± 4.8% of total vessels recorded (P < 0.001; Fig. 3E, F).

RDEA119 and trametinib also had potent inhibitory activity on human SSRBC adhesion and vasoocclusion compared with vehicle-treated nude mice (Fig. 3A, C, D).
Figure 3. MEK inhibitors reduce human SSRBC adhesion and vasoocclusion in nude mice in vivo. Anesthetized TNF-α–treated nude mice with dorsal skin-fold window chamber implants were treated with vehicle, U0126 at 0.2 or 2 mg/kg, RDEA119 at 0.2 or 2 mg/kg, or trametinib at 0.3 or 1 mg/kg before infusion of fluorescence-labeled human RBCs (protocol 1). A, B) Representative images of postcapillary venules (20× magnification) from the nontreated and treated nude mice are presented. Vessels without adherent cells appear gray, due to the rapidly moving fluorescence-labeled RBCs. Adhesion of human SSRBCs in inflamed vessels and vasoocclusion are indicated with arrows. A) Animals nontreated or treated with vehicle showed marked human SSRBC adhesion and vasoocclusion, whereas treatment of animals with 0.2 and 2 mg/kg U0126, 0.2 or 2 mg/kg (continued on next page)
In 0.2 mg/kg RDEA119-treated animals, adherent SSRBCs occupied 33 ± 6.6% of the total vessels recorded with a mean diameter of 28 ± 5 μm (P < 0.0001) (Fig. 5D), and obstructing only 5% of total recorded vessels (P < 0.0001) (Fig. 3E, F). RDEA119 at 2 mg/kg also reduced by 89% SSRBC adhesion (mean vessel diameter, 27 ± 3.7 μm) compared with vehicle-treated animals (P < 0.0001) (Fig. 3A, C, D). As a result, blood flow was restored in 76 ± 6.2% of the vessels compared with vehicle-treated mice showing 45 ± 4.9% of vessels with blood flow (P < 0.001) (Fig. 3E, F). Trametinib at 0.3 and 1 mg/kg inhibited SSRBC adhesion by 93 and 96%, respectively, compared with vehicle treatment (P < 0.0001 for each 0.3 and 1 mg/kg trametinib) (Fig. 3A, C, D). Trametinib at 0.3 and 1 mg/kg reestablished blood flow in 77 ± 8.6% and 98.5 ± 1.2% of total vessels recorded, respectively (Fig. 3F). These data strongly suggest that MEK inhibitors can prevent inflammation from precipitating and/or exacerbating vasoocclusion by at least down-regulating human SSRBC adhesive function via MEK-dependent ERK1/2 signaling inhibition.

MEK inhibitor RDEA119 reduces human SSRBC organ trapping in nude mice in vivo

We examined the effect of RDEA119 on SSRBC sequestration in the lungs, kidneys, spleen, and liver, typically damaged in SCD. Human SSRBC trapping was extensive in the lungs, liver, and spleen of vehicle-treated nude mice (Fig. 4A, B). However, injection of 0.2 mg/kg RDEA119 significantly decreased SSRBC sequestration in those organs. Human SSRBC trapping in the kidneys was similar in vehicle-treated and RDEA119-treated nude mice (Fig. 4A, B).

RDEA119 at 0.2 mg/kg also increased the percentage of human SSRBCs circulating in the microvasculature of nude mice as opposed to vehicle treatment (Fig. 4C). The percentage of circulating human SSRBCs following RDEA119 treatment was 2.5-fold higher 2 h after human SSRBC infusion compared with vehicle treatment (P = 0.0189). The increased number of SSRBCs in the bloodstream of animals reflects the fact that these SSRBCs did not adhere to ECs and were not sequestered in organs, and suggests that this may lead to alleviation of organ damage in SCD.

MEK inhibitors reverse human SSRBC adhesion in nude mice in vivo

The ability of RDEA119 and trametinib to reverse human SSRBC adhesion and established vasoocclusion in nude mice was assessed. To accurately evaluate the effect of the drugs on established vasoocclusion, we compared the intensity of cell adherence and vasoocclusion in the exact same vessel segments prior to and following drug administration. Using protocol 2 (Fig. 1) and once again, before drug infusion, SSRBCs adhered avidly to vessels promoting vasoocclusion with apparent blood stasis (Fig. 5A, C, F–I, and Supplemental Movies 1 and 2). Vehicle treatment (Post-vehicle) of animals had no effect on SSRBC adhesion (Fig. 5B, F, G and Supplemental Movie 3). Conversely, SSRBC adhesion and vasoocclusion were reduced within 5 min of 1 mg/kg RDEA119 (Post-RDEA119) infusion into animals and progressively decreased thereafter (Fig. 5D–F and Supplemental Movie 4) compared with before drug administration (P < 0.0001) (Fig. 5C, F). SSRBC adhesion and the percentage of vessels occupied by adherent cells were reduced by 75 and 72%, respectively, compared with before RDEA119 treatment (P < 0.001; Fig. 5F, G). This led to restored blood flow in 50% of the vessels (P < 0.001 for 15–60 min post-RDEA119 infusion vs. prior to RDEA119 infusion) (Fig. 5H, I). Human SSRBC adhesion and established vasoocclusion in nude mice were affected similarly by administration of 2 mg/kg, i.e., RDEA119 and 0.3 and 1 mg/kg, i.v. trametinib (data not shown).

Using protocol 3 (Fig. 1), treatment by oral gavage with trametinib also reversed SSRBC adhesion and vasoocclusion with average observation times of 4 h (Fig. 6). In vehicle-treated nude mice, SSRBC adhesion and vasoocclusion increased over time when comparing the same vessel locations (Fig. 6, A1 vs. A2 and A3 vs. A4), and after 210 min, SSRBC adhesion and vasoocclusion were dramatic (Fig. 6, A5). In contrast, SSRBC adhesion and vasoocclusion were reduced progressively over time in 0.3 mg/kg trametinib-treated animals (Fig. 6, B1 vs. B2 and B3 vs. B4), and 210 min later, SSRBC adhered poorly to inflamed vessels (Fig. 6, B5). Trametinib at 1 mg/kg had a much stronger inhibitory effect than at 0.3 mg/kg. SSRBC adhesion and vasoocclusion were all but absent within the first 30 min following drug administration. The effect of the drug was sustained over time (Fig. 6, C1 vs. C2 and C3 vs. C4). SSRBC adhesion was decreased by 65 and 87% in 0.3 and 1 mg/kg trametinib-treated mice, respectively, compared with vehicle-treated animals (n = 4; P < 0.01) (Fig. 6D). Trametinib at 0.3 and 1 mg/kg reestablished blood flow in 95% of total occluded vessels and all vessels, respectively (Fig. 6F). These data suggest that MEK inhibitors reverse existing vasoocclusion by at least affecting SSRBC adhesion.

MEK inhibitors reverse adhesion of activated leukocytes after the inflammatory trigger of vasoocclusion in sickle mice in vivo

In sickle mice, RBCs bind to adherent leukocytes in inflamed venules producing vasoocclusion of cremasteric
vessels (3). We therefore evaluated the therapeutic benefits of RDEA119 and trametinib in reversing activated leukocyte adhesion in sickle mice, by infusing the MEK inhibitor 120 min after TNF-α injection (Fig. 1, protocol 4), a time at which leukocytes have already been recruited and adhered, and an acute vasoocclusive crisis is established. Leukocyte adherence in inflamed vessels was visualized in vehicle-treated TS mice promoting vasoocclusion (Fig. 7A). However, 0.025, 0.05, and 0.1 mg/kg RDEA119 reversed leukocyte adhesion to activated ECs, which was reduced by 73, 99, and 97%, respectively ($P < 0.001$; Fig. 7A). Leukocyte adhesion was abrogated within the first 10 min of 0.025, 0.05, and 0.1 mg/kg RDEA119 administration compared with vehicle treatment ($P < 0.05$), and adhesion further decreased thereafter ($P < 0.05$ regardless of the time following drug administration; Fig. 7B).

RBCs also adhered markedly in vehicle-treated animals (Fig. 7C). RBC adherence occurred in 41 ± 12.4% of small inflamed vessels with a diameter ≤25 μm, which led thereafter to blood stasis in larger vessels (diameter up to 50 μm). In contrast, 0.025, 0.05, and 0.1 mg/kg RDEA119 reversed RBC adhesion, which was inhibited by 76, 99, and 98%, respectively ($P < 0.0001$ regardless of the dose of RDEA119; Fig. 7C). RDEA119 even at the lowest dose (0.025 mg/kg) reversed RBC adhesion within the first 10 min of drug administration compared with vehicle ($P < 0.05$), and this effect was sustained over time (Fig. 7D). Reversal of adhesion of both leukocytes and RBCs led to restored blood flow in 57% of vessels recorded ($P < 0.05$ for each RDEA119 dose) (Fig. 7D). Adhesion of leukocytes (Fig. 7F) and RBCs (Fig. 7G), and established vasocclusion, were also reversed by 0.05 mg/kg trametinib. This led to 88 ($P < 0.001$) and 71% ($P < 0.01$) reduction in adhesion of leukocytes and RBCs, respectively (Fig. 7F, G). Together, our in vivo data suggest that the antiadhesive activity of MEK inhibitors on RBC-endothelium, leukocyte-endothelium and RBC-leukocyte interactions in both nude and sickle mice was rapid and persistent, and these compounds may have potential therapeutic benefits in reducing acute vasoocclusive crises in SCD.
Figure 5. Intravenous RDEA119 reverses human SSRBC adhesion after onset of vasoocclusion in nude mice in vivo. Anesthetized TNF-α–treated nude mice with dorsal skin-fold window chamber implants were treated with vehicle or 1 mg/kg RDEA119 as per protocol 2. (A–E) Representative images of postcapillary venules (×20 magnification) from nude mice immediately after infusion of fluorescence-labeled human SSRBCs and before (A) treatment with vehicle (before vehicle infusion) and (C) RDEA119 (before RDEA119 infusion) and after (B) treatment with vehicle (postvehicle) and (D, E) 1 mg/kg RDEA119 (post-RDEA119) are presented. The same vessel segments are presented in (A) (before vehicle infusion) and (B) (27 min postvehicle infusion)
MEK inhibitors reverse adhesion of SS leukocytes and SSRBCs to microvascular ECs via shedding of endothelial and leukocyte adhesion molecules

SSRBCs adhere to endothelium via Landsteiner-Wiener blood group antigen (intercellular adhesion molecule 4) (LW-αβ3 and CD44-CD44 interactions (4, 22) and to endothelial P-selectin (23). Leukocytes bind via β2 integrin and CD44 to endothelium, and ECs involve E-selectin, P-selectin, αβ3 integrin, CD44, or fibronectin to interact with leukocytes (15, 24–27). We therefore examined the expression of these adhesion molecules on HMVECs-d, SS leukocytes, and SSRBCs after reversal of adhesion by RDEA119. Activated HMVECs-d RDEA119 treated and activated HMVECs-d vehicle treated expressed E-selectin, P-selectin, CD44, αβ3, and fibronectin similarly (Fig. 8A). However, exposure of activated HMVECs-d to SSRBCs or leukocytes, followed by RDEA119 treatment, decreased endothelial E-selectin, P-selectin, and αβ3 expression with no change in endothelial CD44 and fibronectin expression compared with activated HMVECs-d RDEA119 treated (Fig. 8A). It is noteworthy that the cleaved endothelial E-selectin, P-selectin, and αβ3 were found bound to SS leukocytes detached from the endothelium, along with a decrease in leukocyte CD44 and β2 expression (Fig. 8B). In contrast, no changes in SSRBC CD44 and LW expression and no endothelial adhesion molecules were detected on SSRBCs (Fig. 8C). These data suggest that detachment of SSRBCs and SS leukocytes from the endothelium by MEK inhibitor causes shedding of endothelial E-selectin, P-selectin, and αβ3 integrin, and leukocyte CD44 and β2 integrin but does not affect SSRBCs.

The safety of trametinib in sickle mice in vivo

The toxicity on Hb and platelets of trametinib in sickle mice was assessed because 1) patients with SCD are anemic; 2) a daily dose of 2.5 mg trametinib for 28 d can cause thrombocytopenia in patients with cancer, although a daily dose of 100 mg RDEA119 had no effect on platelets; 3) trametinib is a more potent inhibitor than RDEA119; and 4) trametinib is already approved by the U.S. Food and Drug Administration (28–32). During hospital care, an acute vasoocclusive crisis usually subsides within 7–10 d. We examined the effects on Hb and platelet counts of trametinib at 0.1 and 0.3 mg/kg when given daily for 14 d to sickle mice. Trametinib at 0.1 and 0.5 mg/kg had a similar and no real effect on Hb in TS mice over time compared with vehicle treatment (n = 7 per treatment group; Fig. 9A). Platelet count was in the normal range in TS mice before treatment and remained normal over the period of 14 d treatment with either vehicle or trametinib at 0.1 or 0.3 mg/kg (Fig. 9B). These data suggest that 0.3 mg/kg trametinib is well tolerated because it did not exacerbate anemia or cause thrombocytopenia in sickle mice.

DISCUSSION

In SCD, acute vasoocclusive episodes remain a major scientific obstacle that calls for new therapeutic approaches. We now propose a novel potential therapeutic alternative that is effective in treating acute vasoocclusive crises in vivo. We provide a proof of principle that existing MEK inhibitors reverse adhesion of both sickle cells and leukocytes, and vasoocclusion triggered by inflammation, and restore blood flow in 2 mouse models of an acute sickle cell vasoocclusive crisis.

MEK inhibitors prevent precipitation and/or progression of vasoocclusion in nude mice in vivo

Numerous mechanisms of adhesion of SSRBCs and leukocytes to the vascular endothelium and vasoocclusion have been demonstrated in in vitro and ex vivo models, but there are only limited data regarding the contribution of specific factors and signaling mechanisms in SSRBCs initiating or inducing those interactions in vivo (4, 8, 15). We have recently shown that MEK-dependent ERK1/2 signaling in SSRBCs plays a key role in adherence of these cells to the vascular endothelium in vitro and in vivo and in the ability of SSRBCs to activate neutrophil adhesion (8). Our data now reveal a potential therapeutic benefit of MEK inhibitors in SCD evidenced by the ability of these compounds to prevent the precipitation and/or progression of a vasoocclusive process. We provide functional evidence that treatment of nude mice with MEK inhibitors inhibits human SSRBC adhesion to inflamed venules and vasoocclusion in vivo. Our in vitro data further show that MEK inhibitors affect adhesion of both SS reticulocytes and mature SSRBCs. Vehicle, however, showed no effect on SSRBC adhesion in vivo, which occurred both in inflamed vessels, predominantly in small and large postcapillary venules promoting vasoocclusion, and in arterioles in nude mice (33). In contrast, normal RBCs and normal reticulocytes did not adhere to activated microvascular ECs in vitro and/or inflamed vessels in vivo (Fig. 3). This might be explained by the fact that the kinase ERK1/2 was never found active in either normal reticulocytes (Fig. 2) or total normal RBCs (5). Our data also suggest that human SSRBC adhesion and vasoocclusion were not due to...
Figure 6. Oral trametinib reverses human SSRBC adhesion and established vasoocclusion in nude mice in vivo. Anesthetized TNF-α–treated nude mice with dorsal skin-fold window chamber implants were treated with vehicle or 0.3 or 1 mg/kg trametinib by oral gavage as per protocol 3. A1–C4) Representative images of postcapillary venules (20X magnification) from nude mice 30 min after treatment with vehicle (A1–A5), 0.3 mg/kg trametinib (B1–B5), and 1 mg/kg trametinib (C1–C4) are presented. The same vessel segments are presented in (A1) and (A2) and in (A3) and (A4) for vehicle-treated mice, in (B1) and (B2) and in (C1) and (C2) for 0.3 mg/kg trametinib treated mice, and in (C3) and (C4) for 1 mg/kg trametinib treated mice. (continued on next page)
nonspecific binding caused by the large size of human RBCs relative to the size of murine RBCs because normal human RBCs failed to both adhere to the endothelium and induce vasoocclusion in nude mice in vivo (Fig. 3B) (4, 8). Our previous data have also shown that in the absence of inflammation, murine sickle RBCs do not promote vasoocclusion in the nude mouse model (4).

In our animal model, administration of TNF-α to nude mice before SSRBC infusion can trigger activation of both murine neutrophils and platelets (34) and binding of neutrophils to both the endothelium (3) and platelets (35). Therefore, it is possible that SSRBCs adhered to activated ECs, leukocytes, and platelets promoting vasoocclusion, and MEK inhibitors affected all these SSRBC cell-cell interactions, and subsequent vasoocclusion. Interspecies RBC-EC and RBC-leukocyte interactions have been shown to exist. Human SSRBCs can adhere to human, murine, and rodent microvascular ECs in vitro, ex vivo, and in in vivo animal models, and murine leukocytes in in vivo animal models (4, 8, 36).

**MEK inhibitors reverse adhesion of sickle RBCs in both nude mice and sickle mice in vivo**

In addition to their preventive action on the progression of vasoocclusion, the MEK inhibitors, RDEA119 and trametinib, given intravenously or orally were effective in reversing adhesion of both human and murine sickle RBCs, and established vasoocclusion, and restored blood flow in a large number of occluded vessels. In our system, the action of RDEA119 and trametinib on sickle cell adhesion and established vasoocclusion was rapid and efficient because these drugs reversed human SSRBC and murine RBC adhesion within the first 5–30 min of drug administration and at very low doses. Decreased RBC adhesion was sustained over time in vivo (37). In nude and sickle mice, MEK inhibitors likely affect the adhesive interactions of SSRBCs with both the vascular endothelium and adherent leukocyte. We further surmise that in SCD, MEK inhibitors may also inhibit activation of ECs by down-regulating MEK-dependent ERK1/2 signaling. These inhibitors may in addition alleviate vascular and organ injuries both at the site of SSRBC-induced vasoocclusion and in organs because these drugs reduced cell adhesion in the microvasculature and SSRBC organ sequestration. MEK inhibitors have been shown to reduce apoptosis and inflammation: they inhibit activation of caspase-3 and proinflammatory cytokine production such as TNF-α and IL-1β; reduce organ injury; and enhance tissue resistance to ischemic injury in other animal models (12, 13, 30, 38, 39).

**MEK inhibitors reverse adhesion of leukocytes in sickle mice in vivo**

There is a growing body of evidence that activated neutrophils (8, 40), activated monocytes (41), and lymphocytes (15) adhere to the endothelium and contribute to the vasoocclusive processes in SCD (3). Turhan et al. (3) have shown that treatment of sickle mice with TNF-α caused adhesion of activated leukocytes to inflamed vessels, initiating a vasoocclusive event in the cremasteric vessels in vivo. Leukocyte recruitment and adhesion to activated ECs are an extremely dynamic process in which most adherent leukocytes remain adherent, some continuously crawl along the venular endothelium, whereas others detach to return in the circulation. Extravasated leukocytes account for a relatively minor subset of leukocytes that have adhered. Long after the inflammatory challenge was initiated and occurrence of vasoocclusion, MEK inhibitors not only attenuated RBC adhesion in sickle mice but also efficiently reduced the number of adherent leukocytes, which led to improved blood flow. The reversal effect of MEK inhibitors on leukocyte adhesion was similar to the drug’s effect on RBCs and was rapid and efficient. These inhibitory compounds reversed leukocyte adhesion at very low doses and within the first 10–30 min of drug administration, and leukocyte adhesion further decreased over time. Thus, MEK-dependent ERK1/2 signaling activation in leukocytes also plays a crucial role in their recruitment and adherence to the vascular endothelium and initiation of vasoocclusion in sickle mice in vivo. Studies have found that the MEK-dependent ERK1/2 pathway in neutrophils is essential in the regulation of their adhesive responses to endothelin-1, chemoattractants, arachidonic acid, or peroxynitrite (9, 42–45). ERK1/2 in neutrophils seems to have a specific, nonmitotic signaling function (43, 46, 47). Together, our studies highlight the importance of MEK-dependent ERK1/2 signaling in adhesion of at least SSRBCs and leukocytes, and the dynamic nature and potential reversibility of adhesion of these cells after targeted intervention with MEK inhibitors under the conditions studied herein. We further show for the first time that MEK inhibitors cause detachment of human SS leukocytes and human SSRBCs from activated vascular ECs in vitro via shedding of at least the endothelial adhesion molecules E-selectin, P-selectin, and αvβ3 integrin, which surprisingly were found bound to SS leukocytes, but not to SSRBCs. This phenomenon was accompanied by leukocyte CD44 and B2 integrin cleavage but, and as expected, did not affect RBC adhesion molecule expression. Although cleavage of adhesion molecules from leukocyte and cytokine-activated ECs has been

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(B3) and (B4) for 0.3 mg/kg trametinib-treated mice, and in (CI) and (C2) and in (C3) and (C4) for 1 mg/kg trametinib-treated mice. Human SSRBC adhesion and vasoocclusion are indicated with arrows. Human SSRBCs adhered markedly to vessel walls, and adhesion increased progressively over time promoting vasoocclusion in vehicle-treated animals. Treatment of animals with 0.3 mg/kg trametinib diminished SSRBC adhesion and vasoocclusion over time. Trametinib at 1 mg/kg had a stronger effect than the lower dose (0.3 mg/kg) and reversed human SSRBC adhesion and vasoocclusion within the first 30 min of drug administration. Scale bars, 50 µm. D and E) Still images showing vessel segments of animals were used to quantify fluorescence-labeled human SSRBC adhesion in venules and arterioles recorded, and blood flow, FU, fluorescence unit. The values of at least 60 segments of vessels were analyzed and averaged among groups of animals. Error bars show SEM of 4 different experiments for each treatment group. *P < 0.01 compared with vehicle treatment and regardless of the vessel diameter within the ranges specified.
Figure 7. MEK inhibitor administration after the inflammatory challenge reverses adhesion of murine sickle RBCs and leukocytes and vasoocclusion in TS mice in vivo. Anesthetized TS mice were treated with 0.025, 0.05, or 0.1 mg/kg RDEA119, 0.05 mg/kg trametinib, or vehicle as per protocol 4. A–G) Still images of vessel and arteriole segments of animals were used to quantify adhesion of fluorescence-labeled murine RBCs and leukocytes [fluorescence unit (FU)] and percentage of occluded vessels. Error bars show SEM of 4 different experiments for each treatment group. A and C) *P < 0.0001 compared with vehicle-treated animals regardless of the vessel diameter. B) Adhesion of leukocytes (FU) presented as a function of time. *P < 0.05 compared with vehicle-treated animals.) D) Adhesion of murine RBCs (FU) presented as a function of time. *P < 0.01 compared with vehicle-treated animals. E) Data are presented as percent (%) of occluded vessels. *P < 0.05 compared with vehicle-treated animals regardless of the vessel diameter. F, G) *P < 0.001 and *P < 0.01, respectively, compared to vehicle-treated animals regardless of the vessel diameter.
described to occur by anti-inflammatory drug-related compounds (48, 49), in our system, SS leukocyte and SSRBC separation from the endothelium may implicate MEK/ERK1/2 signaling in regulating cleavage of endothelial E-selectin, P-selectin, and αvβ3, and leukocyte β2 and CD44, when these proteins are engaged in firm cell-cell binding. Also, this may hinder SSRBCs and SS leukocytes from readhering to ECs and SSRBC-leukocyte from interacting.

Although the sequence of events leading to suddenly symptomatic vasoocclusion in humans with SCD is still poorly understood, the fact that MEK inhibitor-targeted therapy leads to reversibility of SSRBC and leukocyte adhesion triggered by inflammation in the face of both such a severe sickle mouse model and in nude mouse model of vasoocclusion portends for at least the potential clinical relevance of such intervention. Our data also argue that existing MEK inhibitors may be safe compounds if used acutely in patients with SCD to treat vasoocclusion because daily administration for 14 d of trametinib used at a dose 12-fold higher than the lowest effective dose of RDEA119 (28, 30) did not exacerbate anemia or cause thrombocytopenia in sickle mice. Additionally, shorter-term use (≤7 d) of these drugs during acute pain crises in SCD should minimize any drug-associated side effects.

Figure 8. MEK inhibitor reverses SSRBC and SS leukocyte adhesion via endothelial and leukocyte adhesion molecule shedding. Surface expression of adhesion molecules on ECs (A), human SS leukocytes (B), and human SSRBCs (C) 1 h after RDEA119 treatment. Washed TNF-α-activated HMVECs-d were coincubated or not with human SSRBCs or human SS leukocytes for 10 min, followed by exposure to fluid flow to wash off nonadherent cells before treatment with vehicle or the MEK inhibitor RDEA119. Washed TNF-α-activated HMVECs-d treated with vehicle served as a positive control. Data represent means ± SEM (n = 4). *P < 0.05 vs. RDEA119-treated activated HMVECs-d (A); *P < 0.05 vs. SS leukocytes (B).
Finally, whereas MEK inhibitors have been suggested to treat a diverse range of pathologies, this is the first report showing that these drugs can reverse adhesion of both SSRBCs and leukocytes in 2 different models of an acute vasoocclusive crisis. Thus, existing MEK inhibitors, including RDEA119 and trametinib, may be used as a potential therapeutic approach for treating acute vasoocclusive episodes in SCD.

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