Vascular endothelial hyperpermeability induces the clinical symptoms of Clarkson disease (the systemic capillary leak syndrome)

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Abbreviations: SCLS-Systemic Capillary Leak Syndrome; VEGF-vascular endothelial growth factor; Ang-Angiopoietin; VE-cadherin-vascular endothelial cadherin; HMVEC-human microvascular endothelial cells; Tie2-TEK tyrosine kinase, TEER-transendothelial electrical resistance.

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Abstract

The systemic capillary leak syndrome (SCLS) is an extremely rare disorder characterized by transient episodes of hypotensive shock and anasarca thought to arise from reversible microvascular barrier dysfunction. Although the high prevalence of a monoclonal gammopathy of unknown significance (MGUS) in SCLS suggests a pathogenic contribution of endogenous immunoglobulins, the mechanisms of vascular hyperpermeability remain obscure. Herein, we report clinical and molecular findings on 23 subjects, the largest SCLS case series to date. Application of episodic SCLS sera, but neither the purified immunoglobulin fraction nor sera obtained from subjects during remission, to human microvascular endothelial cells caused vascular endothelial cadherin (VE-cadherin) internalization, disruption of inter-endothelial junctions, actin stress fiber formation, and increased permeability in complementary functional assays without inducing endothelial apoptosis. Intravenous immunoglobulin (IVIG), one promising therapy for SCLS, mitigated the permeability effects of episodic sera directly. Consistent with the presence of endogenous, non-immunoglobulin, circulating permeability factor(s) constrained to SCLS episodes, we found that two such proteins, vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang2), were elevated in episodic SCLS sera but not in remission sera. Antibody-based inhibition of Ang2 counteracted permeability induced by episodic SCLS sera. Comparable experiments with anti-VEGF antibody (bevacizumab) yielded less interpretable results, likely due to endothelial toxicity of VEGF withdrawal. Our results support a model of SCLS pathogenesis in which non-immunoglobulin humoral factors such as VEGF and Ang2 contribute to transient endothelial contraction, suggesting a molecular mechanism for this highly lethal disorder.

Introduction

In 1960, Dr. Bayard Clarkson described a patient who experienced sporadic bouts of hypovolemia, hypotension, and edema. SCLS, also called Clarkson syndrome, is now known as a disorder of unknown etiology characterized by transient but severe hypotension resulting in vascular collapse and shock, hemoconcentration, and ultimately anasarca due to accumulation of fluids and macromolecules (up to 900 kDa) in tissues. The most typical presenting signs are the triad of hypotension, elevated hemoglobin and hematocrit, and hypoalbuminemia. The
symptoms reverse almost as quickly as they arise, with massive fluid re-mobilization from tissues into circulation, resulting in diuresis.

The most common treatment modality during episodes is judicious use of intravenous fluids and vasopressors to maintain perfusion to the brain and other vital organs. Although no more than one hundred cases of SCLS were reported in the literature from 1960-2006, the nonspecific nature of the presenting signs and symptoms and high mortality rate during episodes may have resulted in considerable under-diagnosis. Fifty new cases of SCLS were reported from 2006-2011, suggesting that there may be increased awareness of this disorder. The 5-year survival rate is approximately 75%, and deaths are most commonly related to acute SCLS events.

A monoclonal gammopathy of unknown significance (MGUS), typically of the IgG class, is present in a large majority of SCLS cases. While paraprotein levels in SCLS are uniformly below 1 g/dL, recent case reports of symptom resolution following treatment of the underlying plasma cell dyscrasia and a small cohort study demonstrating efficacy of intravenous immunoglobulin administration for prevention of SCLS episodes have suggested a pathogenic role for the monoclonal IgG in the recurrent episodes of vascular leakage. Although early studies using serial measurements of infused radiolabeled albumin established the link between marked, but transient vascular hyperpermeability and the clinical manifestations of SCLS episodes, little is known about the molecular events leading to the episodic hyperpermeability of SCLS. The only molecular clues come from the original description by Clarkson, who reported that plasma drawn during an episode from an index case induced a shock-like syndrome when injected into rats and contained heparin-precipitable protein.

One such heparin-precipitable protein, VEGF, was reported in 1983, and at that time this protein was named “vascular permeability factor” for its ability to induce rapid leakage from
blood vessels. VEGF is secreted by a variety of cells including fibroblasts, keratinocytes, and mast cells, and binds receptor tyrosine kinases expressed on the surface of vascular endothelial cells. An analogous endothelial pathway regulating vascular barrier function, the Angiopoietin-Tie2 signaling axis, was first described in 1996. While studies in rodent and cell culture models have clarified the mechanisms by which VEGF and Angiopoietins regulate permeability, the importance of these molecules in human disorders of vascular leakage has only recently been appreciated with the introduction of neutralizing biotherapeutic agents.

Previous mechanistic studies on SCLS have been limited for two reasons—1) the rarity of the condition, resulting in experiments performed on only 1-2 subjects, and 2) limited prior efforts to adapt cellular models of endothelial barrier function for use with SCLS biological material. Here, we assembled and studied blood samples from 20 subjects who met the criteria for “classic acute” SCLS and 3 subjects classified as “chronic” SCLS. In a subset of patients, we were also able to capture blood samples at or near the onset of their episode, including serial samples collected daily over a one-week period in one patient. Using these materials, we performed studies on the functional and structural effects SCLS sera exert on human microvascular endothelial cells (HMVEC) and measured levels of candidate permeability mediators. We studied the barrier-defending effect of a standard SCLS treatment, IVIG, and evaluated the potential benefits of inhibiting specific factors (VEGF and Ang2).

Methods

Subjects

Patients were classified according to established criteria by at least one episode of reversible hypotension, hemoconcentration, and hypoalbuminemia, or chronic edema and
hypoalbuminemia in the absence of secondary causes\textsuperscript{4, 5}. Although a total of 23 patients were evaluated (Tables 1-2), experimental studies of sera from the 3 “chronic” SCLS patients were not included for the sake of uniformity. Experiments on paired basal and episodic sera (Figs. 1 and 6) are labeled with the patient number designated in Tables 1-2. All patients were seen at the Clinical Center of the National Institutes of Health during an asymptomatic period. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki as reflected in a priori approval from the Institutional Review Board of the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIH). Age-, gender-, and race-matched serum and plasma samples were obtained from the NIH Blood Bank.

**Reagents and cells**

Human adult dermal microvascular endothelial cells (HMVEC) were purchased from Lonza (cat# CC-2811) or obtained from the CVBR Core Facility at Beth Israel Deaconess Medical Center and cultured in endothelial growth medium EGM-2 containing growth factors per the manufacturer’s instructions. Caspase-Glo 3/7 assay system was purchased from Promega. Recombinant human TNF\textalpha was purchased from PeproTech and staurosporine from Sigma. The Lab-TekII Chamber Slide System coated with CC2 was obtained from Nalge Nunc International. AnnexinV-FITC fluorescence microscopy kit was purchased from BD Biosciences. Bevacizumab (Avastin) was obtained from Genentech/Roche; IVIG (Gamunex) was from Talecris; recombinant human VEGF-165 and Tie2-Fc were from R & D Systems.
Caspase-Glo 3/7 apoptosis assay

HMVEC were seeded in 96-well plates overnight in EGM-2 medium. Cells were starved for 6 h in EBM-2 basal medium without growth factors followed by incubation with 10% serum or apoptosis-inducing reagents in basal medium overnight. In some experiments, cells were incubated with medium containing both test sera and TNFα (1.25 ng/ml). Caspase 3/7 activity was assayed using the Caspase-Glo 3/7 luciferase system according to the manufacturer’s guidelines. Luciferase activity was measured using POLARstar Optima luminescence plate reader (BMG LabTech, Offenburg, Germany).

Annexin V staining

HMVEC were seeded in Chamberwell slides for 24 h in EGM-2 medium, followed by incubation with basal EBM-2 medium containing 10% test serum or reagents (staurosporine) overnight. Cells were then stained with Annexin V-FITC according to the manufacturer’s protocol, fixed in Annexin V buffer containing 1% paraformaldehyde, and mounted with glass coverslips using ProLong Gold anti-fade reagent with DAPI (Invitrogen). Images were collected on a Leica SP5 inverted confocal microscope (Leica Microsystems).

ELISA

Serum or plasma cytokine levels were determined using Quantikine ELISA kits (R & D systems) according to the manufacturer’s protocols. Absorbance was determined using a GENios plate reader at 450 nm using a wavelength of 550 nm as reference.
IgG purification

Total IgG was purified from individual patient serum samples using the Melon Gel IgG Purification Kit (Thermo Scientific) and pooled prior to use in functional assays. Purity was evaluated by SDS-PAGE and Coomassie blue staining, and concentration was calculated based on measurement of OD$_{260}$ and comparison of values obtained to a standard curve generated using bovine serum albumin (BSA) of known concentrations.

Transwell permeability assay

Confluent HMVEC monolayers were grown on collagen-1 coated Costar Transwell membranes (polyester 0.4 µm filter, Corning) and permeability determined by measurement of fluorometric signal in the luminal and abluminal chambers at the indicated time points after luminal addition of 1 mg/ml FITC-labeled human serum albumin (Sigma) as described previously$^{14}$. Relative fluorescence units (RFUs) were used in the following equation to determine the permeability coefficient of albumin ($P_a$):

$$ P_a/hr = \frac{[A]}{[L]} \times \frac{V}{tA} $$

where [A] is abluminal concentration; [L] is luminal concentration; V is volume of abluminal chamber; t is time in hr; A is area of membrane in cm$^2$.

Transendothelial electrical resistance assays

Raw resistance (transendothelial electrical resistance, TEER) values across HMVEC monolayers were recorded at 4000 Hertz (Hz) amplitude using an electrical cell-substrate impedance sensing system (ECIS) (Applied BioPhysics, Inc.). A cut-off resistance of 1500 ohms indicative of confluency was required prior to commencing an experiment. Human subject serum (5% final vol/vol concentration) was added at time zero to confluent cells in basal medium containing 1%
fetal bovine serum (FBS). Serial changes in TEER were recorded thereafter over a period of several hours. To enable comparisons across experiments, raw resistance values were divided by the mean time-zero raw resistance for the given experimental run. Data were generated from 3-6 replicates per condition. In experiments with IVIG (1.25 mg/ml), Avastin (10 μg/ml), or Tie-2 Fc (1 μg/ml), these reagents or equivalent concentrations of BSA or control IgG were applied to HMVECs 1 h prior to addition of episodic SCLS sera (5% final vol/vol concentration). The raw resistance value at the time of SCLS serum addition was again taken as the time-zero data point for normalizing subsequent readings. We used the normalized resistance at 2.5 hours after addition of sera for statistical analyses.

**Immunofluorescence**

HMVEC were grown to confluence on glass coverslips coated with collagen. Cells were serum starved with 1% serum-containing growth medium for 3 h prior to addition of patient serum at a 5% (vol/vol) final concentration for 2.5 h. Cells were fixed for 10 min in 2.5% paraformaldehyde and permeabilized for 5 min in PBS containing 0.2% Triton X-100. Cells were incubated overnight at 4°C in blocking buffer (PBS containing 1% BSA, 0.2% Triton X-100, sodium azide) followed by incubation with anti-VE-cadherin antibody (BD Biosciences) for 12 h. Cells were washed several times with PBS followed by incubation with DyLight 488-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson Immunoresearch) and Alexa Fluor 594-conjugated phalloidin (Invitrogen). In experiments using purified IgG, cells were incubated with purified IgG (final concentration of 300 μg/ml) in growth medium followed by fixation and immunostaining with AlexaFluor 594-conjugated anti ZO1 antibody (Invitrogen). After a second round of washing, cells were mounted onto coverslips with ProLong Gold anti-fade/DAPI. Cells
were visualized using a Zeiss LSM510 META confocal system at 63x magnification. All images were obtained using identical laser power, gain, and offset instrument settings.

**Statistical analysis**

Data were analyzed using the GraphPad Prism 5 software package. For Transwell and TEER assays, Student $t$ tests for 2 groups and 1-way ANOVA for multiple groups were used to analyze functional replicates from individual patients. Mann-Whitney or Wilcoxon tests (for pairwise comparisons) or Kruskal-Wallis and ANOVA (for multiple groups) were used for grouped cytokine analyses as non-parametric distributions were assumed due to small sample size. $P$ values $< 0.05$ were considered significant.

**Results**

**Clinical and laboratory characteristics of study subjects**

Table 1 reports patient demographics and disease characteristics, and Table 2 reports laboratory evaluations obtained at the time of study enrollment. 20/23 subjects had bona fide classic acute SCLS as defined by at least one episode meeting 3 or more of the diagnostic criteria recently established by Gousseff et al. (edema with acute weight gain of $> 1$ kg in less than one week; systolic blood pressure $< 100$ mm Hg or mean blood pressure $< 70$ mm Hg; Hgb elevation; hypoalbuminemia) in the absence of secondary causes. 61% of the subjects were male; 22 patients were Caucasian, and one patient was African-American. The median age at disease onset was 52 years (range, 41 to 67 years). 26% of patients were classified as having moderately frequent attacks ($> 2$/year), while 26% of the total cohort had frequent attacks (defined as $> 6$/yr, ranging from every other month to biweekly). 100% of the patients with classic acute SCLS
experienced at least one “severe” episode based on the Gousseff criteria\(^4\). Well-defined events occurring prior to attacks could be identified in only 22% of patients, which included seropositive influenza in one subject (two separate instances) and seropositive West Nile virus infection in another. 3 patients met the criteria for “chronic” SCLS in that they experienced non-cyclical peripheral edema and hypoalbuminemia in the absence of secondary causes of edema. 90% of the patients were receiving prophylactic treatment at the time of evaluation, including theophylline + terbutaline or IVIG. One patient died of a severe SCLS attack during the study. No patient reported a family history of SCLS.

97% of study subjects had a monoclonal gammopathy, typically IgG, with the exception of an IgA M-spike in one patient. The mean serum monoclonal IgG level was 0.36 ± 0.05 g/dL, and M-spike isotypes were characterized by kappa light chains in 61% of subjects. 32% of patients had a skewed serum free light chain ratio indicative of excess circulating free light chains. In the vast majority of patients, routine laboratory evaluations including Hgb/Hct, albumin, C-reactive protein, erythrocyte sedimentation rate, C1 esterase inhibitor level and function, tryptase, and complement components were within the normal ranges at the time of their initial evaluation during remission.

**SCLS serum induces endothelial permeability in vitro**

Although there have been major assumptions made regarding the etiology of SCLS, there has been no prior demonstration that this disease actually results directly from endothelial hyper-permeability. We hypothesized that SCLS serum would increase microvascular endothelial barrier dysfunction and permeability. To test this, we applied serum obtained from a subject during either an acute attack (episodic) or a quiescent period (basal) to confluent primary human
microvascular endothelial cells (HMVEC) and measured macromolecule flux and electrical resistance across the cell monolayer. These measurements have been previously shown to provide a highly sensitive biophysical assay that indicates the state of endothelial cell shape and focal adhesion as described\textsuperscript{14}. FITC-labeled albumin migrated at an equivalent rate across confluent HMVEC treated with basal SCLS serum and serum pooled from age- and gender-matched healthy controls (Fig. 1A). In contrast, FITC-albumin migrated at a significantly faster rate across monolayers treated with episodic SCLS serum, mimicking the signs and symptoms of vascular leakage observed clinically. In agreement with this finding, application of the same episodic serum progressively decreased the electrical resistance of confluent HMVEC compared to matched basal serum, indicating increased endothelial permeability (Fig. 1B). As resistance increased transiently (mostly likely due to mechanical disruption of the monolayer induced by pipetting) followed by divergence under the two conditions at 2-3 hours, we used resistance values at 2.5 hours after application of episodic and basal sera from several subjects to perform the comparison. In each pair, serum obtained during an episode decreased endothelial resistance significantly more than its basal counterpart (Fig. 1C). While prior heat inactivation of serum affected absolute resistance values, the pattern of reduction in TEER by episodic serum relative to basal serum was similar to that observed with untreated serum, suggesting that a major contribution of serum complement is unlikely (supplemental Fig. 1). Together, these data strongly suggest that circulating factor(s) present in SCLS serum during an acute crisis provoke vascular leak symptoms by eliciting endothelial hyper-permeability.
SCLS serum does not induce endothelial apoptosis

Prior work demonstrated apoptosis of endothelial cells treated with serum from two subjects experiencing an acute SCLS attack, and endothelial apoptosis could be one of several explanations for the barrier dysfunction we observed in Fig. 1. Therefore, we tested whether treatment of endothelial cells with serum from diseased or control subjects affected cell viability. We measured the activity of caspases 3 and 7 in growth factor-deprived HMVEC, which reflects activation of both the intrinsic and extrinsic apoptosis pathways.

Growth factor withdrawal induced a 4-fold increase in caspase activity compared to untreated cells while exposure to staurosporine, a known proapoptotic agent, elicited a 20-fold increase in caspase 3/7 activity (Fig. 2A). By contrast, neither serum from SCLS subjects (basal or episodic) nor serum from healthy donors elicited any increased caspase activity compared to growth factor deprivation alone (Fig. 2B). To sensitize cells further to pro-apoptotic stress, we tested the effect of study sera on growth factor-deprived cells in the presence of TNFα. Overnight incubation of HMVEC with TNFα in the presence or absence of staurosporine induced robust caspase 3/7 activity (Fig. 2C). Addition of basal SCLS sera elicited caspase activity comparable to that induced by TNFα alone (Fig. 2D). Interestingly, HMVEC treated with both TNFα and episodic SCLS sera had significantly reduced caspase activity compared to cells exposed to serum from asymptomatic SCLS subjects or healthy controls (Fig. 2D). These results suggest that serum factor(s) present during acute attack phase of SCLS may have an anti-apoptotic effect on endothelial cells in vitro.

In accordance with the prior study, we also evaluated apoptosis independently using Annexin V staining. Annexin V binds to translocated phosphatidylserine on the plasma
membrane outer surface of cells undergoing apoptosis. We visualized Annexin V-FITC staining and cell morphology by immunofluorescence and confocal microscopy after overnight treatment with pro-apoptotic compounds or patient sera in growth factor-deprived cells. Staurosporine elicited prominent Annexin V-FITC staining in most cells, which was accompanied by visible changes in morphology including membrane blebbing and fragmentation (Fig. 3A). We detected comparable (minimal) Annexin V membrane staining in growth factor-deprived HMVEC after application of control or episodic SCLS sera (Fig. 3B). Furthermore, we did not observe gross morphological changes indicative of necrotic death, such as cell detachment, even after prolonged incubation with either sera. These results do not support the hypothesis that a circulating factor elicits permeability in SCLS by inducing endothelial apoptosis.

**SCLS serum elicits disruption of endothelial adherens junctions and cell retraction**

Having observed that endothelial apoptosis could not account for barrier dysfunction induced by episodic sera, we asked whether episodic serum promoted structural changes that would favor paracellular movement of water and solutes. To test this, we applied 5% study subject serum or purified IgG to confluent HMVEC for 2.5 hours, fixed them, and stained for F-actin and VE-cadherin or the tight junction-associated protein ZO-1. F-actin is a critical component of the cytoskeleton, known to rearrange into stress fibers that enable retraction of cell boundaries following cellular exposure to diverse permeability mediators. VE-cadherin and ZO-1 are vascular endothelial-specific transmembrane proteins, whose calcium-mediated homotypic interactions between adjacent cells are indispensable for endothelial barrier function. Application of episodic serum, but not its basal counterpart, induced prominent actin stress fibers and attenuated the junctional localization of VE-cadherin (Fig 4A). Disrupted paracellular
junctons and cell retraction was also observed when sera pairs from other study subjects were used (Figs. 4B-C). In contrast, incubation of HMVEC with the purified IgG fraction of SCLS sera alone failed to induce morphological changes or cell-cell junctional disruption (supplemental Fig. 2). Together with the albumin flux, electrical resistance, and apoptosis assays, these data promote the interpretation that episodic SCLS serum, but not basal serum, contains factors other than IgG or complement that augment permeability and disrupt quiescent endothelial cell structure without inducing cell death.

**Elevated VEGF and Ang2 levels in SCLS**

We evaluated levels of soluble factor(s) that could potentially contribute to acute SCLS symptoms by eliciting transient endothelial permeability. We measured levels of VEGF and Ang2 as abnormalities in these cytokines have been described in disorders associated with vascular leakage including sepsis, POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes), and hemorrhagic fevers caused by infections with hantaviruses. A recent case report demonstrated acutely elevated serum VEGF in one SCLS patient experiencing active symptoms, which correlated with the clinical course. In our series of 20 classic acute SCLS patients, serum VEGF and Ang2 levels were significantly elevated in the SCLS group during acute episodes compared to asymptomatic SCLS subjects and healthy controls (Figs. 5A-B). Notably, serum Ang2 was also elevated in SCLS subjects during asymptomatic periods relative to the control group (Fig. 5B), which may render them more susceptible to vascular leakage. Based on a case report of SCLS episodes in 1-2 patients being associated with elevated markers of inflammation, we analyzed prototypical “inflammatory” cytokines associated with vascular hyperpermeability. We found modestly increased TNFα.
levels in acute SCLS serum samples relative to the basal and control groups (Fig. 5C). However, although direct comparisons to previously reported values are difficult to interpret due to methodological differences in cytokine analysis, the values we measured were considerably lower than those described\textsuperscript{23, 24}. Neither IL-2 nor IL-8 was elevated in SCLS subjects compared to controls (supplemental Fig. 3 and data not shown). Given the distinct clinical profile of subjects who experienced chronic, continuous symptoms rather than transient, reversible episodes (n=3), we excluded them from this cytokine analysis. Although VEGF and Ang2 levels were also significantly higher in the chronic subset of SCLS than in healthy controls, inclusion or exclusion of these values did not affect statistical differences between groups one way or another (data not shown).

We also examined factors known to modify VEGF and Ang2 activity. Although Ang1 shares a common receptor with Ang2 (TEK tyrosine kinase 2, Tie 2), Ang1 occupation of Tie2 receptors is thought to promote endothelial barrier function, vascular development, and angiogenesis\textsuperscript{17}. Ang1 levels were similar in asymptomatic or symptomatic SCLS subjects and healthy controls without SCLS (Fig. 5D). Soluble Tie2 and VEGF receptors (VEGFR2) may be shed from activated or damaged endothelial cells and inhibit the activity of Ang1-2 or VEGF, respectively, by acting as decoys for the circulating pool\textsuperscript{25, 26}. However, soluble Tie2 and VEGFR2 levels were similar in serum of SCLS subjects and healthy controls (Figs. 5E-F).

**Evaluation of IVIG and experimental inhibition of VEGF and Ang2 on permeability**

We compared individuals’ samples obtained during an SCLS crisis with those from the same patients during a well-demarcated, asymptomatic period. In these matched specimens, serum VEGF and Ang2 increased significantly during the acute SCLS attack compared to the baseline.
value (Figs. 6A-B). Based on the permeability induced by episodic, but not basal, serum in the TEER assay (Fig. 1C), we determined the contribution of VEGF or Ang2 in these sera to the phenotype using antibody-based neutralization of these factors. As a positive control for these experiments, we evaluated the effect of a promising SCLS treatment, IVIG. Using the episodic samples of 4-6 subjects, we found that IVIG pre-treatment mitigated serum-induced permeability significantly as measured by electrical resistance ($P = 0.002$, 2-way ANOVA). Similarly, anti-Ang2 pre-treatment counteracted the hyperpermeability elicited by these episodic sera ($P = 0.0192$). Surprisingly, anti-VEGF antibody (bevacizumab) demonstrated no protective effect ($P = 0.4387$). Representative data are shown Fig. 6C. However, application of bevacizumab alone to endothelial monolayers in the presence of culture medium containing VEGF progressively weakened electrical resistance over a several hour period (supplemental Fig 4). Dead and floating cells observed at the end of these experiments suggested that the toxicity of anti-VEGF in this assay might have confounded our ability to evaluate its barrier-fortifying effect against episodic sera.

**Convergent findings in a single SCLS patient**

We collected serial samples from a single patient during a severe SCLS episode characterized by profound hypotension, hemoconcentration as evidenced by a rapid rise in Hgb level to over 20 g/dL (Fig. 7A), and anasarca, with a nearly 20 kg weight gain in a period of three days (Fig. 7B). This patient required massive fluid resuscitation, hemodynamic support by vasopressors, prolonged mechanical ventilation, and fasciotomy in all four extremities. Compared to the patient’s baseline values 3 months prior to the episode during an asymptomatic period, serum and plasma VEGF were markedly elevated at the start of the episode but declined rapidly upon
admission to intensive care (Fig. 7C). At the beginning of the “post-leak” phase, which is characterized by massive fluid remobilization from tissues into the intravascular space and diuresis, VEGF levels had already returned to baseline. Circulating Ang2 was only slightly higher than basal values at the beginning of the leak phase, increasing at a slower rate than VEGF. Ang2 levels peaked several days into the hospitalization after VEGF levels had normalized and remained elevated during the post-leak phase relative to pre-episode values (Fig. 7C). We applied serum from the peak of this subject’s clinical illness (day 1) and from the post-leak resolution phase (day 3) on confluent HMVEC. Consistent with the results obtained using other SCLS sera (Figs. 1B-C), endothelial resistance was substantially depressed by peak illness serum and restored to a normal level as the flare resolved (Fig 7D). Furthermore, peak serum from this patient disrupted cytoskeletal and adherens junctions whereas day three serum did not (Fig 7E).

Discussion

Although it has long been speculated that the transient episodes of hypotension, intravascular volume depletion, and anasarca characteristic of SCLS are caused, at least in part, by a massive, reversible endothelial barrier breach, this hypothesis has never been formally tested. We show for the first time that episodic SCLS sera directly induce endothelial hyperpermeability and barrier breakdown whereas matched sera from asymptomatic periods or the IgG fraction of sera has no such effect. Furthermore, we identified two mediators of permeability, VEGF and Ang2, whose induction in the circulation is associated with SCLS episodes across our study population and whose deflection tracks the clinical course in an index patient.
One feature that distinguishes SCLS from more common clinical scenarios associated with vascular leak, such as sepsis and chemotherapeutic toxicity, is the lack of inflammation. For example, perivascular mononuclear infiltrates have been detected in only a few cases of SCLS while the majority of skin biopsies done in these patients have been reported as normal at the light microscopic level\textsuperscript{27-33}. These studies are consistent with our finding that soluble serum factors present in SCLS sera induce endothelial hyper-permeability without a requirement for accessory immune cells (e.g. leukocytes) to elicit an inflammatory vasculitis. The absence of substantial acute inflammation also makes involvement of traditional inflammatory mediators of vascular leakage such as TNF-\(\alpha\), platelet activating factor (PAF), thrombin, histamine, and IL-8 unlikely.

Limited ultrastructural studies of skeletal muscle endothelial cells from one SCLS patient suggested apoptosis without intercellular gap formation\textsuperscript{34}. In contrast to these findings and other prior studies suggestive of endothelial apoptosis in SCLS\textsuperscript{15}, we found no evidence that serum or plasma from SCLS subjects induced apoptosis of HMVEC under any conditions, including in the absence of growth factors or in the presence of TNF\(\alpha\). The reason(s) for this discrepancy are unclear. Notably, both subjects reported in the previous study had experienced a flu-like illness prior to their hypotensive SCLS crisis, and serum from subjects with sepsis and pancreatitis induced a similar degree of apoptosis as SCLS serum did (as detected by Annexin V staining)\textsuperscript{15}. These results suggest that systemic inflammation, perhaps due to viral infection, rather than factor(s) unique to SCLS, might account for these results.

The most significant aspect of our study is the finding that sera from subjects experiencing an acute SCLS episode induce endothelial permeability through remodeling of endothelial cell-cell junctions. Although all study patients were seen initially during asymptomatic periods...
(Table 2), we were successful in obtaining several samples taken at the onset of an acute capillary leak episode, which were mailed to the NIH clinical center for evaluation. All episodic samples tested increased endothelium monolayer permeability (Figs. 1, 6, 7) and induced disruption of endothelial adherens junctions (Figs. 4, 7), suggesting a common mechanism underlying the vascular hyperpermeability characteristic of acute SCLS. Endothelial shape change, rather than apoptosis of endothelial cells or pericytes\textsuperscript{35, 36}, may mediate the transient symptoms of SCLS. Vascular endothelial barrier function is maintained by intercellular contacts primarily through VE-cadherin, whose transcellular interactions fortify adherens junctions\textsuperscript{37}. Notably, the only treatments shown to reduce the frequency and severity of SCLS attacks, aside from IVIG, is a regimen of theophylline (phosphodiesterase inhibitor) and terbutaline (β-adrenergic agonist)\textsuperscript{6, 38}, which are known to promote endothelial barrier function by stabilizing VE-cadherin-mediated adhesive junctions\textsuperscript{39-41}. Thus, our results could account for the effectiveness of these therapies for SCLS.

The permeability mediators in SCLS we identified, VEGF and Ang2, also regulate endothelial barrier function through VE-cadherin. VEGF promotes internalization of VE-cadherin by inducing its tyrosine phosphorylation\textsuperscript{42} while Ang2 disrupts adherens junctions through myosin light chain phosphorylation\textsuperscript{43}. In our cohort of 20 classic acute patients, VEGF and Ang2 levels were significantly higher than healthy subjects without SCLS, which could account for the disrupted adherens junctions observed. In agreement with individual case reports, our patients had elevated serum VEGF levels at the onset of an acute SCLS episode compared to their baseline, and in one patient VEGF returned to baseline rapidly upon presentation with a severe hypotensive crisis\textsuperscript{10, 22}. Our results show that a short interval of elevated VEGF (and possibly other permeability factors) may exist in SCLS.
Ang2 promotes vascular permeability by inhibiting its receptor, Tie2, whose tonic activation otherwise stabilizes VE-cadherin at adherens junctions\textsuperscript{43, 44}. Whereas Ang2 levels were significantly increased in patients experiencing an acute SCLS attack, levels of the Tie2 agonist ligand, Ang1, were similar in symptomatic and asymptomatic patients. Elevated Ang2 in SCLS basal sera relative to healthy controls may increase the susceptibility of these patients to subsequent vascular leakage. Further increases in Ang2 during acute episodes, together with VEGF, could worsen or prolong the leak state. High circulating Ang2 has been reported in several conditions associated with endothelial hyperpermeability such as sepsis, adult respiratory distress syndrome, and toxicity induced by therapeutic IL-2 administration\textsuperscript{45, 46}.

Several questions are suggested by the current findings. First, what physiological and molecular processes trigger the induction of leak-promoting mediators such as VEGF and Ang2 in SCLS? Although clinical risk factors may include viral infections and psychological stressors, genetic predisposition could also be important. Hemodynamic and metabolic factors including tissue hypoxia\textsuperscript{47}, cardiac function\textsuperscript{48}, and shear stress\textsuperscript{49} also influence VEGF and Ang2 levels. Second, our results suggest that the TEER assay is a useful tool for mediator discovery and evaluation of therapies in SCLS. To assess the contribution of Ang2, we used Tie2-Fc, a commercial reagent previously shown to prevent Ang2-mediated destabilization of confluent endothelium\textsuperscript{50}. In the future, Ang2-targeted antibodies currently under pharmaceutical development may become available for specific testing in this assay\textsuperscript{51}. Our results suggest additional therapeutic avenues for future exploration such as c-Src inhibition\textsuperscript{52, 53}, which would be expected to stabilize VE-cadherin.

Third, future refinements to the presented methodology should improve the ability to discriminate between basal and episodic specimens and control v. candidate treatments. In this
regard, our evaluation of anti-VEGF antibody may have been confounded by the endothelial toxicity of VEGF withdrawal (supplemental Fig. 4). Endothelial hyperpermeability \textit{in vitro} can arise from regulated mechanisms (e.g. thrombin, lipopolysaccharide exposure), which are typically transient and reversible upon stimulus removal, or endothelial cell injury (e.g. following hydrogen peroxide treatment), which is irreversible and develops over a longer period of time. Patients receiving anti-VEGF therapy may develop endothelial injury, which manifests as hypertension, proteinuria, and thrombotic microangiopathy\textsuperscript{54}. Although it has long been established that VEGF induces endothelial hyperpermeability \textit{in vitro}\textsuperscript{42} and \textit{in vivo}\textsuperscript{11}, it also stimulates cellular proliferation over time. As confluent monolayers proliferate, cells become more tightly packed, leading to an increase in electrical resistance that is not related to barrier function \textit{per se} but is an artifact of cellular proliferation. As this situation may not mirror the \textit{in vivo} setting and because of VEGF’s unique dichotomous effects on electrical resistance (based on whether one is looking at the first few minutes or over several hours), our interpretation of TEER assays in the presence of anti-VEGF is thereby limited at this point.

Although the majority of our study cohort has MGUS, further studies are needed to determine whether or not the paraprotein contributes to disease pathogenesis. Based on the limited experience with IVIG and reports of SCLS resolution following treatment of the underlying plasma cell dyscrasia, we speculate that the monoclonal gammopathy is related to the clinical manifestations of SCLS. However, our results suggest that the paraprotein may function indirectly or upstream of vascular permeability mediators as the direct application of IgG isolated from patient sera could not recapitulate the effects of episodic sera on HMVEC structure. This result is also consistent with the observation that serum paraprotein levels do not substantially fluctuate between episodes and remissions.
Notably, although application of IVIG to endothelial monolayers lessened the permeability-inducing effects of acute episodic sera, it is unknown whether this mechanism accounts for the clinical efficacy of IVIG for the treatment of SCLS. IVIG is also thought to possess numerous immunomodulatory properties, including regulation of cell receptor and adhesion molecules, suppression or neutralization of cytokines (by specific antibodies present in the IVIG preparation), activation of regulatory macrophages and/or dendritic cells, and accelerated clearance and/or blockade of autoantibodies\(^{55, 56}\). We are actively investigating whether the monoclonal IgG present in SCLS sera contains specific permeability-enhancing activity. For example, might the paraprotein activate a permeability-promoting receptor or neutralize a permeability-fortifying substance? It is entirely possible that antibodies present in IVIG preparations could bind and inhibit permeability-promoting factor(s) present in acute SCLS serum, including the monoclonal IgG itself.

In summary, our results provide the first demonstration that episodes of SCLS, but not asymptomatic periods, are associated with a circulating activity that promotes microvascular endothelial barrier breakdown. VEGF and Ang2 appear to be contributors to this process. Further study of the mechanism of endothelial barrier dysfunction in SCLS may not only result in novel targeted therapeutic approaches to this under-diagnosed disease, but may also inform our understanding of more common clinical disorders of vascular leak including diabetic retinopathy, lupus nephritis\(^57\), and infection with malaria and Ebola/Marburg viruses\(^58\), among others. Our results offer an explicit framework for the development of drug targets for SCLS and may eventually lead to the identification of new therapeutic strategies for other diseases associated with aberrant vascular function.
Acknowledgements

This study was supported in part by the Intramural Research Program of the NIH, NIAID (grant no. AI001083 LAD to K.M.D.) and N.I.H. grants to S.M.P. (R01HL093234, R01HL093234-01S1, and K08DK06916).

Authorship contributions

Z.X., C.C.G., and R.P., designed and performed experiments; S.I. processed research samples; D.G., C.N., N.J., and P.R.G. recruited and cared for patients; S.M.P. and K.M.D. conceived and directed the project and wrote the paper.

Conflict of interest disclosures

None

References


Table 1. Clinical characteristics of study subjects

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*Note: several patients experienced episodes consistent with SCLS prior to formal diagnosis.
Table 2. Laboratory evaluation of study subjects

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*Laboratory reference values are indicated in parentheses. **Not done.
Figure Legends

Figure 1 Sera from SCLS subjects elicit permeability of endothelial monolayers. (A) Migration of FITC-albumin across HMVEC monolayers was determined as outlined in the Methods after addition of medium containing 5% serum from an SCLS subject during an asymptomatic interval (“basal”) or during an acute attack (“episodic”) or sera pooled from healthy controls without SCLS (n=8); *P = 0.02, 1-way ANOVA. (B-C) Transendothelial electrical resistance (TEER) of HMVEC monolayers after application of matched basal and episodic SCLS serum from 3 subjects with SCLS. Values in (C) are end-point resistance values at 2.5 h (mean ± S. E. M.; *P < 0.03; ***P = 0.0003, paired t test).

Figure 2 Sera from SCLS subjects fail to induce endothelial apoptosis. (A) HMVEC monolayers were cultured in the presence (“basal”) or absence of growth factors or after addition of staurosporine (1 μM) overnight followed by measurement of caspase 3/7 activities by luciferase assay. Data are mean ± S. E. M. of 2 or more experiments measured in duplicate. (B) Growth factor-deprived HMVEC were incubated overnight with sera from control or SCLS subjects (basal or episodic) at 10% (vol/vol) concentrations. Data are mean ± S. E. M. (C-D) Same experiments as in A-B except that growth factor-deprived cells were cultured with TNFα (1.25 ng/ml). Data are mean ± S. E. M.; *P = 0.03, Mann-Whitney test.

Figure 3 Detection of endothelial apoptosis by Annexin V staining. (A) HMVEC were incubated overnight in the presence (bottom panel) or absence (middle panel) of
staurosporine. Cells were left untreated (top panel) or stained with FITC-labeled Annexin V antibody (green) followed by fixation and visualization by confocal microscopy. DAPI (blue) was used to identify nuclei. (B) Annexin V staining in growth factor-deprived HMVEC incubated overnight with sera pooled from healthy donors (“control”) or individual subjects with SCLS (10% vol/vol concentrations).

**Figure 4 SCLS sera disrupt endothelial adhesive junctions and elicit retraction.** (A-C) Matched basal or episodic serum from 3 subjects with SCLS was applied to HMVEC monolayers followed by immunostaining with VE-cadherin antibody (green) and phalloidin (red) to identify F-actin. Bar scale = 20 μm.

**Figure 5 Serum VEGF and Ang2 are increased in acute SCLS.** (A-F) Serum VEGF (A), Ang2 (B), TNFα (C), plasma Ang1 (D), serum soluble VEGFR2 (E), or serum soluble Tie2 (F) in SCLS subjects without symptoms (“basal”), during acute attacks (“episodic”), or healthy controls without SCLS were determined by ELISA. Horizontal bars depict the median value; in A-B, \( P < 0.0003 \), Kruskal-Wallis test for all across-group comparisons; \( *P < 0.05; **P < 0.005; ***P < 0.0005 \), Dunn’s post test.

**Figure 6 Permeability-modifying factors in SCLS.** (A-B) Comparison of basal and episodic VEGF or Ang2 in individual SCLS subjects. (VEGF: \( P = 0.015 \); Ang2: \( P =0.008 \), Wilcoxon signed rank test. (C) The barrier-defending roles of IVIG, anti-Ang2 (Tie2-Fc), and Anti-VEGF (bevacizumab) or equivalent concentrations of control IgG (for anti-Ang2 and Avastin) or BSA (for IVIG) against episodic sera from 4-6 subjects was evaluated in
the TEER assay. Shown is the response to Patient 1’s episodic sera (n = 3-5 replicates per condition). * P < 0.05, **P < 0.01.

Figure 7 Elevations in circulating VEGF and Ang2 correlate with progression of an acute SCLS episode and induction of endothelial permeability. (A-B) Clinical course of a patient with a severe SCLS crisis characterized by hypotension, hemoconcentration (elevated Hgb) (A) and a 20 kg weight gain in 3 days (B). (C) Serum and plasma VEGF and Ang2 in serial samples taken during the course of the illness measured by ELISA. Baseline values were obtained 130 days prior to the episode during an asymptomatic period and at the beginning of the leak (ICU admission) and post-leak (diuresis) phases as indicated by arrows. (D-E) Serum obtained at the peak of symptoms (“peak”) but not serum from the post-leak resolution phase (“resolved”) induces endothelial permeability as assessed by decreased TEER (D) and reorganization of adhesive junctions and cell retraction (E); **P = 0.003, paired t test.
Fig. 1

A

B

C

Pt 1 basal
Pt 1 episodic
Normalized resistance

Patient serum added

Normalized resistance

Pt 1 basal
Pt 1 episodic

Pt 1
Pt 2
Pt 3
Fig. 2

- **A**

  - **- TNFα**

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Fig. 3

A

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<tr>
<th>DIC</th>
<th>Annexin V</th>
<th>DAPI</th>
<th>Overlay</th>
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<tbody>
<tr>
<td>Unstained</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
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<tr>
<td>Basal</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
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<tr>
<td>Staurosporine</td>
<td>[Image]</td>
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<td>[Image]</td>
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</tbody>
</table>
Fig. 3

B

control

<table>
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<tr>
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SCLS

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Fig. 4A

Pt 1 basal

VE cadherin

Actin

Merge DAPI

Pt 1 episodic
Fig. 4B

Pt 2 basal

VE cadherin

Actin

Merge DAPI

Pt 2 episodic
Fig. 5

A

VEGF (pg/ml)

control SCLS basal SCLS episodic

***

B

Ang2 (pg/ml)

control SCLS basal SCLS episodic

***

*
Fig. 5 cont.

C

D

E

F

TNFα (pg/ml)

Ang1 (pg/ml)

soluble VEGFR2 (pg/ml)

soluble Tie2 (pg/ml)

control  SCLS basal  SCLS episodic

control  SCLS basal  SCLS episodic

control  SCLS basal  SCLS episodic

control  SCLS basal  SCLS episodic
Fig. 6

A

\[ \text{VEGF (pg/ml)} \]

\[ \begin{align*}
\text{basal} & \quad \text{episodic} \\
\text{Pt 1} & \quad \text{Pt 2} & \quad \text{Pt 3} & \quad \text{Pt 4} & \quad \text{Pt 5} & \quad \text{Pt 20} & \quad \text{Pt 22} & \quad \text{Pt 23}
\end{align*} \]

B

\[ \text{Ang2 (pg/ml)} \]

\[ \begin{align*}
\text{basal} & \quad \text{episodic} \\
\text{Pt 1} & \quad \text{Pt 3} & \quad \text{Pt 4} & \quad \text{Pt 5} & \quad \text{Pt 20} & \quad \text{Pt 22} & \quad \text{Pt 23}
\end{align*} \]
Fig. 6 cont.

C

Pt 1

<table>
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<th>Normalized resistance</th>
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<tr>
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<td>0.90</td>
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<tr>
<td>1.10</td>
</tr>
<tr>
<td>1.15</td>
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<td>1.20</td>
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** IVIG anti-Ang2 anti-VEGF

control  treatment

* P < 0.05

** P < 0.01

n.s. P > 0.05

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Fig. 7D

- Normalized resistance vs. Hr
- Graph showing trend of normalized resistance over time for two conditions:Resolved and Peak.

- Bar chart for Pt 4:
  - Peak vs.Resolved comparison with error bars and statistical significance indicated by **.
Fig. 7E

Pt 4 resolved

VE cadherin  Actin  Merge DAPI

Pt 4 peak

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Vascular endothelial hyperpermeability induces the clinical symptoms of Clarkson disease (the systemic capillary leak syndrome)