Dysregulation of Angiopoietin 1 and 2 in Escherichia coli O157:H7 Infection and the Hemolytic-Uremic Syndrome

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Escherichia coli O157:H7-associated hemolytic-uremic syndrome (HUS) is characterized by profound prothrombotic abnormalities. Endothelial dysfunction, manifested as dysregulation of angiopoietins 1 and 2 (Ang-1/2), could underlie HUS pathophysiology. We measured Ang-1/2 in 77 children with HUS pathophysiology. We measured Ang-1/2 in 77 children with E. coli O157:H7 infection. Ang-1, Ang-2, and the Ang-2/Ang-1 ratio were significantly different in HUS vs the pre-HUS phase of illness or uncomplicated infection. Angiopoietin dysregulation preceded HUS and worsened as HUS developed. In vitro exposure of human microvascular endothelial cells to Shiga toxin recapitulated the in vivo observations. Angiopoietin regulation is profoundly affected before and during HUS, reflecting that subclinical endothelial dysfunction precedes overt microangiopathy.

Keywords. angiopoietin 1; angiopoietin 2; E. coli O157:H7; hemolytic-uremic syndrome; endothelial dysfunction.

Shiga toxin (Stx)–producing Escherichia coli (STEC) can cause diarrhea, bloody diarrhea, and/or the hemolytic-uremic syndrome (HUS), a disorder in which there is profound nonimmune hemolytic anemia, thrombocytopenia, and renal failure [1]. Shiga toxins are enzymatic ribosome-inactivating proteins at high concentrations, but at lower concentrations more typical of human infection, Stxs alter endothelial cell gene expression [2, 3]. Although Stxs have been shown to induce a prothrombotic, vasoconstrictive, and adhesive endothelial cell phenotype [3], the mechanism by which they mediate endothelial cell activation/dysfunction in HUS is still largely undetermined.

Angiopoietins (Ang) 1 and 2 are important regulators of endothelial cell function and competitive ligands of the endothelial Tie-2 receptor [4]. Ang-1 is produced constitutively and promotes endothelial cell quiescence. Ang-2 is stored in endothelial cells and leads to endothelial activation upon release by noxious or inflammatory stimuli. In health, circulating concentrations of Ang-1 exceed those of Ang-2, and the Ang-2/Ang-1 ratio is low. In infections characterized by endothelial cell dysfunction, including sepsis, streptococcal toxic shock, and cerebral malaria, the Ang-2/Ang-1 ratio increases as a result of decreased Ang-1, increased Ang-2, or both [5–8].

We hypothesized that Ang-1/2 dysregulation (an increased Ang-2/Ang-1 ratio) might also be present in HUS. Here we report serum Ang-1/2 concentrations at various times throughout illness in children with E. coli O157:H7 infection with or without HUS. To explore the role of Ang-1/2 dysregulation in the pathogenesis of HUS, we also investigated the potential of Stxs to alter the Ang-1/2 expression profile of human microvascular endothelial cells in vitro.

METHODS

Clinical Study Design and Enrollment Criteria

Seventy-seven participants were enrolled during a 9.5-year prospective cohort study of children aged <10 years from whom a stool culture for E. coli O157:H7 was obtained on sorbitol-MacConkey agar within the first 7 days of illness [9]. The study was approved by each participating hospital’s institutional review board, and written informed consent was obtained. Day 1 of illness was defined as the first day of diarrhea. HUS was defined as hemolytic anemia (hematocrit <30% with evidence of schistocytes on peripheral blood film), thrombocytopenia (platelet count <150 × 10⁹/L), and azotemia (serum creatinine greater than the age-adjusted upper limit of normal), and uncomplicated infection was defined as not having met criteria for HUS by day 14 of illness. Sera were stored at −80°C until assayed. Eighty-two serum samples were tested: 21 from
Biomarker Assays
Serum concentrations of Ang-1 and Ang-2 were measured by enzyme immunoassays per the manufacturer’s instructions. All samples were assayed in duplicate, in dilutions of 1:20 for Ang-1 and 1:4 for Ang-2. The technical upper and lower limits of detection were 10,000 and 9.77 pg/mL, respectively, for Ang-1 and 2520 and 2.46 pg/mL, respectively, for Ang-2.

Cell Culture and Gene Expression Analysis
Primary human neonatal dermal microvascular endothelial cells were cultured in EGM-2MV growth medium on 0.2% gelatin-coated plates. Stx-2 was purified from recombinant E. coli as described [3] and added to confluent endothelial cell monolayers during passages 5/6 at concentrations of 10 or 1000 fM for 24 hours.

Total cellular RNA was purified by CsCl ultracentrifugation. cDNA was synthesized from DNase-treated total cellular RNA per the manufacturer’s protocol. Quantitative real-time polymerase chain reaction assays were performed in triplicate using the ABI 7900HT Sequence Detection System with the following cycle parameters: 10 minutes at 95°C; 40 cycles of 10 seconds at 95°C and 1 minute at 60°C. Ang-1, Ang-2, and GAPDH mRNA were measured using SYBR Green and the following primers: Ang-1: 5′-GACAAGATGCAGAAGGGTGGG-3′ and 5′-AGC TACCACCCAAAAGGTG-3′; Ang-2: 5′-TGCCACGGTGAA TAATTGAG-3′ and 5′-TTTCTTTTACCAACAGTGG-3′; GAPDH: 5′-GAAGGTGAAGGTCGGAGTCA-3′ and 5′-GAA GATGGTGATGGGATTTC-3′ [10]. Ang-1 and Ang-2 mRNA were normalized to GAPDH mRNA and expressed relative to levels in vehicle-treated cells using the ΔCT method [11].

Statistical Analysis
Statistical analysis was performed using GraphPad Prism version 4.03 and Instat version 3.06. The statistical significance of between-group differences was calculated using the χ² test (categorical variables) and the 1-way analysis of variance or Kruskal–Wallis test followed by Dunn test for multiple comparisons (continuous variables). Values in matched pre-HUS and HUS samples were compared using the Wilcoxon signed rank test. Receiver operating characteristic curves were generated using pre-HUS patients as cases and those with uncomplicated infection as controls, with the null hypothesis that the area under the curve equaled 0.5. All tests were 2-tailed.

RESULTS
Ang-1/2 dysregulation (decreased Ang-1, increased Ang-2, and increased Ang-2/Ang-1 ratio) was associated with illness severity. The median serum Ang-1 concentrations in patients with uncomplicated infection (76,015 pg/mL; interquartile range [IQR], 51,252–114,218 pg/mL) and in the pre-HUS phase of illness (46,982 pg/mL; IQR, 28,077–79,301 pg/mL) were significantly greater than in patients with HUS (9419 pg/mL; IQR, 3464–22,261 pg/mL; P < .001 and P < .05, respectively; Figure 1A). Conversely, the median serum Ang-2 concentration was significantly lower in those with uncomplicated infection or in the pre-HUS phase of illness than in those with HUS (1155 pg/mL; IQR, 851–1536 pg/mL and 1035 pg/mL, IQR, 802–1404 pg/mL vs 2348 pg/mL; IQR, 1940–3154 pg/mL; P < .001 and P < .01, respectively). Finally, the Ang-2/Ang-1 ratio was 0.015 (IQR, 0.011–0.024) in patients with uncomplicated infection, 0.024 (IQR, 0.012–0.052) in patients in the pre-HUS phase of illness, and >10-fold higher, at 0.29 (IQR, 0.12–0.69) in those with HUS (P < .001 and P < .01 for the comparison between uncomplicated infection and HUS and pre-HUS and HUS, respectively).

The serum Ang-1 concentration early in illness partly discriminated between 2 difficult-to-predict outcomes: (1) infected individuals who did not progress to HUS (uncomplicated infection), and (2) those who did (pre-HUS), with an area under the receiver operating characteristic curve of 0.722 (95% confidence interval [CI], 0.573–0.870; P = .03; Figure 1B).

The difference in serum Ang-1 concentrations between patients in the pre-HUS phase of illness and those with uncomplicated infection was not attributable to either thrombocytopenia or timing of serum sampling. Median platelet counts did not differ significantly between patients in the uncomplicated infection (327 × 10⁹/L; IQR, 266–383 × 10⁹/L) or pre-HUS groups (305 × 10⁹/L; IQR, 218–328 × 10⁹/L; P = not significant; Figure 1C and Supplementary Table 1), and the majority of children in both groups underwent phlebotomy on or before day 4 of illness (Supplementary Table 1).

In paired samples from individual patients, Ang-1/2 dysregulation worsened as microangiopathy ensued. Specifically, serum concentrations of Ang-1 fell and the Ang-2/Ang-1 ratios rose as patients progressed from the pre-HUS to the HUS phase of illness (Figure 1D).

Finally, Stx-2 affected microvascular endothelial cell expression of Ang-1/2 in vitro. In a dose-dependent manner, Stx-2 downregulated Ang-1 mRNA and, conversely, upregulated Ang-2 mRNA (Figure 2).
Figure 1. Serum angiopoietin concentrations in *Escherichia coli* O157:H7 infection. A, Angiopoietin 1 (Ang-1), angiopoietin 2 (Ang-2), and the Ang-2/Ang-1 ratio in children with uncomplicated *E. coli* O157:H7 infection (n = 51), children before the diagnosis of hemolytic-uremic syndrome pre-HUS (n = 10), and children at the time of diagnosis of HUS (n = 24), compared using the Kruskal–Wallis test followed by Dunn test for multiple comparisons. B, Receiver operating characteristic (ROC) curve comparing Ang-1 levels among children with uncomplicated infection and those in the pre-HUS phase of illness, with the null hypothesis that the area under the curve is 0.5. *P* = .03. C, Platelet counts in children with uncomplicated infection, children before the diagnosis of HUS, and children at the time of diagnosis of HUS. D, Ang-1, Ang-2, and the Ang-2/Ang-1 ratio in matched serum samples from individual patients at the time of diagnosis of *E. coli* O157:H7 infection (pre-HUS) and at the time of diagnosis of HUS. *P* = not significant. *P* < .05; **P* < .01; ***P* < .001; o = outlier (1.5 × interquartile range [IQR]); = extreme outlier (3 × IQR). Abbreviations: Ang-1, angiopoietin 1; Ang-2, angiopoietin 2; HUS, children at the time of diagnosis of hemolytic-uremic syndrome; pre-HUS, children before the diagnosis of hemolytic-uremic syndrome; UC, children with uncomplicated *E. coli* O157:H7 infection.
DISCUSSION

We show for the first time that Ang-1/2 homeostasis is disrupted in complicated E. coli O157:H7 infection. Ang-1/2 dysregulation (a relatively low serum Ang-1 concentration and high Ang-2/Ang-1 ratio) was present in children in the pre-HUS stage of illness and worsened as HUS ensued. This novel finding is consistent with the known biology of Ang-1 and Ang-2 and their predicted impact on endothelial cell function during E. coli O157:H7 infection. Ang-1 and Ang-2 compete for binding to the endothelial Tie-2 receptor [4]. In healthy individuals, high serum concentrations of Ang-1 facilitate binding to the Tie-2 receptor and subsequent endothelial cell quiescence through activation of prosurvival pathways and inhibition of proinflammatory pathways [4]. The high serum Ang-1 concentrations during uncomplicated infection are comparable with those in healthy children and adults and consistent with minimal clinical evidence of endothelial activation in these patients [5, 7]. During periods of inflammation, there is a relative increase in the serum concentration of Ang-2 as a result of decreased Ang-1 release, increased Ang-2 release, or both [4]. Consequently, Ang-2 outcompetes Ang-1 for binding to the Tie-2 receptor, thereby freeing proinflammatory and prothrombotic pathways from Ang-1–mediated negative regulation [4]. The end result is endothelial cell activation and dysfunction. In children with HUS, we observed a frank deficit of Ang-1 and a relative excess of Ang-2 as reflected by the elevated Ang-2/Ang-1 ratio. In keeping with this finding, thrombotic microangiopathy is present in these children and represents the clinical manifestation of endothelial cell dysfunction in HUS.

Our observations of Ang-1/2 dysregulation are similar to those made in other disorders of endothelial cell function, including sepsis, cerebral malaria, and streptococcal toxic shock, in which there is a direct association between the degree of Ang-1/2 dysregulation and severity of illness, likely reflecting progressive endothelial dysfunction [5–8]. This is also true in HUS: the Ang-1 concentration decreases and the Ang-2/Ang-1 ratio increases commensurate with disease severity.

The mechanism for Ang-1/2 dysregulation in E. coli O157: H7 infection is unknown. The decline in serum Ang-1 levels through progressive stages of illness may reflect direct, Stx-induced injury to the pericytes (an important source of Ang-1) or frank thrombocytopenia [12]. However, serum Ang-1 is also reduced in the pre-HUS phase of illness when platelet counts are preserved, suggesting that thrombocytopenia is not solely responsible. Similarly, Chandler et al demonstrated that children in the pre-HUS phase of illness have normal platelet counts yet show evidence of early vascular injury [9]. By demonstrating Ang-1/2 dysregulation in the pre-HUS phase of illness, our work supports the concept that subclinical endothelial dysfunction precedes the development of overt HUS and can be detected before classically recognized markers of disease, such as thrombocytopenia, hemolysis, and azotemia.

Endothelial dysfunction in the pre-HUS phase of illness further suggests that therapeutic strategies that target the endothelium warrant study in complicated E. coli O157:H7 infection, for which no specific therapy has emerged [1]. We have shown that Ang-1/2 dysregulation, reflecting endothelial dysfunction, exists at presentation in children with complicated E. coli O157:H7 infection and worsens throughout illness. Therefore, factors that promote endothelial stabilization or quiescence, such as exogenous Ang-1 [13], recombinant Slit proteins [14], and the sphingosine-1-phosphate receptor agonist FTY720 (fingolimod) [15], should be considered as novel approaches to the treatment of complicated E. coli O157:H7 infection.

There are some important limitations to our study. We cannot exclude the impact of renal failure on Ang-1/2 dysregulation in HUS. Nonetheless, renal function was intact in children in the
pre-HUS phase of illness, and therefore, this limitation should not detract from the finding that vascular injury precedes overt HUS. We also wish to caution that despite the statistically significant receiver operating characteristic curve comparing Ang-1 concentrations in children with uncomplicated infection and those in the pre-HUS phase, the degree of overlap precludes the use of a single measurement of Ang-1 to determine prognosis. However, our study of paired, sequentially obtained sera from these patients illuminates an aspect of angiopoietin biology also relevant to other infections, including malaria and sepsis. In such acute and evolving disorders it is often impossible to assess, on clinical grounds, the phase of illness at the time of patient presentation. Paired measurement of Ang-1/2 might classify a patient as improving, stable, or deteriorating based on the kinetics of these markers of endothelial cell function and injury. Finally, we note that endothelial dysfunction might not have reached its peak at the time of serum sampling in our study (within 24 hours of the diagnosis of HUS).

In conclusion, this study is the first to demonstrate Ang-1/2 dysregulation in E. coli O157:H7 infection, in which the degree of dysregulation is associated with disease severity. These findings illustrate that subclinical endothelial dysfunction is present in children with complicated E. coli O157:H7 infection before the onset of HUS and can be detected before the classic triad of hemolytic anemia, thrombocytopenia, and renal failure.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** K. C. K. and W. C. L. are listed as inventors on a pending patent application regarding the use of Ang-1/2 as prognostic biomarkers in critical illness, including the hemolytic-uremic syndrome. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**