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Angiopoietin-2 is a potential mediator of endothelial barrier dysfunction following cardiopulmonary bypass

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Abstract

Introduction—Endothelial activation leading to vascular barrier dysfunction and organ failure is a well-recognized complication of cardiovascular surgery with cardiopulmonary bypass (CPB). The endothelial-specific angiopoietin–Tie2 ligand–receptor system has been identified as a non-redundant regulator of endothelial activation. Binding of angiopoietin-2 (Ang-2) to the Tie2 receptor antagonizes Tie2 signaling and renders the endothelial barrier responsive to pro-inflammatory cytokines. We aimed to study the time course and potential triggering factors of Ang-2 release after CPB, as well as the association of Ang-2 changes with surrogates of increased vascular permeability, organ dysfunction, and outcome.

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Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cyto.2012.04.002>.

Methods—Serum levels of Ang-2 from 25 adult patients (140 screened) were measured before and at 0, 12, and 24 h following CPB procedure by in-house immuno-luminometric assay (ILMA), and compared with indices of organ dysfunction, duration of mechanical ventilation (MV), length of stay (LOS) in the intensive care unit (ICU), and hospital mortality. The effect of Ang-2 was studied *in vitro* by incubating high Ang-2 patient serum with endothelial cells (EC).

Results—Ang-2 levels steadily increased from 2.6 ± 2.4 ng/mL at 0 h up to 7.3 ± 4.6 ng/mL at 24 h following CPB ($P < 0.001$). The release of Ang-2 correlated with the duration of CPB, aortic cross-clamp time, and post-CPB lactate levels. Changes in Ang-2 during follow-up correlated with partial pressure of oxygen in arterial blood (PaO_2)/fraction of inspired oxygen (FiO_2) ratio, alveolar–arterial oxygen tension difference (AaDO_2), hemodynamics, fluid balance, and disease severity measures. Ang-2 levels at 12 h predicted the duration of MV, ICU-LOS, and hospital mortality. High Ang-2 patient sera disrupted EC architecture *in vitro*, an effect reversed by treatment with the competitive Tie2 ligand angiotensin-1 (Ang-1).

Conclusions—Collectively, our results suggest that Ang-2 is a putative mediator of endothelial barrier dysfunction after CPB. These findings suggest that targeting the Ang/Tie2 pathway may mitigate organ dysfunction and improve outcome in patients undergoing CPB.

Keywords

Cardiopulmonary bypass; Endothelium; Endothelial activation; Angiotensin-2; Tie2

1. Introduction

The vascular endothelium constitutes a key player in the pathogenesis of organ dysfunction [1]. It is particularly sensitive to the side effects of cardiopulmonary bypass (CPB), which include complement and platelet activation as well as consumption, and the release of a multitude of proinflammatory cytokines [2,3]. As a result, the phenotype of the vascular endothelium changes from a quiescent, anticoagulant state to an activated, procoagulant state, which is paralleled by disassembly of adherence junctions, myosin driven cell contraction and subsequent inter-endothelial gap formation [4]. This highly regulated cascade of events lead to net extravasation of fluid, which contributes to hypovolemia, tissue edema, and eventually organ dysfunction. Consistently, devastating organ injuries such as acute kidney injury (AKI) or acute lung injury (ALI) are among the most frequent complications after on-pump cardiac surgery [5–8].

Angiotensins are angiogenic factors essential for vascular development, maturation, and inflammation [9–12]. As circulating or matrix-bound molecules, angiotensin-1 (Ang-1) and angiotensin-2 (Ang-2) bind to the extracellular domain of the tyrosine kinase receptor Tie2, predominantly expressed on endothelial cells [13,14]. Operational Ang-1/Tie2 signaling prevents endothelial cells apoptosis by activating the phosphoinositol 3-kinase/Akt survival pathway [34,36]. Moreover, Ang-1 decreases vascular permeability through coordinated and opposite effects on the Rho GTPases Rac1 and RhoA which in turn restrict the number and size of gaps that form at endothelial cell junctions in response to various leakage-inducing agents such as bradykinine, thrombin, VEGF, or $\text{TNF-}\alpha$ [12,15,16]. Constitutive Ang-1 expression by vascular mural cells, and low-level Tie2 phosphorylation, probably

represent a non-redundant control pathway that maintains vessel integrity, prevents endothelial hyperpermeability and inhibits leukocyte–endothelium interactions [9,17]. Upon a variety of stimuli the endogenous context-specific Tie-2 antagonist, Ang-2, is rapidly released by the activated endothelium from so-called Weibel-Palade bodies [18] and disrupts constitutive Ang-1/Tie2 signaling by preventing Ang-1 from binding to the receptor [13,18,19]. Cellular experiments have shown that Ang-2-mediated endothelial destabilization results from complex formation between Tie2 and $\alpha v\beta 3$ with subsequent integrin internalization and degradation [20].

We and others have shown that Ang-2 levels in plasma from critically-ill septic patients correlate with the extent of pulmonary vascular leak and acute lung injury [21,22], increase with the severity of multiple-organ dysfunction syndrome [23,24], and independently predict mortality in the intensive care unit (ICU) [23–28]. Recently, Giuliano et al. were the first to show that plasma Ang-2 levels increased early after CPB in children [29]. We therefore hypothesized that Ang-2 release following CPB correlates with surrogates of capillary leakage and outcome in adults. In addition, we performed *in vitro* experiments with patients' serum before and after CPB to test the contributory role of Ang-2 on endothelial dysfunction.

2. Methods

2.1. Study design and patient population

From May to June 2009 we screened 140 patients scheduled for elective heart surgery with CPB at Hannover Medical School. To ensure a well-defined and homogenous study population several inclusion and exclusion criteria were defined as follows. Inclusion criteria were age ≥ 18 years, scheduled major cardiac on-pump surgery, and willingness to provide written informed consent. Exclusion criteria included conditions that are associated with elevated Ang-2 levels *per se*, such as emergency surgery, severe congestive heart failure (ejection fraction $< 20\%$), active malignancy [30], previous organ transplantation or any disease that requires immunosuppressive drugs during the past 6 months, active or uncontrolled viral infection (Hepatitis/HIV), active acute infectious disease and/or severe chronic infectious disease requiring antibiotic treatment [23,31], advanced chronic kidney disease (estimated GFR [MDRD formula] < 30 mL/min or requiring any kind of dialysis) [27] and pregnancy [32].

Enrollment was performed in a consecutive fashion after obtaining written informed consent from the patients or their legal representatives. The study was performed in accordance with the declaration of Helsinki and approved by the institutional review board.

2.2. Evaluation

Patient demographics, including age at surgery, weight, diagnosis, duration of CPB, duration of aortic cross-clamp, peri-operative fluid balance at 24 h following CPB (the total fluids in – the total fluids out), duration of mechanical ventilation (MV), ICU-LOS, and dose of inotropic substances were prospectively collected. Routine laboratory data were determined twice daily after surgery. Sequential Organ Failure Assessment (SOFA) score [33] and

Simplified Acute Physiology Score (SAPS II) score [34] were calculated at 0, 24, and 48 h after ICU admission. For spontaneously breathing patients in whom the arterial line was removed, the PaO₂/FiO₂ ratio was set >300 mmHg to complete the score calculations at 48 h after CPB.

2.3. Intraoperative management

Operations were performed according to in house standard operating procedures. In brief, sodium thiopental, fentanyl and pancuronium bromide were administered to all patients. All patients underwent routine median sternotomy, in coronary artery bypass graft patients the left internal mammary was prepared. Prior to CPB, patients received heparin (300 U/kg) and an activated clotting time of more than 400 s was maintained thereafter. CPB was established *via* cannulation of the ascending aorta and right atrium using a heparin-coated circuit, a roller pump (Stockert Instrumentation, Munich, Germany) and a membrane oxygenator (Monolyth; Sorin Biomedica, Munich, Germany). In case of mitral valve surgery, double venous cannulation was established in the superior and inferior vena cava. During CPB a mean arterial pressure of 50 to 70 mmHg and moderate hypothermia (30–32 °C) was maintained. For cardioplegia, St. Thomas' solution (1–1.5 L) was infused through the aortic root or direct ostial cannulation to achieve myocardial preservation during cross-clamping. At the completion of surgery, patients were warmed to a minimum of 36.5 °C before CPB was weaned off, after which heparin was reversed completely. Upon completion of the operation, all patients were immediately transported to the surgical intensive care unit for recovery.

2.4. Sampling and quantification of circulating angiopoietin-2 and endothelial adhesion molecules

Baseline blood samples were collected within 24 h before the surgical procedure. Additional blood samples were obtained at 0, 12 and 24 h after the surgical procedure. Immediately after procurement, blood samples were centrifuged at 2.000 G for 10 min, divided into aliquots and stored at –80 °C. Routine chemistry test and blood gas analyses were performed in parallel. Serum Ang-2 levels were measured by in-house immuno-luminometric assay (ILMA) using human Ang-2 monoclonal Ang-2-antibody and anti-Ang-2-antibody Ang-2 monoclonal methodology (R&D, Oxon, UK) as described previously [24,30]. The assay had a detection limit of 0.2 ng/mL. Inter-assay and intra-assay imprecision is 4.6% and 5.2%, respectively. All measurements were performed in duplicates at the same day by the same investigator blinded to patients' characteristics and outcome. Mean serum Ang-2 levels in 29 apparently healthy subjects (59 ± 18 years of age) were 1.0 ± 0.5 ng/mL. Serum levels of soluble vascular-cell adhesion molecule-1 (sVCAM-1) and soluble E-selectin were quantified by bead-based flow cytometry assay (FlowCytomix, eBioscience, Frankfurt, Germany) according to the manufacturer's instructions.

2.5. Cell culture

Passage five dermal human microvascular endothelial cells (HMVEC) (Lonza, Basel, Switzerland) were cultured in EBM-2 media (Lonza, Basel, Switzerland) supplemented with

5% fetal bovine serum (FBS) and growth factors according to the manufacturer's instructions at 37 °C and 5% CO₂. Before experimental treatments, HMVECs were starved for 2 h in EBM-2 containing 1% FBS to increase specificity of changes in morphology after rhAng-1 treatment.

2.6. Immunofluorescence and confocal microscopy

HMVECs were grown to confluence on glass coverslips coated with collagen type I. After starvation the cells were incubated for 30 min with EBM-2 media supplemented with 5% pre-filtered human serum collected from two patients at two time points, 0 and 24 h after CPB, respectively. Serum from a healthy individual not subjected to CPB served as a control. In some experiments, HMVECs were first treated with 500 ng/mL recombinant human Ang-1 (rhAng-1, R&D systems, Minneapolis, MN, USA) or phosphate-buffered saline (PBS) 90 min prior to media supplementation with 5% patient serum. Cells were fixed for 10 min in 2.5% paraformaldehyde and permeabilized for 5 min in 0.2% Triton X-100 in PBS. Cells were blocked overnight at 4 °C with a blocking buffer (1% BSA, Triton, sodium azide), then incubated for 12 h with primary antibody, serial washes in PBS, then 60 min incubation with secondary Alexa-antibody and phalloidin. The coverslips were mounted using ProLong Gold/DAPI. All images were taken by a Zeiss LSM510 META confocal microscope at 63× with the same laser power, gain, and offset conditions.

2.7. Statistical analysis

Data are presented as absolute numbers, percentages and means with corresponding standard deviations (SD) unless otherwise stated. Ang-2 levels of CPB patients at baseline and controls subjects were compared using the two-sided Student's t-test. A repeated measures analysis of variance (ANOVA) was used to compare changes in Ang-2 over time. Changes in Ang-2 (or -Ang-2) were calculated by subtracting the 0 h value (measured immediately post-OP) from the defined time points (see below). The relationship between Ang-2 (or -Ang-2) and clinicopathologic variables was investigated using Pearson's product-moment correlation. Adjusted linear and Cox regression models were used to identify predictors of MV time and ICU-LOS respectively. Selection of variables to be included in the multivariable models were performed a priori, based on theoretical considerations. In all parametric tests, preliminary analysis and transformation were performed to ensure no violation of the assumption of normality, linearity and homoscedasticity. The distributions of the time-to-event variables were estimated using the Kaplan–Meier method with log-rank testing. All tests were two-sided and significance was accepted at $P < 0.05$. Data analysis was performed using SPSS (SPSS Inc, Chicago, Illinois, USA). Figures were prepared using the GraphPad Prism (GraphPad Prism Software Inc, San Diego, California, USA).

3. Results

3.1. Study population

In total, 140 patients were prospectively assessed for eligibility for this study. Of those, 110 patients were excluded (13 patients denied informed consent, 10 patients had a history of malignoma, 3 patients had a severe congestive heart failure, two had an active infectious disease, 57 patients were enrolled in other studies and 20 had other reasons). After study

inclusion, five patients dropped out, one due to early sepsis, one due to extracorporeal membrane oxygenation and three patients for other reasons. Finally, longitudinal serum samples from 25 patients (mean age 69 ± 10 years) were prospectively obtained and analyzed. Patient characteristics, types of surgical procedures, and clinical data of the final study cohort are summarized in Table 1.

3.2. Ang-2 levels increase after CPB

Following CPB we detected a significant increase in circulating Ang-2 levels from 2.6 ± 2.4 ng/ml at 0 h up to 7.3 ± 4.6 ng/mL at the end of the 24 h study period ($P < 0.001$) (Fig. 1A). However, post-OP Ang-2 values at 0 h (2.6 ± 2.4 ng/mL) were non-significantly lower than pre-OP values (3.6 ± 4.0 ng/mL), probably due to dilution caused by intra-operative volume administration (5.2 ± 2.5 L). Thus, we used both, absolute Ang-2 levels, as well as relative changes of Ang-2 (Δ Ang-2), shown in Fig. 1B, for further analyses.

3.3. Ang-2 release is associated with duration of CPB and tissue hypoxia

Parametric correlation using the Pearson test revealed strong positive correlations of Ang-2 values at 12 h and 24 h with CPB duration ($r = 0.64$, $P < 0.001$; $r = 0.53$, $P < 0.01$) and aortic cross clamp duration ($r = 0.57$, $P < 0.001$; $r = 0.48$, $P = 0.02$) as shown in Fig. 2A and B. Postoperative lactate levels (0 h), representing a surrogate marker for tissue hypoperfusion and microcirculatory tissue hypoxia, correlated directly with Ang-2 at 12 h following CPB ($r = 0.79$, $P < 0.0001$) (Fig. 2C). Similar associations were found for Ang-2 levels measured at 24 h after CPB, as well as for Δ Ang-2 values throughout the study period (data not shown). Ang-2 levels did not correlate with soluble VCAM-1 and E-selectin levels (data not shown). Collectively, these results suggest that Ang-2 release after CPB correlates tightly with the duration and extent of ischemia/reperfusion injury induced by CPB.

3.4. Surrogates for capillary leakage correlate with Ang-2 release

Previous studies have indicated that the release of Ang-2 should preferentially affect lung permeability – where Tie-2 expression is highest [14]. Elevated Ang-2 levels measured at 12 h correlated inversely with the ratio of oxygen partial pressure in arterial blood (PaO_2) to the fraction of inspired oxygen (FiO_2) at 24 h after surgery, indicating a defect in oxygen diffusion from the alveolar space into the capillary bed ($r = -0.45$, $P < 0.01$). Similarly, Ang-2 measured at 12 h correlated positively with the alveolar-arterial oxygen tension difference (AaDO_2) determined at 12 h ($r = 0.65$, $P < 0.001$) and 24 h ($r = 0.46$, $P < 0.005$) following CPB. Of note, Ang-2 measured at 0 h did not correlate with any of the aforementioned pulmonary parameters at 12 h (data not shown), indicating that the cumulative Ang-2 release after reperfusion determines the severity of pulmonary dysfunction. Changes in Ang-2 (0 vs. 12 h) correlated positively with 24 h-perioperative fluid balance ($r = 0.4$, $P < 0.005$) and the heart rate to mean arterial pressure index ($r = 0.51$, $P < 0.015$) at 24 h after CPB. Moreover, serum Ang-2 levels at 12 h were directly associated with severity measures at 24 and 48 h as assessed by the SOFA ($r = 0.73$, $P < 0.001$; $r = 0.74$, $P < 0.001$) and SAPS II score, respectively ($r = 0.50$, $P < 0.05$; $r = 0.53$, $P < 0.01$). Similar results were obtained in a modified SOFA score that did not contain the variables

central nervous system and *hepatic system* to exclude bias from sedation and hemolysis-derived bilirubin ($r = 0.71$, $P < 0.001$, $r = 0.65$, $P < 0.001$). Detailed correlations of Ang-2 and -Ang-2 with the aforementioned variables are given in Supplementary data file 2.

3.5. Ang-2 is associated with duration on mechanical ventilation and outcome after CPB

To test whether the sum of the aforementioned correlations of elevated Ang-2 values with (sub-) clinical organ impairment entail a longer duration of mechanical ventilation and ICU-LOS we performed linear regression analyses. Ang-2 values measured at 12 h predicted the duration of mechanical ventilation ($\beta = 0.62$, $P < 0.001$) and ICU-LOS ($\beta = 0.52$, $P < 0.01$), even after adjustment for age, gender, and type of surgical procedure ($\beta = 0.52$, $P < 0.01$ and $\beta = 0.61$, $P < 0.001$). Similar positive correlations were found for adjusted -Ang-2 values at 12 h (MV: $\beta = 0.62$, $P < 0.001$) (ICU-LOS: $\beta = 0.65$, $P < 0.001$). Median duration of MV (10 vs. 36 h) and ICU-LOS (1 vs. 4.5 days) was significantly different between patients with Ang-2 values (12 h) vs. >median. The corresponding Kaplan–Meier curves for death-censored duration of mechanical ventilation ($P < 0.005$) and ICU-LOS ($P = 0.02$) are shown in Fig. 3. Finally, Ang-2 levels at 12 h were significantly higher in non-survivors compared to survivors (12.5 ± 3.3 vs. 4.0 ± 0.5 ng/mL, $P < 0.0001$), and predicted hospital mortality in Cox regression analysis (HR 1.29 [95% CI 1.09–1.53] per 1 ng/mL increase; $P = 0.003$), even after adjustment for age, gender, and type of surgical procedure (HR 1.55 [95% CI 1.10–2.19] per 1 ng/mL increase; $P = 0.013$).

3.6. High Ang-2 in serum from CPB patients disrupts endothelial integrity in vitro

Parikh et al. have previously shown that in sepsis, excess Ang-2 diminishes Tie2 activation which in turn leads to paracellular gap formation – a process driven by actin–myosin-based cell contraction of the cytoskeleton [35]. Such gaps facilitate macromolecular flux across the microvasculature, thus represent a structural change that might correlate with endothelial hyperpermeability. Given the close correlation of rising Ang-2 levels with surrogates for capillary leakage and duration of mechanical ventilation in our patients, we hypothesized that a possible barrier-disrupting effect of patient serum on cultured endothelial cells should correlate with the respective serum Ang-2 concentration. Therefore we added serum from a patient taken at two time points, 0 and 24 h following CPB, to HMVECs to test exemplarily the effect of higher Ang-2 levels on endothelial barrier function. We performed fluorescent immunocytochemistry for F-actin (a structural protein of the cytoskeleton) and VE-cadherin (a main constituent of endothelial adherens junctions). As shown in Fig. 4, incubation (30 min) of HMVECs with control serum resulted in a compact, confluent cell layer with cortical actin filaments and localization of VE-cadherin to cell–cell junctions. When confluent HMVEC monolayers were incubated with patient serum obtained at 0 h after CPB surgery (Ang-2: 3.2 ng/mL), the endothelial architecture was similar to control with minimal actin stress fiber (ASF) formation and normal VE-cadherin expression, suggesting intact cell–cell contacts. However, incubating the same patient's serum collected at 24 h after CPB surgery (Ang-2: 12.2 ng/mL) with HMVEC monolayers disrupted endothelial barrier integrity, as evidenced by increased ASF and distinct endothelial gap formation. We then performed an experiment to test the specificity of Ang-2 in contributing in endothelial barrier breakdown rather than other proinflammatory cytokines. Exposure of the same patient's serum collected at 24 h after CPB surgery (Ang-2: 12.2 ng/mL) to HMVECs after

pre-incubation with the specific Tie2 agonist Ang-1 completely reversed the formation of ASF and interendothelial gaps. These results were reproducible with sera from a second patient (data not shown). However, blocking Ang-2 with a monoclonal Ang-2 antibody had a less protective effect on endothelial-cell monolayer morphology than Ang-1 stimulation (data not shown).

4. Discussion

To our knowledge this is the first prospective study on circulating Ang-2 in *adult* patients undergoing heart surgery with CPB. Our data indicate that Ang-2 release after CPB depends on the duration of CPB and the extent of tissue hypoxia. We provide evidence that post-operative Ang-2 elevation correlates with impaired oxygenation, greater fluid needs, longer MV, ICU-LOS, and hospital mortality. Last, we demonstrate that the negative effects of serum from patients undergoing CPB on endothelial cytoskeletal architecture are associated with Ang-2 level and can be reversed by activating the Tie2 receptor with the recombinant endogenous ligand (i.e. angiopoietin-1). Collectively, our results suggest that Ang-2 is a potential mediator of endothelial barrier dysfunction after major onpump heart surgery in adults. These findings therefore raise the question whether targeting the Ang/Tie2 pathway may mitigate organ dysfunction and improve outcome in patients undergoing on-pump heart surgery.

CPB elicits a complex host response characterized, at least in part, by the activation of the endothelium. Triggering factors for the release of Ang-2 after CPB remain elusive. As a Weibel-Palade body-stored protein, however, Ang-2 is rapidly released and induced upon various stimuli by a multitude of factors, including cytokines, thrombin, activated platelets and leucocytes, and changes in blood flow or oxygenation [9,36,18]. It is thus conceivable to assume that particularly the latter two conditions provoke Ang-2 release in the setting of CPB, as suggested by a close correlation of Ang-2 with CPB time and post-operative lactate levels in our patients.

The average Ang-2 level in our cohort was markedly lower compared to the levels reported in adults with sepsis. This likely reflects a greater severity of endothelial injury in patients with sepsis compared to patients following CPB. However, the magnitude of Ang-2 release was comparable to that initially observed by Giuliano and colleagues in children after cardiac surgery with CPB [29].

Given that Tie-2 mRNA and protein are most abundant in the pulmonary vasculature [14], we assume that the lung is not only uniquely dependent on Tie2 signaling to maintain endothelial integrity, but that it may also constitute the primary source of Ang-2 during CPB. Thus, protective Tie2 signaling in the ischemic pulmonary vasculature is probably inhibited by excess of locally released Ang-2. Then, with termination of CPB and reperfusion of the heart and lungs, pulmonary-derived Ang-2 would be expected to accumulate in the systemic circulation and become accessible for quantification. Consistent with this notion, post-operative Ang-2 elevation was associated with pulmonary dysfunction, as evidenced by impaired oxygenation, a greater AaDO₂, and prolonged mechanical ventilation in our preliminary and small cohort. Moreover, changes in post-

operative Ang-2 levels correlated with surrogates of systemic capillary leakage, such as fluid requirements and hemodynamics, suggesting that pulmonary-derived Ang-2 might also act as a promoter of endothelial activation and subsequent barrier disruption throughout the entire vascular tree. As in our previous studies on patients with sepsis [23,27,31], Ang-2 seemed to predict outcome – *i.e.* the duration of MV, ICU-LOS, and mortality - following CPB. At this end, our results extend previous findings on Ang-2 levels in children following CPB [29], in which pulmonary dysfunction and mortality were not assessed.

Previous studies could demonstrate that systemic administration of recombinant Ang-2 *in vivo* can provoke pulmonary vascular leak and congestion in healthy mice [21,37], whereas administration of recombinant Ang-1 counteracts sepsis-induced pulmonary vascular leakage [38]. Moreover, Mammoto and Parikh earlier provided mechanistic evidence, that Ang-2 causes endothelial hyperpermeability by regulating the endothelial cytoskeleton through coordinated and opposite effects on the Rho GTPases Rac1 and RhoA *in vitro* [35]. In the current study, we were able to show that high Ang-2 patient serum, obtained 24 h after CPB, disrupted EC architecture *in vitro*, whereas low Ang-2 serum from the same patient immediately post surgery (at 0 h) had no such effect. Of note, many pro-inflammatory cytokines are released during the surgery and already start to decline shortly thereafter. This finding supports the concept by Fiedler et al., whereby Ang-2 acts as an important regulator of vascular responsiveness exerting a permissive role for the activities of proinflammatory cytokines [39]. Consistent with this notion, the cytoskeletal changes induced by high Ang-2 patient serum could be rescued with specific antagonism by Ang-1 despite abundantly present cytokines and leak proteins. Thus, in an *in vitro* model (without immune cells and pericytes), endothelial barrier damage from CPB sera is attenuated by Tie2 phosphorylation without the addition of other agents. This makes the Tie2 receptor pathway a potentially attractive therapeutic target in patients undergoing CPB. However, blocking circulating Ang-2 by neutralizing antibodies [40] may be less potent than approaches that activate Tie-2 since exogenous receptor activation offers greater titration control. Indeed, we and others have shown that viral over-expression of human Ang-1 [41,42], acute administration of recombinant Ang-1 protein [35], or acute administration of a novel synthetic PEGylated Tie2 agonist peptide [43,44] had potent preventative effects against lung vascular leakage, tissue injury, and mortality in mouse models of sepsis.

Our study is hypothesis generating in nature but has limitations. First, due to the strict exclusion criteria, this prospective cohort is rather small. Second, we only analyzed blood samples up to 24 h following CPB. Therefore, the temporal kinetics of Ang-2 beyond 24 h remains unknown. Third, the clinical correlates of endothelial injury and capillary leak are difficult to define. However, it was not feasible to specifically assess vascular leak (*e.g.* by imaging of extravasated radiolabeled macromolecules [22]) in the current study. Fourth, we do not assume that Ang-2 alone is responsible for vascular barrier dysfunction, but that Ang-2 acts as a facilitator of endothelial responsiveness that primes the endothelium towards pro-inflammatory mediators [13,16,21]. Thus, it would be highly desirable to correlate Ang-2, in the context of multiple cytokines, with a variety of outcomes in a much larger prospective multi- center study.

5. Conclusions

Collectively, our data suggest that the release of Ang-2 after CPB is both, biologically and clinically relevant. It is intriguing to speculate that pre-treatment with Tie2 agonists may have potential as an endothelium-targeted therapy to stabilize the endothelium and prevent organ injury in patients undergoing CPB. If Ang-2 is confirmed as a relevant biomarker of organ injury and adverse outcome, then a rising slope of serial measurements could identify patients that would particularly benefit from therapy with agents directed against vascular leak itself. Further studies pertaining to the role of Ang-2 in the pathophysiology of endothelial barrier dysfunction following CPB are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CPB	cardiopulmonary bypass
Ang-2	angiopoietin-2
ILMA	immuno-luminometric assay
SAPS II	Simplified Acute Physiology Score II
SOFA	Sequential Organ Failure Assessment
LOS	length of stay
ICU	intensive care unit
EC	endothelial cells
AKI	acute kidney injury
ALI	acute lung injury
Ang-1	angiopoietin-1
MV	mechanical ventilation
SD	standard deviation
ASF	actin stress fibers

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Fig. 1. Bar charts showing (A) absolute and (B) relative (n-fold vs. 0 h) changes of Ang-2 after CPB during the 24 h study period (SD + SEM). Individual changes of Ang-2 are provided in Supplementary data file 1.

Fig. 2.

Scatter plots showing Pearson's product-moment correlation of Ang-2 (12 h after CPB) with (A) duration of CPB, (B) duration of aortic cross-clamp duration, and (C) post-CPB lactate levels. Dots indicate individual cases.

Fig. 3. Kaplan-Meier curves showing (A) the duration of mechanical ventilation and (B) ICU-LOS in patients stratified to Ang-2 vs. >median at 12 h after CPB.

Fig. 4.

Confocal microscopy images showing immunofluorescence staining for VE-cadherin (green), F-actin (red), and DAPI (blue) was performed on 100% confluent P5 HMVECs. Cells were treated with 500 ng/mL recombinant human Ang-1 or PBS 90 min prior to challenge with EBM-2 media supplemented with 5% pre-filtered human serum. Serum was collected from a patient at two time points, 0 h (Ang-2: 3.2 ng/mL) and 24 h (Ang-2: 12.2 ng/mL), after CPB surgery, respectively. White arrows point to inter-endothelial gaps ($n = 2$ independent experiments per condition). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Baseline characteristics.

Variables	Values
Subjects (No.)	25
Age (years)	69 ± 10
Weight (kg)	75 ± 11
Male gender (No. (%))	13 (52)
Procedures	
CABG (No. (%))	9 (36)
Valve (No. (%))	12 (48)
CABG + Valve (No. (%))	2 (8)
Valve + aortic arch replacement (No. (%))	2 (8)
CPB duration (min)	117 ± 71
Aortic cross-clamp duration (min)	70 ± 44
SOFA score at 24 h (pts.)	3.6 ± 4.1
SOFA score at 48 h (pts.)	3.4 ± 4.0
SAPS II score at 24 h (pts.)	40 ± 13
SAPS II score at 48 h (pts.)	28 ± 14
24 h fluid balance (L)	11.1 ± 2.7
Duration of mechanical ventilation (h)	41 ± 65
ICU LOS (days)	4 ± 4
Hospital mortality (No. (%))	4 (16)

Abbreviations: CABG, coronary artery bypass graft; CPB, cardio pulmonary bypass; SOFA, Sequential Organ Failure Assessment score; SAPS II score, Simplified Acute Physiology Score; ICU, intensive care unit; LOS, length of stay.