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Angiopoietin-2: A Key to Understanding Sepsis and Its Pulmonary Sequelae?

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Abstract

Sepsis remains a major cause of morbidity and mortality especially in the older individual. In the US alone, sepsis occurs in approximately 750,000 individuals per year and ranks as the tenth leading cause of death. A major complication of sepsis is organ failure, with the lung being one of the first organs to fail. Moreover, sepsis is the most common risk factor for Acute Lung Injury (ALI) and approximately 50% of individuals with sepsis subsequently develop ALI. Despite its importance, the pathophysiology of sepsis remains unclear. Angiopoietin-2 (Ang-2) is a component of pathways involved in endothelial survival and maintenance of a quiescent state of the vascular system. The functional significance of Ang-2 remains to be fully elucidated, but the evidence thus far suggests that it may be key to a better understanding of the vascular dysfunction and associated organ failure that are so devastating in sepsis. However, the assessment of the cellular release of Ang-2 is not without difficulty. In this brief review, we discuss the relevance of Ang-2 to the endothelial dysfunction associated with the severe inflammatory response in sepsis, and why Ang-2 may not be just a biomarker, but may play a critical role in the pathology. In addition, we discuss some of the reasons why particular sepsis models may present confusing data with respect to Ang-2 involvement.

Introduction

Sepsis has been recognized since ancient times [1], and the gross pathology of pulmonary edema in the absence of cardiac insufficiency was described in the first quarter of the 19th century [2]. Yet, sepsis and Acute Lung Injury (ALI) remain major sources of morbidity and mortality especially in the older individual [3-6]. Indeed, approximately 50% of individuals with severe sepsis develop ALI [7,8]. While there have been significant advances in the treatment of both sepsis and ALI, particularly in the areas of antimicrobial [9,10], resuscitative [11-13], and ventilation therapies [14,15], the disappointing lack of major advances in therapeutic interventions, highlights the need for a better understanding of the molecular mechanisms underlying these conditions. Here we examine the altered pulmonary endothelial activity that results in altered vascular integrity and hemodynamic instability. Angiopoietin 2 (Ang-2) [16] is a naturally occurring ligand for the endothelial receptor tyrosine kinase-2(Tie-2) [17]. In view of the significant correlations of Ang-2 levels with clinical features of sepsis [18-25], and the characteristic of the Ang/Tie system having control over vascular system homeostasis [18,26,27], this review will focus on the importance of Ang-2 in the better understanding of sepsis/ALI pathophysiology.

Sepsis and Acute Lung Injury

Sepsis is a progressive, injurious, systemic response of the host to an infection [28]. In the initial response to a local infection, the interaction with bacterial toxins can drive proximal cells to release several mediators involved in the proinflammatory response [29-31]. However, the excessive, unrestrained release of these mediators can result in the development of an overwhelming systemic response, characteristic of sepsis, leading ultimately to cardiovascular collapse [32,33]. A common feature of both sepsis and ALI is the development of increasing vascular leakage leading to extravascular fluid accumulation, intravascular volume depletion, circulatory and respiratory failure [34-36].The failure is due in part to the unresolved lung inflammation that results in loss of endothelial barrier integrity. These changes lead to an imbalance between an increased oxygen demand as a result of increased cellular metabolism, and decreased oxygen transport, resulting from myocardial depression, and inefficient oxygen extraction due to changes in the peripheral microvasculature [37,38]. The respiratory failure tends to be severe enough to require ventilatory support in approximately 85% of cases [39]. Furthermore, approximately fifty percent of the septic individuals requiring ventilatory support progress to the acute respiratory distress syndrome [39]. The mortality associated with ALI is greater than 38%, and the mortality rate greatly increases as a function of age [5].

Death in sepsis most likely results from the collective burden of the sequential failures of organ systems [34,40]. Although the starting points are the same, simple local infection and complicated sepsis have very different disease courses. Most simple local infections can be eradicated by normal functions of the immune system and sometimes with the help of anti-bacterial [41-43], -viral [44-46], or –fungal [47-49] agents or thermal interventions [50]. During the disease course, even after etiologic organisms are controlled, it still takes time until inflammation resolves. For this process to be successful, an optimal environment of proper oxygenation and circulation by an intact vasculature is critical. If it fails, the healing process can be delayed or complicated as can be seen in patients with diabetes or peripheral vascular disease who often have chronic non-healing wounds [51,52]. In severe sepsis, the same principles apply because of the fundamental problem of the vascular system failure. Systemic inflammation or generalized vascular inflammation can result in vascular dysfunction. In inflamed dysfunctional vessels, activated Endothelial Cells (ECs) secrete a variety of cytokines, recruit immune cells and activate coagulation cascades.

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Most importantly, the vessels are highly permeable. Leaky vessels with decreased tone not only destabilize the overall hemodynamics, but also create increased interstitial spaces making oxygen and nutrient supply to the target tissues difficult. Considering the vascular system as one big web of highways to every single cell in the body, it is not surprising that any organ can be affected and eventually fail in sepsis, in a state of global vascular inflammation and dysfunction. This is one of the reasons why it is difficult to predict the efficacy of any medication given in sepsis. In short, sepsis is a life-threatening disease because it is a state of systemic vascular inflammation. It can involve any organ that gets oxygen supply by the vascular system which means all organs in the body are potentially vulnerable in sepsis. Vascular inflammation and dysfunction makes the healing process fundamentally impaired, triggering a vicious cycle of worsening inflammation, more organ damage and dysfunction, ultimately resulting in shock and multiple organ failure.

There is considerable evidence that microvascular dysfunction is a critical event in sepsis [53-55]. A major function of the microcirculation is to provide appropriate oxygen delivery for parenchymal cell requirements in each organ. In addition, this network of small blood vessels plays roles in modulation of inflammation and coagulation. In the healthy state, the microvasculature responds to changes in both blood flow and metabolic demands. However in sepsis these functions, which are predominantly controlled by endothelial cells [56], become dysfunctional [57], and there is evidence that the endothelium is predominantly a target rather than a source of systemic inflammation [58]. Furthermore, data from animal and human studies suggest that improving endothelial function, and in particular reducing endothelial barrier dysfunction, can significantly improve sepsis outcome [59,60].

In view of the critical importance of altered endothelial barrier function during severe sepsis, there is great interest in the factors that could mediate these changes. In particular, interest has been drawn to the angiopoietin-Tie2 system, a signaling pathway involved in the control of microvascular permeability [61,62].

Angiopoietin-2

Ang-2, a molecule implicated in the pathogenesis of critical illness [63,64], is a member of the Ang/Tie (angiopoietin/tyrosine kinase with immunoglobulin-like and EGF-like domains) system. The system consists of four ligands, Ang-1, 2, 3 and 4 and two receptors, Tie-1 and 2. Ang-1 and Ang-2 (both of which bind to Tie-2 [16,65-67] are believed to be more clinically relevant and better studied than others. Ang- 2 [16] was originally identified from its homology to the angiogenic factor Angiopoietin-1 (Ang-1), with which it shares significant amino acid identity [16,68]. Although it was initially recognized for its anti-angiogenic properties, over the intervening years much emphasis has focused on its role in modulating vascular permeability, particularly in inflammatory lung disease [69], sepsis [19,26,70-72] and acute lung injury [21,24,25,73]. Furthermore, the simplistic assessment that Ang-1 is a Tie-2 receptor agonist that phosphorylates and activates downstream pathways to promote EC survival and maintenance of the quiescent state of the vascular system; and that Ang-2 antagonizes Ang-1 activity to increase apoptosis, inflammation and subsequently endothelial permeability, is somewhat limited. Recent studies suggest that Ang-2 has complex, context-dependent variable actions which remain somewhat ill-defined [26,27]. Figure 1 shows the effects of Ang/Tie-2 interactions on endothelial permeability.

Angiopoietin-2 and the Weibel-Palade Body

Ang-2 is synthesized and stored, primarily, in endothelial cell-specific organelles (known as Weibel-Palade bodies (WPB)) which can, in the presence of various inflammatory stimuli, be released rapidly by exocytosis [74,75]. The dynamics and plasticity of WPB have been reviewed previously by Rondaij et al. [74]. The WPB also contain other proteins including von Willebrand factor (VWF) [76], endothelin [77], interleukin-8 [78], p-selectin [79], and tissue-type plasminogen

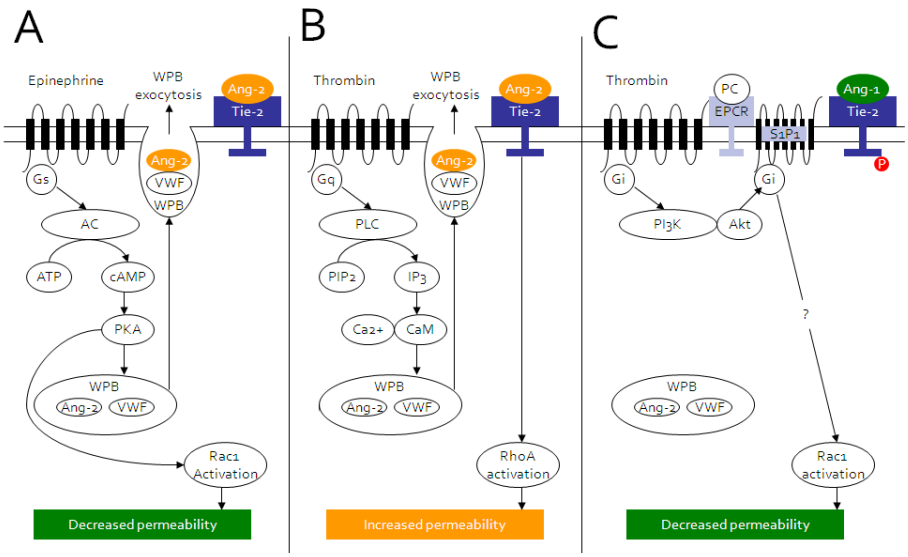

Figure 1: Permeability change by induced secretion of Ang-2: opposing effects depending on different secretary agonists.
A. Although epinephrine induces WBP exocytosis, thus Ang-2 secretion, it rather decreases EC permeability by a direct effect on Rac1 activation mediated by cAMP-PKA pathway. B. Thrombin induces increased EC permeability by PLC-Ca^{2+} mediated Ang-2 exocytosis which results in RhoA activation. C. Thrombin, in the presence of protein C in EPCR, induces decreased EC permeability mediated by Gi protein-PI3K-Akt and/or transactivation of S1P1 and by increasing expression of Ang-1-Tie-2, subsequently resulting in Rac1 activation. Gs, Gq, Gi, G protein –coupled receptors; AC, adenylyl cyclase; PKA, protein kinase A; PLC, phospholipase C; PIP2, phosphatidylinositol biphosphate; IP3, inositol (1,4,5) triphosphate; CaM, calmodulin; EPCR, endothelial protein C receptor; S1P1, sphingosine 1-phosphate receptor; PI3k, phosphatidylinositol 3-kinase; Akt [74,141].

activator (t-PA) [80]. However, many of the components of the WPB are not unique to this organelle and are also stored elsewhere in the cell [81,82]. Therefore while many of the endothelial cell proteins stored in the WPB may be secreted together from endothelial cells by both constitutive and regulated secretion [74], their release may also be induced separately [83]. The situation is further complicated by the substantial heterogeneity of endothelial cells from different sources, even from a single organ [84-86]. Thus, the structure and function of the microcirculation differs between organs and depends upon the function of that organ [87]. Similarly the endothelial cells that line the vessels are not homogeneous throughout the cardiovasculature. There is heterogeneity between micro- and macro-vascular endothelial cells as well as between microvascular endothelial cells from different vascular beds [88]. They are morphologically different (continuous, fenestrated, and discontinuous) and have structural differences related to macromolecular transport between blood and tissues [85]. This is perhaps exemplified by the presence or absence of WPB [89].

Understanding WPB exocytosis-mediated Ang-2 secretion is very important in studying sepsis for many reasons. First, it is known that various bacterial compounds induce WPB exocytosis, so it is highly likely that in the septic state there is a high degree of WPB activation [90,91]. Second, in addition to Ang-2, WPB contains many molecules involved in inflammation and coagulation pathways including VWF, P-selectin and t-PA [74,92], although the effects of these molecules are not limited to either inflammation or coagulation. For example, VWF is an important mediator of coagulation, but it is also known to promote leukocyte extravasation [93]. Thus, uncontrolled activation of the inflammatory and coagulant pathways associated with sepsis could be explained by endothelial activation and rapid deposition of WPB contents into the blood stream.

Since the storage and release of Ang-2 is complex, there are many issues to be considered in regard to Ang-2 activity, including release, plasma accumulation, and the presence of other mediators. Each of these areas has been studied, and the data obtained may sometimes appear conflicting. Some of the studies are outlined below, and some limitations of the types of studies discussed.

Clinical Studies

Of the many markers of endothelial activation known to be elevated in sepsis [94,95] Ang-2 is a useful marker of organ dysfunction and mortality in severe sepsis [72,96-98]. In addition, four studies illustrate the possible importance of Ang-2 as a marker in sepsis. First, a prospective clinical study of 61 septic patients found Ang-2 concentrations to have a positive linear relationship with plasma TNF-α and IL-6 concentrations as well as the severity of sepsis assessed by Acute Physiology and Chronic Health Evaluation (APACHE) II and Sequential Organ Failure Assessment (SOFA) scores [99] . Second, in a study of 124 individuals, Ang-2 was elevated in sepsis and correlated with plasma IL-6 concentration but not TNF-α which was below the assay lower limit of detection in most cases [100]. In this study, Ang-2 correlated with organ failure. The study also revealed increased Ang-2 concentrations to be related to decreased reactive hyperaemia-peripheral arterial tonometry (RH-PAT), a measure of nitric oxide bioavalability. The authors suggested that the decreased bioavalability of nitric oxide may be directly involved in the increased, extra-cellular accumulation of Ang-2. Third, in 9 recent randomized trial of 931 patients with ALI, compared to VWF, Ang-2 had a differential prognostic value for mortality depending on the infection status and the levels were affected by the type of fluid therapy suggesting its specificity as a marker of overall vascular status and sepsis pathophysiology [23]. The fourth report describes a prospective, observational study carried out at a tertiary care center pediatric intensive care unit [101]. The authors of the study measured both plasma Ang-1 and Ang-2 and showed that the Ang-2/1 ratio was elevated in the first three days of sepsis, with the peak at day 2.

In spite of the close correlation between disease severity and serum level, whether Ang-2 is a marker of disease or one of the important etiologic factors in disease progression, remains unclear. In addition, although increased levels can give clues to possible mediators of the disease, they do not necessarily explain the mechanisms involved. Thus, if Ang-2 is a mere marker of endothelial dysfunction or a critical factor to the disease pathogenesis remains to be fully elucidated.

In vivo Animal Studies

In vivo studies reveal data consistent with the earlier concept of Ang-1 and Ang-2 as simple agonist and antagonist, respectively. To evaluate the interactions, the expression of Ang-1 and -2 has been manipulated in murine models. These studies show that Ang-1-overexpressing mice are resistant to vascular leakage caused by inflammatory agents [102], while Ang-2 knockout mice have impaired leukocyte adhesion to activated endothelial cells in response to TNF-α [103]. In addition, mice genetically deficient in Ang-2 showed decreased vascular leakage in response to histamine, bradykinin or VEGF administration [104]. Consistent with these findings, Ang-2 heterozygous mice develop less vascular inflammation, organ dysfunction and superior survival in cecal ligation and puncture sepsis model [105]. In addition, overexpression of Ang-2 in mice resulted in a sepsis-like phenotype including hyperpermeability, hypercirculatory hypotension, cardiac hypertrophy, and fibrosis which are prevented by increasing Ang-1 or inhibiting Ang-2 [106]. Other investigators have taken the approach of evaluating the effects of injecting exogenous Ang-2. Local injection of recombinant Ang-2 induces rapid edema formation in the mouse paw in a dose-dependent manner peaking at 30 min and resolving by 4 h [107]. However, systemic administration of Ang-2 induces vascular hyper-permeability and pulmonary congestion within 3 hours which progresses over 48 hours in a dose dependent manner [108]. Furthermore, in a mouse model, blocking Ang-2 exocytosis is associated with improved pulmonary function following Gram-positive challenge [109]. Lomas-Neira et al. [110] have also investigated a "two-hit" model of indirect-acute lung injury induced by hemorrhagic shock and sepsis [110]. The authors showed that Ang-2 was elevated in this model, and that direct interactions between neutrophils and the endothelium significantly contributed to the extracellular accumulation of Ang-2. Importantly, they found that suppression of Ang-2 activity by injecting the mice intravenously with an siRNA targeting Ang-2 production, decreased inflammatory lung injury, neutrophil recruitment, and the plasma concentrations of both IL-6 and TNFα.

Based on these results, elevated Ang-2 levels shown in clinical studies are consistent with Ang-2 as a pro-inflammatory and permeability inducing factor. However, some *in vitro* studies raise questions challenging this concept.

In vitro studies

VWF and Ang-2 are co-localized within WPB and are depleted concurrently within 15 min upon uric acid stimulation of HUVEC [111]. However, peptidoglycan, a component of the bacterial cell wall, induces secretion of VWF selectively, and not that of Ang-2 [112]. This implies that for each specific stimulus of WPB exocytosis, there may be different molecules co-secreted which could affect the final action.

Second, although t-PA is known to be stored in WPB, at least one study showed that VWF and t-PA are stored separately, VWF in large tubular WPB and t-PA in smaller discrete punctate structures [92]. The authors demonstrated many different sizes of granules going through exocytosis along with WPB with variable thresholds of activation in response to stimulation. WPB can also be exocytosed without exogenous stimulation [113]. Other studies show that there is constitutive release of Ang-2 into cell culture medium even in the absence of defined stimulation [114,115]. Taken together, the data suggest that it is highly possible that Ang-2 is stored not only in WPB but also in other secretary granules that can have a variety mechanisms of release. One interesting finding, revealed by electrophysiological studies of WPB exocytosis, is that calcium induced exocytosis is accompanied by active endocytosis, especially after an initial rapid exocytosis phase [113]. If endocytosis overrides exocytosis, this may explain the phenomenon of certain situations where there is a decrease, rather than an increase, in extracellular Ang-2 level in culture medium after stimulation [114].

Permeability Change by Manipulation of Tie-2 Receptor

Transfection of endothelial cells with Tie-2 specific siRNA significantly increases the permeability of the EC layer [108,115]. This suggests that Ang-1 has a protective effect on cell layer permeability. However, it does not necessarily offer any useful information regarding an Ang-2 effect on cell permeability, since Ang-2 effects on Tie-2 receptor are variable and context-dependent [116].

Ang-2 is synthesized and secreted by ECs, and acts on Tie-2 in an autocrine manner [16,117]. On the other hand, Ang-1, which is believed to occupy the same receptor, and result in phosphorylation of Tie-2 when vessels are in a quiescent state, is synthesized and secreted by pericytes and smooth muscle cells. Studies of the effects of on Tie-2 receptor phosphorylation indicate that Ang-1 alone can induce Tie-2 phosphorylation at 15 and 30 min, and that Ang-2 blocks this response. However, Ang-2 alone does not change the Tie-2 phosphorylation state [114]. Other studies have shown that high concentrations of Ang-2 itself can lead to Tie-2 phosphorylation. Additionally, Ang-2 initiation of the pathways activated downstream of Tie-2 phosphorylation are slightly different from those activated by Ang-1 [118]. Overall, the data suggest that Ang-2 has an antagonistic effect against Ang-1-Tie-2 interaction rather than against the Tie-2 receptor itself, and that Tie-2 phosphorylation status is not a single confirmatory marker to show the activity of Ang-2.

Permeability Changes Induced by Direct Addition of Recombinant Ang-2

For reasons similar to those discussed previously, studies of the effect of recombinant Ang-2 on permeability are not simple to interpret. One study showed that addition of neither Ang-2 nor Ang-1 affects the basal passage of Horseradish Peroxidase (HRP) or Transepithelial Electrical Resistance (TEER) at concentrations ranging from 5 to 400 ng/ml at 30min to 5 hours in human pulmonary microvascular endothelial cells (HPMVEC) [114]. In this study the authors observed that Ang-1 enhanced the basal activity of Rac1 and Ang-2 had a statistically non-significant opposite trend to reduce the activity. However, a study by Parikh et al. [108] showed recombinant Ang-2 (100ng/ml) induces thick actin stress fibers and intercellular gap formation and increases permeability measured by FITC-labeled albumin passage in human microvascular endothelial cells (HMVECs, from neonatal dermis) [108]. Although the results from the second study may seem to be more reflective of the *in vivo* and clinical situation, considering the cell type used, the study of van der Heijden may be more relevant to sepsis pathophysiology (Figure 2).

Limitations of *in vitro* Systems for the Study of Ang-2

Endothelial cells are known to be highly heterogeneous [117,119]. Even among the same cell types, WPB content depends on the passage number and the state of confluence [113]. WPB may not be observed in newly plated endothelial cells until after at least 24h. Thereafter, the numbers of WPB per cell may increase to reach a steady level by 48-72h. Hence, even if the cells reach confluence, but the culture time is not long enough for the cells to develop mature WPB (to produce a burst reaction in response to stimulus), the same stimulus could induce different results. In addition, isolated EC culture systems are not ideal to study the Ang/Tie-2 system because there are no pericytes or smooth muscle cells which are the physiologic sources of Ang-1 and natural environments of the system. It is known that ECs and mural

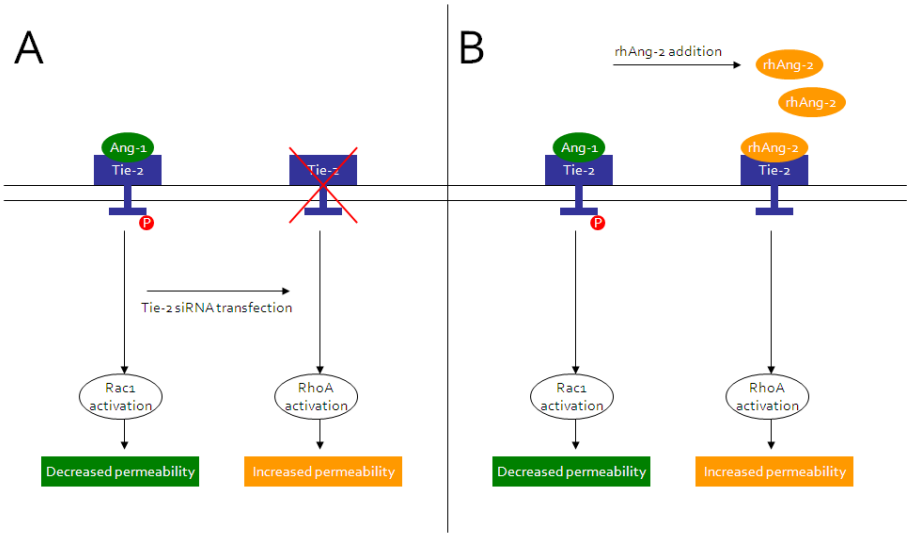

Figure 2: Manipulating endothelial permeability.
A. Ang-1 has a protective effect on cell layer permeability. siRNA transfection of Tie-2 has an opposite effect on permeability. B. Direct addition of recombinant Ang-2 induces increased permeability showing the antagonistic effect of Ang-2 on Ang-1-Tie-2 induced protective effect on permeability.

cells are highly interactive [120-123]. As a result, in monocultures, critical factors, including Ang-1, are missing, which could confound the assessment of Ang-2 effects. Thus, it is reasonable to consider co-cultures or pre-treatment with Ang-1 for *in vitro* studies of Ang/Tie-2 system. In addition, ECs are known to communicate with other hematopoietic cells[124], and there is evidence suggesting that EC Ang-2 may recruit Tie-2 positive monocytes to inflammatory sites [125]. This being the case, it is highly likely that in sepsis ECs actively communicate with other inflammatory cells. Thus, studying EC-inflammatory cell communication could also give new insights into the role of the Ang/Tie-2 system.

There are many artificial environmental factors that can potentially affect study results of in *vitro* culture systems. For example, it is known that WPB exocytosis is affected by pH [126] and Ang-2 expression is up regulated by hyperoxia [127]. To complicate the situation further, *in vitro* culture conditions, do not reflect the physiological conditions and the generally accepted culture terminology can be misleading. For example, the terms, "normoxic" and "hypoxic" conditions used in cell culture [128] may not be representative of the *in vivo* situations. Cell cultures are conventionally maintained with the oxygen level equivalent to that of room air (approx 20% oxygen). While this is normally referred to as "normoxic", it is not the physiologic environment that cells would experience *in vivo.* In addition, the oxygen concentration at the cellular level in static *in vitro* culture changes greatly depending on the amount of culture medium and the cell number [129-134]. Since hypoxia is a known triggering factor of WPB exocytosis [135] and Ang-2 dynamics are affected by both hypoxia and hyperoxia, the effect of significantly different oxygen levels generated by slight differences in cell culture environment should not be ignored. In addition, there are other unanswered fundamental questions in cell culture systems. There is consensus that the passage number of primary cells should be kept low so that they are studied before possible changes in phenotype [136]. However, during the processes of cell isolation and freeze- thawing, the cells are subject to considerable stress. The rate of cell replication, intracellular pathway activation, secretion of cytokines, and the expression of the surface molecules can be altered as a result of this stress [137-140]. Thus, it is often difficult to assess which cell phenotype best reflects the *in vivo* situation. Clearly, *in vitro* cultures only model the *in vivo* situation. However, efforts to isolate and minimize confounding factors are essential especially in a system as complicated, dynamic, and inter-dependent as Ang/Tie-2-WPB. As in many systems, due to dynamic interactions, no one molecule can be examined separately and efforts should be made to compensate for the deficient conditions.

Conclusion

Multiple clinical studies have consistently shown close correlation between Ang-2 level and sepsis severity and its pulmonary sequelae. Thus, Ang-2 is most likely an important mediator, or at least a clear marker of disease severity, in sepsis pathophysiology. So far, *in vitro* studies have shown that Ang-2 induces or enhances EC permeability in a context dependent manner and *in vivo* and clinical studies suggest Ang-2 as a powerful permeability inducing factor. Taken together, Ang-2 is most likely an effective permeability triggering or enhancing factor only in the presence of other cofactors normally present in physiologic conditions. This makes the mechanisms involved in Ang/Tie-2 interactions key to a better understanding of the pathogenesis of sepsis and its pulmonary sequelae. It also makes the Ang/Tie-2 system an interesting therapeutic target to improve tissue oxygenation and hemodynamics, and significantly alter the pathological course of sepsis.

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