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CIRCULATING ANGIOPOIETIN 2 CORRELATES WITH MORTALITY IN A SURGICAL POPULATION WITH ACUTE LUNG INJURY/ADULT RESPIRATORY DISTRESS SYNDROME

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Abstract

There are few blood biomarkers predictive of mortality in adult respiratory distress syndrome (ARDS), and none that currently serve as therapeutic targets. Here, we ask whether a circulating protein angiopoietin 2 (Ang2) correlates with severity of lung injury and mortality in a surgical intensive care unit cohort with acute lung injury (ALI)/ARDS. Tie 2 is a tyrosine kinase receptor expressed on endothelial cells. One ligand, angiopoietin 1, phosphorylates Tie 2 and stabilizes adult vasculature. An alternate ligand, Ang2, serves as a context-dependent antagonist and disrupts barrier function. Previously, our laboratory detected high circulating Ang2 levels in septic patients and a correlation with low PaO₂/Fio₂. In this study, daily plasma was collected in 63 surgical intensive care unit patients. Eighteen patients met clinical criteria for ALI or ARDS. The median Ang2 at admission in patients who never developed ALI/ARDS was 3.7 ng/mL (interquartile range [IQR], 5.6; n = 45). The Ang2 on the day a patient met criteria for ALI/ARDS was 5.3 ng/mL (IQR, 6.7) for survivors (n = 11) and 19.8 ng/mL (IQR, 19.2) for nonsurvivors (n = 7; *P* = 0.004). To explore the mechanism of high Ang 2 leading to increased permeability, plasma from patients with ALI was applied to cultured lung endothelial cells and found to disrupt normal junctional architecture. This effect can be rescued with the Tie 2 agonist angiopoietin 1. A patient's convalescent (low Ang2) plasma did not disrupt junctional architecture. Although further studies with larger sample sizes will be needed to confirm these results, high Ang2 in critically ill

patients with ALI/ARDS is associated with a poor outcome. These data, coupled with our cell culture experiments, suggest that antagonism of Ang2 may provide a future novel therapeutic target for ARDS.

Keywords

Angiopoietin; ALI; ARDS; endothelium; surgical intensive care unit

INTRODUCTION

The endothelial receptor Tie 2 and its ligands angiopoietin (Ang) 1 and Ang2 are known to be critical in postnatal vessel maturation and stabilization (1). Their function(s) in adult vasculature is an area of intense investigation. Classically Ang1, which phosphorylates the Tie 2 receptor, is regarded as vessel stabilizing and anti-inflammatory (2-4). Conversely, Ang2, which blocks Tie 2 phosphorylation [context dependent (5)], is considered vessel destabilizing and proinflammatory (6, 7). It had previously been shown that Ang1 can attenuate leak in the adult murine vasculature (8, 9) via closing interendothelial gaps (10). We have shown that Ang2 causes endothelial paracellular gap formation *in vitro*, and that Ang2 infusion produces pulmonary leak in murine models. We have also shown Ang2 to be present in circulating blood of septic subjects, and that higher Ang 2 levels correlate with a PaO₂/Fio₂ (P/F) less than 200 (6). In a recent collaboration published in this journal, we also found elevated Ang2 in a pediatric cohort with septic shock (11). Additional support for the role of Ang2 in lung injury comes from Bhandari et al. (12), who demonstrated that Ang 2-/- mice or Ang2 siRNA (intratracheal)-treated mice are protected from hyperoxic lung injury. Further evidence for the involvement of the Ang/Tie 2 axis in acute lung injury (ALI) comes from McCarter et al. (13, 14), who found Ang1 therapy (via either cell-based gene transfer or gain-of-function transgenic) significantly attenuated inflammation in a rat model of LPS-induced lung injury.

The mechanism for Ang2 inducing vasopermeability is believed to be through blockade of normal phosphorylation of the Tie 2 receptor. This leads to up-regulation of RhoA, a protein in the GTPase family, which in turn increases phosphorylation of the myosin light chain with resultant actin stress fiber formation. Such cytoskeletal changes lead to cellular contraction with disruption of endothelial junctional proteins such as vascular endothelial cadherin (VE-cadherin) and the formation of interendothelial gaps with increased permeability (6).

This study asks whether Ang2 is elevated in a cohort of critically ill surgical patients with ALI, whether Ang2 levels correlate with mortality, and, finally, whether ALI patient plasma itself can disrupt endothelial junctional architecture.

MATERIALS AND METHODS

Sample collection

During a 2-month period at Beth Israel Deaconess Medical Center, under an institutional review board-approved protocol, we prospectively collected plasma samples from all

patients in the general surgical intensive care unit (SICU). There were no comorbidities that led to exclusion, and patients with chronic obstructive pulmonary disease, end-stage liver disease, end-stage renal disease, and isolated head injury are included. Samples were immediately processed by centrifuging at 8,000 rpm, aliquoted, and then stored at -80°C . All identifying information was removed from patient samples, and data were encoded to protect patient privacy. Clinical data were collected on each patient by chart review.

By review of charts and radiographs, two study investigators blinded to Ang2 values classified patients as cases of adult respiratory distress syndrome (ARDS) using the North American European Consensus Conference criteria of (1) acute hypoxemia with P/F ratio less than 200, (2) chest x-ray with bilateral opacities consistent with pulmonary edema, and (3) no clinical evidence of left atrial hypertension or pulmonary capillary wedge pressure less than 18 mmHg (15). Acute lung injury was defined with the same criteria except for P/F less than 300. Lung injury scores were calculated as previously described with chest roentgenogram score, P/F, positive end-expiratory pressure, and compliance when available (16).

Analyte assessment

Enzyme-linked immunosorbent assay—Angiopoietin 1 and Ang2 were measured in plasma samples from patients by sandwich enzyme-linked immunosorbent assay (ELISA) using the human Ang1 and Ang2 ELISA kits (R and D Systems, Minneapolis, Minn).

Cell culture

Human microvascular endothelial cells from lung (HMVEC-L; Cambrex Bio Science Walkersville, Inc., Walkersville, Md) were cultured in endothelial basal medium 2 (Cambrex) supplemented with 5% fetal bovine plasma and growth factors according to the manufacturer's instructions. Passages 4 to 8 were used for all experiments.

Immunostaining

Human microvascular endothelial cells from lung were grown to confluence on glass coverslips coated with 1% gelatin. The cells were fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline and incubated for 5 min in 0.5% Triton X-100 in phosphate-buffered saline. After blocking, the monolayers were processed for staining with anti-VE-cadherin monoclonal antibody (BD Biosciences Pharmingen, San Diego, Calif) and Alexa Fluoro 488 goat antimouse immunoglobulin G, rhodamine phalloidin (Molecular Probes, Eugene, Ore) for F-actin staining and TOPRO-3-iodine (Molecular Probes) for nuclear staining. Fluorescence images were obtained using a BioRad magnetic resonance cholangiography confocal fluorescence microscope. For experiments using cells treated with plasma from patients, plasma Ang2 concentration was first measured by ELISA. Then, patient plasma was diluted to 10% with endothelial basal medium 2 and filtered with low-protein-binding polyvinylidene difluoride membrane (0.22 μm , Millipore) before a 60-min incubation with HMVEC-L monolayers.

Statistical analysis

In consideration of the fact that Ang2 is not known to be normally distributed and the small sample size, summary statistics are presented as median (interquartile range [IQR]). Mann-Whitney test (or Wilcoxon rank sum test) was used for comparisons between groups. Logistic regression was used to evaluate the association of Ang2 and survival status.

RESULTS

Sixty-three patients were enrolled over the 2-month period. Table 1 reveals baseline characteristics for all subjects. Forty-five patients were considered non-ALI/non-ARDS, and the median Ang2 at admission was 3.7 ng/mL (IQR, 5.6). Of note, 30 of these 45 patients were mechanically ventilated, suggesting that ventilatory support alone does not lead to elevated Ang2. This Ang 2 level is on par with prior studies we have performed where hospitalized nonseptic patients had Ang2 levels of 3.5 ng/mL (6).

Eighteen subjects were identified as ALI/ARDS defined by North American European Consensus Conference criteria ("Materials and methods"). Table 2 details the day of SICU admission when ALI diagnosis was made, each individual's Ang2, P/F, acute physiology and chronic health evaluation (APACHE) II scores, and lung injury scores.

As shown in Figure 1, the Ang2 on the day a patient met criteria for ALI/ARDS was 5.3 ng/mL (IQR, 6.7) for survivors ($n = 11$) and 19.8 ng/mL (IQR, 19.2) for nonsurvivors ($n = 7$; $P = 0.004$). Angiopoietin 2 levels did not correlate with positive end-expiratory pressure or Fio_2 as surrogates for extent of ventilatory support. Of note, Ang1 levels were similar between all subjects enrolled (0.4 ng/mL; IQR, 3.0).

Consistent with prior investigations (17), in this study, neither the P/F ratio nor the lung injury score was correlated with mortality. Prior studies have shown the primary risk factors for death from ARDS are age, multiple organ failures, and sepsis as the inciting ARDS event (18, 19). A recent study by Luecke et al. (20) found that APACHE II was the sole clinical predictor of mortality in an ARDS cohort. In our cohort, we found that an APACHE II greater than 16 predicted death with a sensitivity of 85.7% and a specificity of 66.7%. By contrast, the sensitivity of Ang2 greater than 9.1 ng/mL predicting death due to ALI/ARDS was 100%, with a specificity of 81.8% and an accuracy of 88.9%. Logistic regression showed that with a 1-ng/mL increase in Ang2, the odds of dying increase by 1.29 ($P = 0.03$; 95% confidence interval [CI], 1.021 – 1.63). When each of these variables was separately used in the logistic regression model, Ang2 yielded an area under the plasma drug concentration versus the time curve (AUC) value of 0.91 (SE, 0.68; 95% CI, 0.78 – 1.04; Hosmer and Lemeshow goodness of fit, 0.38). Acute physiology and chronic health evaluation II yielded an AUC value of 0.82 (SE, 0.12; 95% CI, 0.58 – 1.06). Inclusion of APACHE II in a model already containing Ang2 did not increase the AUC, whereas inclusion of Ang2 in a model already containing APACHE II increased the AUC by 0.13, suggesting that Ang2 has more discriminatory ability than APACHE II.

Because Ang2 is known to phosphorylate the Tie 2 receptor, which in turn leads to myosin light chain phosphorylation, contraction of actin cytoskeleton, and paracellular gaps, we

performed immunostaining experiments to assess the impact of high Ang2 ALI/ARDS plasma on endothelial cells. Based on a previously published protocol (21), cultured monolayers of HMVEC-L were bathed in a 1:10 dilution of patient plasma, and the effect on cell structure was examined. As outlined in the “Materials and methods,” cells were fixed and stained for actin and for VE-cadherin. As shown in Figure 2A, (1) when confluent HMVEC-L monolayers were incubated for 60 min with high Ang2 ARDS plasma (Ang2, 17.1 ng/mL), actin stress fibers and endothelial gaps are present. Vascular endothelial–cadherin staining is markedly altered, suggesting a disruption of endothelial barrier integrity. (2) As an internal control, HMVEC-Ls exposed to the same patient’s convalescent plasma from 1 week later (Ang2, 3.3 ng/mL) did not disrupt cell architecture. The plasma from patients with ALI/ARDS contains not only elevated Ang 2 but also many other inflammatory proteins. To determine whether the endothelial barrier breakdown might be mediated by Ang2 specifically, we exposed the cells to the endogenous Ang2 competitor Ang1. (3) Human microvascular endothelial cells from lung monolayers were bathed with high Ang2 patient plasma along with Ang1 (final concentration, 100 ng/mL). Under these conditions, there was significant attenuation in stress fiber formation and maintenance of VE-cadherin cell junction expression. In Figure 2B, the same experiment was performed with a different ARDS patient plasma containing an even higher level of Ang 2 (49.5 ng/mL). Stress fibers formed, VE cadherin staining was disrupted, and interendothelial gaps appeared, but these effects were significantly attenuated when cells were simultaneously treated with Ang 1 in addition to the high Ang2 plasma.

DISCUSSION

This study demonstrates that Ang2 is elevated in SICU patients with ALI/ARDS, and it is the first to demonstrate that higher Ang2 levels on the day a patient meets clinical criteria for ALI/ARDS correlates with mortality.

The search for biomarkers in ARDS that correlate with mortality has a long history (22). Meduri et al. found multiple elevated markers in subjects who died with ARDS but only IL-1 β (sensitivity, 83%; specificity, 78%) and IL-6 (sensitivity, 75%; specificity, 78%) levels at the time of disease development were predictive of death (23, 24). Other well-validated markers of mortality include von Willebrand factor (25), protein C (26), surfactant protein D (27), and soluble TNF receptors I and II (28). In our preliminary and small cohort, an elevated Ang2 level at presentation of ALI/ARDS also correlates with mortality and warrants further investigation.

In our *in vitro* experiments, we were able to demonstrate a potential mechanism for increased pulmonary vascular leak by demonstrating that high Ang2 ALI/ARDS patient plasma disrupts endothelial barrier integrity by inducing actin stress fibers and altering VE cadherin cell-cell contacts, whereas convalescent low Ang 2 plasma from the same patient has no such effect. Additionally, these cytoskeletal changes induced by high Ang2 ALI/ARDS plasma can be “rescued” with specific antagonism by Ang1 to appear similar to convalescent-treated cells.

There are limitations to this small study. We had previously examined a septic mobile ICU cohort and seen high Ang2 correlated with low P/F ratio, but that study was not designed to examine the correlation with the North American European Consensus Conference diagnosis of ARDS. We now sought to determine if Ang 2 was elevated in a defined ALI/ARDS population, and more specifically, in an ALI induced by causes other than sepsis. We hypothesized that a SICU cohort would be dominated by trauma and possibly massive transfusion as causes of ARDS. Indeed, trauma was the predominant diagnosis (there were no cases of massive transfusion), and only two patients had concomitant sepsis with postoperative ARDS. This confirmed the correlation between high Ang2 and ALI even in cases not due to sepsis. One can argue that by lung injury scores, the patients did not as a group have the most severe forms of ARDS. However, as previously discussed, lung injury scores alone have never been shown to be predictive of mortality (17). It may also be argued that ARDS due to trauma does not itself have as high a mortality as ARDS due to other causes (29), and that the generalizability of our findings are limited to SICU cohort. Although this is accurate, even with this population's lower mortality, the correlation of Ang2 with death is significant. Finally, although we achieved a significant *P* value for mortality, the 95% CIs for AUC are still wide—potentially due to the very small sample size. Coupled with our sepsis findings, we believe a larger multi-institutional study looking at the ability of Ang2 to predict ARDS mortality in a large mobile ICU and SICU population is warranted.

Many inflammatory markers are known to be elevated in sepsis and ARDS, including vascular endothelial growth factor, TNF- α , von Willebrand factor, Ag, IL-6, IL-8, IL-10, and others (25, 28, 30, 31). In this small pilot cohort, it was not feasible to robustly examine the interaction between Ang 2 and multiple “classic” protein markers in ARDS. However, we do not suggest that Ang2 alone is entirely responsible for leak phenomena, but rather, that it is a crucial player in a cytokine milieu. There is increasing support for the role of Ang2 as an endothelial activator. (32) A recent article by Fiedler et al (33). describes how Ang 2 acts as a “switch” that allows TNF- α to induce cell adhesion molecule expression on endothelial cells and promote inflammation. Indeed, Orfanos et al. (34) confirmed the correlation between high Ang2 and TNF levels in the blood of patients with severe sepsis. In future work with a large national database, we will assay Ang2 levels in combination with other markers, and we think this approach will allow us to more fully characterize disease severity.

Turning to the *in vitro* work, it should be noted that there are many ways to visualize endothelial barrier integrity (35), and microscopy in varying forms is standard (36). Immunostaining with confocal microscopy is one tool we, and others in the field, have used consistently (6, 21, 37). For this protocol, we tested plasma from three ARDS/ALI patients and saw endothelial architectural distortion similar to our prior publications (6, 21). We chose this descriptive, rather than quantitative, assessment of the impact of high Ang2 ARDS blood on cultured endothelial cells, but future work can incorporate quantitative measures, including *in vitro* permeability assays and transendothelial electrical resistance.

Support for the mechanistic significance of the Ang/Tie 2 axis in ALI/ARDS is demonstrated by the Ang 1 rescue experiment. Despite being exposed to sera with numerous

leak proteins, when endothelial cells are given supplemental Ang1—whose action is Tie 2 specific—endothelial junctional integrity is maintained. Thus, in an *in vitro* model (without PMNs and pericytes), endothelial barrier damage from ARDS sera is attenuated by Tie 2 phosphorylation without the addition of other agents. This makes Tie 2 a potentially attractive therapeutic target in ARDS patients. Blocking the effects of Ang2 by exogenous administration of Ang1 to maintain Tie 2 phosphorylation seems logical. Indeed, others have shown that Ang 1 not only stabilizes vasculature but decreases PMN adherence and tissue factor expression (38, 39). However, the use of Ang1 infusions has been hampered by a poor pharmacokinetic profile due to matrix binding and multimerization. Additionally, and with particular relevance to ALI/ARDS, Ang1 overexpression has been correlated with pulmonary hypertension (40-43). Currently, a reagent that specifically neutralizes the activity of Ang2 (but not Ang1) is available, and if humanized, might someday be a reasonable candidate for testing in patients with ALI/ARDS (44). However, this will require intensive investigation with more detailed information on Ang 2 kinetics to optimize therapeutic intervention. Our initial data set would suggest that an elevated and rising Ang2 is most concerning, but we need to examine more patients to determine if it is best to intervene with anti-Ang2 therapy before at-risk patients develop respiratory distress, once the threshold of ALI/ARDS is met, or if they fail to improve in a finite time period.

In conclusion, Ang 2 is both a biologically relevant and a potentially targetable circulating protein in ALI/ARDS. Although additional studies with larger sample sizes will be needed to confirm these results, high Ang2 in critically ill patients who develop ALI/ARDS seems to predict a poor outcome. These data, coupled with our cell culture experiments, suggest that antagonism of Ang2 can provide a novel future therapeutic target for ARDS.

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Fig. 1.

Box and whisker plot of median Ang2 levels in survivors versus nonsurvivors ($P = 0.004$ by Wilcoxon rank sum).

Fig. 2.

A, Adult respiratory distress syndrome survivor. Plasma from patient on SICU day 2 with high Ang2 (17.2 ng/mL) is incubated with HMVEC-Ls, actin stress fibers form and endothelial gaps develop. Vascular endothelial–cadherin staining is markedly altered, suggesting a disruption of endothelial barrier integrity. As an internal control, HMVEC-Ls exposed to the patient’s convalescent plasma on day 9 (Ang2, 3.3 ng/mL) did not disrupt cell architecture and seems similar to Ang1 rescue experiments. When Ang1, the endogenous agonist to Ang2, is given at the same time as high Ang2 plasma, there is minimal actin stress fiber formation and intact circumferential VE-cadherin cell surface expression, suggesting barrier integrity. This appearance is similar to convalescent sera (B).

B, Adult respiratory distress syndrome nonsurvivor. Plasma from patient with ARDS and high Ang2 (49.5 ng/mL) is incubated with HMVEC-Ls and leads to actin stress fibers, VE-cadherin disruption, and gap formation. Angiopoietin 1 rescue decreases stress fibers, restores VE-cadherin, and attenuates gap formation.

Table 1**Baseline characteristics**

Characteristics (n = 63)	
Age (yrs)	67 +1– 17
Male sex, n (%)	35 (48)
Mechanically ventilated, n (%)	48 (76)
Mortality n (%)	9 (14)
APACHE II	14 (IQR, 10)
Reason for SICU admission	
Trauma	20
Abdominal	11
Vascular	8
Neurosurgery	10
Genitourinary surgery	4
Thoracic	3
Transplant	5
Other	2

Table 2

Acute lung injury/ARDS subset Ang2 and severity of illness

Survivors	Day diagnosis ALI/ARDS	Ang 2 (ng/mL) on day of diagnosis	P/F ratio	APACHE II	Lung injury score	SICU admission
1	1	2.1	190	5	2.0	Trauma
2	1	2.8	218	14	1.3	Trauma
3	1	1.9	189	16	1.3	Vascular
4	1	11	176	17	1.3	Vascular
5	1	18.2	220	14	1.7	Neurosurgery
6	2	5.1	185	15	2.7	Trauma
7	5	4.7	272	17	1.0	Trauma
8	1	7.2	178	15	2.3	Trauma
9	1	5.4	200	6	1.3	Neurosurgery
10	1	1.9	79	16	3.0	Trauma
11	1	16.8	278	33	1.0	Abdominal
Nonsurvivors						
1	3	19.8	288	20	1.0	Abdominal, sepsis
2	2	14.9	180	14	1.7	Vascular
3	4	9.1	150	19	3.0	Thoracic
4	1	39.4	132	32	2.7	Vascular (+sepsis)
5	1	26.1	158	21	1.7	Abdominal
6	1	33.3	153	31	2.0	Orthopedic
7	1	15.3	122	24	1.7	Vascular