

Paper Essay: “The role of Ang-2 in inducing vascular leakage upon acute kidney injury in septic shock”

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Introduction

Critically ill patients are a growing concern in medicine and represent a public health hazard. Among Intensive Care Units (ICUs), medical *shock* is a condition with high morbidity and mortality. Although it may commonly be used with other connotations among the general public (*i.e.* to describe an intense emotional response to something stressful or adverse), shock is a medical condition occurring when the human body is not receiving enough blood flow. It can damage several organs, so it is life threatening, and for that it requires immediate treatment [*MedlinePlus* website]. Given that patients are not getting enough blood flow in their organs, they have extremely low blood pressure. There are 5 major types of shock, and these are based on the variable underlying causes that lead to shock. Cardiogenic shock is associated with a heart condition; hypovolemic shock is related to an inadequate blood volume; anaphylactic shock is caused by an allergic reaction; septic shock is related to an infectious condition and finally, neurogenic shock which is caused by damage to the nervous system. In this report I will focus on septic shock alone. Sepsis is a systemic overwhelming reaction of the body in response to an infection with different microbes (for example, bacteria), causing widespread inflammation and endothelial activation and dysfunction. Any site invaded by bacteria or other organism can cause sepsis, but the most common places are the bloodstream, kidneys, liver, lungs, skin and bowels (*i.e.* peritonitis) [*PubMed Health* website]. The symptoms are not caused by the pathogen itself, but by chemicals the body releases in response to it. Sepsis can lower blood pressure significantly, leading to shock, and septic shock is one of the most severe complications possible. So why target and treat sepsis? Similar to shock, it is a life threatening condition, although mortality has declined in the past 20 years. Among sepsis patients, those with a higher risk for death are the ones that develop either shock or multi-organ dysfunction.

The consequences of sepsis can be systemic, with damage done to more than one organ, and endothelial activation and dysfunction progressively increase the risk to develop multiple organ dysfunction syndrome (MODS). This syndrome is defined as the “development of progressive and potentially reversible physiologic dysfunction in 2 or more organs or organ systems that is induced by a variety of acute insults”, and one of these can be sepsis [*Medscape Reference* website]. MODS affection can vary from a slight level of dysfunction in organs to completely irreversible failure.

Within septic patients, the most common causes of mortality are septic shock and MODS. Severe sepsis (patients with sepsis and also organ dysfunction) has mortality rates of 25 to 30%, while patients with septic shock have higher numbers, 40 to 70% [Russell, 2006].

In shock patients two possible and major complications are acute respiratory distress syndrome (ARDS) and acute kidney injury (AKI, formerly known as *acute renal failure*). ARDS is characterized by escape of fluid out of pulmonary capillaries into alveolar septa and air spaces. It occurs in approximately 40% of patients with sepsis [Parikh *et al*, 2006], and correlates with adverse clinical outcomes and a high risk of mortality. On the other hand, AKI is characterized by the inability of kidneys to excrete wastes, conserve electrolytes, and keep homeostasis and fluid balance. It manifests itself with proteinuria, and it is associated with 35 to 50% of sepsis patients [Hoste *et al*, 2003]. As with ARDS, mortality is higher when both AKI and sepsis are present. Among many, one mechanism that has been proposed to explain AKI is the loss of microvascular integrity and permeability.

Even if ARDS and AKI are not present, an increase in microvascular permeability is evident in septic patients with the typical development of subcutaneous edema in several body cavities. There is capillary leakage, vasodilation and leukocyte influx. Capillary permeability is normally well regulated in microcirculation in all organs, but when altered it can result in an escape of fluid from vascular spaces into different tissues. Since the endothelium forms the inner cellular lining of all blood vessels, capillary permeability involves endothelial activation, and the endothelial barrier integrity is the molecular basis [Parikh *et al*, 2006]. This integrity is thought to be equilibrium between contractile forces of endothelial cells (ECs) and adhesive forces between these cells. Contractile forces allow ECs to create intercellular gaps that can permit leakage, while the adhesive forces between them restrict such gaps.

Vascular permeability and endothelial dysfunction with edema formation represents a major hazard: the accumulation of fluid in the parenchyma and interstitial space is not optimal to organ function. Excessive fluid increases the space required for oxygen diffusion, and microvascular perfusion to different organs is affected by the increase of interstitial pressure [Lee *et al*, 2010]. However, to this day it is not fully understood whether endothelial dysfunction with edema formation is part of the pathophysiology of sepsis or if it is a mere secondary phenomenon. Studying the mechanism behind this phenomenon could lead us to the answer and represent a potential target to improve the prognosis of sepsis and reduce mortality.

Molecular analysis of microvascular permeability has identified the angiopoietin/Tie2 system (Ang/Tie2 system) as one of the regulatory systems of endothelial integrity. Out of the 4 angiopoietins known and studied (1 to 4), Angiopoietin 1 (Ang-1) and Angiopoietin 2 (Ang-2) bind to the Tie2 receptor in ECs and control its integrity, quiescence, growth and permeability.

In this essay I will describe our current knowledge of septic shock, MODS, AKI, and the involvement of the Ang-1 and Ang-2/Tie2 system. Different studies have been performed modulating the expression of both angiopoietins: overexpression or lack of Tie2, Ang-1 and Ang-2 were evaluated on three-dimensional in vivo cell models and mice. Regarding sepsis and human subjects, angiopoietin levels have been measured in different stages of the disease and later correlated with severity and prognosis. All previous work reveals a major role of the Ang/Tie2 system in the endothelium, whether it is keeping the cells in a “quiet” state, or activating them to loose their functions and alter the homeostasis of different organs. Exposing several scientific studies will showcase Ang-1 and Ang-2 and how they act on Tie2 receptors affecting ECs. Both are interconnected and its balance is crucial for the endothelium (as this essay will prove), but the focus will be mainly on Ang-2, given that it has been described as having an important role in endothelial permeability. The question is what exactly is the role of this angiopoietin in vascular leakage upon AKI in septic shock, and whether there is a probability of modulating the Ang/Tie2 system to beneficially affect the condition. Also, other factors and molecules contributing to the pathological mechanism of endothelial dysfunction and related to the Ang/Tie2 system will be elaborated upon in this essay, with the probability of finding some new targets or innovations for possible experiments.

Mechanisms of septic shock and MODS

It was Louis Pasteur, one of the pioneers in medical microbiology, who first revealed bacteria present in blood samples from patients with puerperal septicemia (an infection contracted by women during childbirth or miscarriage). This was around the year 1880, and his statement was that a patient’s body itself can combat the disease and win, given that one of these affected women survived. From this statement comes the notion that sepsis is a systemic response that serves to fight against an infectious agent.

Sepsis represents 2% of hospital admissions, and of sepsis patients 9% progress to severe sepsis and of those 3% progress further to septic shock [Annane *et al.*, 2005].

As stated previously in the *Introduction*, sepsis and septic shock are not the same entities. An International Sepsis Definitions Conference was held in the year 2001 (with various representatives, including the European Society of Intensive Care Medicine and the American College of Chest Physicians), establishing different definitions within the “sepsis” spectrum. *Sepsis* is the presence of infection along with what is defined as systemic inflammatory response syndrome (SIRS). SIRS consists of the appearance of 2 of the 4 following clinical conditions: temperature higher than 38°C

(Celsius) or less than 36°C, heart rate higher than 90 beats per minute, respiratory rate greater than 20 breaths per minute or arterial carbon dioxide tension lower than 32 mmHg (millimeter of mercury), and white blood cell count higher than 12,000 cells/mm³ (cubic millimeter) or lower than 4,000 cells/mm³ or 10% immature forms (also known as band cells: immature polymorphonuclear leukocytes). On the other hand, *septic shock* is defined as the presence of an acute failure in the circulatory system, with either persistent arterial hypotension (low blood pressure) despite fluid reposition or tissue hypoperfusion, which cannot be explained by other causes [Medscape Reference website].

Regarding the causative agents, in the majority of cases septic shock is secondary to an infection with gram-negative aerobic bacteria. The most common pathogens include *Escherichia coli*, *Klebsiella* and *Staphylococcus* species, while viruses and fungi are not frequent [Rackow et al, 1991].

The early hemodynamic profile of septic shock starts with predominant vasodilation and warm extremities in the patient. There is an increased cardiac output (the blood being pumped by the heart), and decreased systemic vascular resistance. Later on, cardiac output is decreased, mainly by hypovolemia (loss of intravascular volume), underlying cardiac disease and myocardial depression. Hypovolemia may be caused by the manifestation that is the topic of this essay: an increase in systemic microvascular permeability. Along with an altered circulatory state, oxygen extraction is extremely reduced in septic shock, hence; there is impairment in oxygen utilization. Tissues become less oxygenated as a consequence of this, so the anaerobic cycle breaks down glucose for energy production, producing lactic acid [Medscape Reference website]. Lactic acidosis arises, with mixed venous saturation normal or increased (this measurement indicates the percentage of oxygen bound to hemoglobin in blood returning to the right side of the heart, basically the remaining oxygen that body tissues do not need or use). This impaired oxygen use may be caused by the overall distributive changes in the systemic and microvascular circulation (*figure 1*).

As previously stated, gram-negative bacteria are the cause of most cases of septic shock. An important mediator of septic shock is endotoxin (used synonymously with the term *lipopolysaccharide*, later helpful in many animal model experiments where sepsis is recreated), a component of the outer membrane of gram-negative bacteria. Endotoxin triggers a systemic response in patients, with plasma reactions and the activation of mediator cells. It is responsible for: activation of the complement system, the coagulation cascade and the kinin system, the release of several cytokines, platelet activating factor, prostaglandins and leukotrienes, and also the stimulation of different cells like macrophages, monocytes, neutrophils and ECs. Among several cytokines released by endotoxin, several are pro-inflammatory such as tumor necrosis factor (TNF), interleukin 1 (IL-1) and interleukin 6 (IL-6).

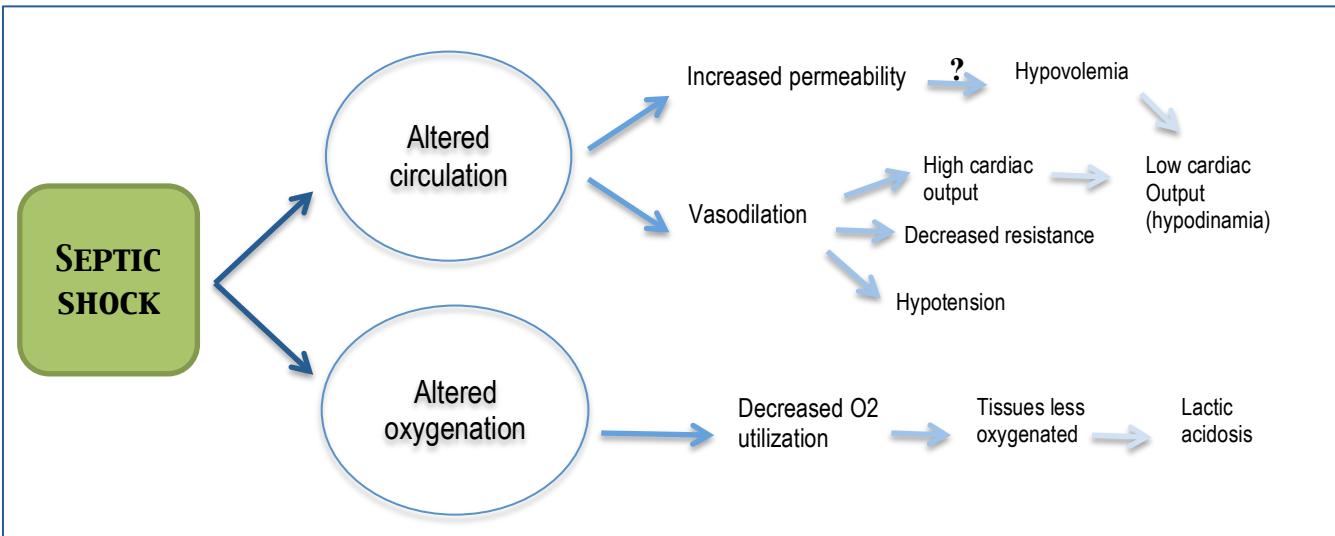


Figure 1- The circulatory and respiratory manifestations in septic shock.

In septic shock, the ECs upregulate adhesion molecules and thus promote adhesion of leukocytes, and migration of these cells into tissues [Annane *et al*, 2005]. Mouse model experiments have proposed that adhesion molecules inherent to either leukocytes or ECs might contribute to tissue damage. Apoptosis of cells in sepsis can be altered or not, depending on the cell type affected. In the case of the endothelium, apoptosis is not abnormal.

MODS is a phenomenon that usually occurs in the second or third week of the course of septic shock, and the majority of shock patients who die usually have this syndrome [Rackow *et al*, 1991]. It represents the terminal phase of the characteristic hypermetabolic profile of septic shock, beginning during the initial phase of shock and resuscitation. An infection represents a local and systemic inflammatory response, which in turn leads to microvascular injury, and eventually results in multiple organ failure. Ischemia and hemorrhagic damage are rarely involved [Annane *et al*, 2005]. Two aggravating factors in this condition are permanent, uncontrolled sepsis and tissue hypoperfusion. There is a pattern, which is seen often in the decline of MODS patients: failure starts with loss of pulmonary function, followed by hepatic and finally renal failure.

AKI in septic shock

AKI is a frequent complication after septic and hemorrhagic shock that is usually life threatening. In sepsis, there is synthesis and release of nitric oxide (NO) resulting in decreased systemic vascular resistance. This causes vasodilation all around in arteries (hemodynamic hallmark of sepsis), and this vasodilation may cause patients to become susceptible in developing AKI, and ultimately increased mortality [Schrier *et al*, 2004].

Endotoxin also affects renal vasculature resistance, and among many effects, it is related to changes in renal hemodynamics: alteration in blood flow, and glomerular filtration rate [Kim *et al*, 2009].

Two phenomena occurring in sepsis that contribute to kidney injury are microvascular injury and peritubular endothelial dysfunction. Also, related to vascular dysfunction and being key in this paper essay, hyperpermeability secondary to capillary leakage is crucial within AKI, causing migration of fluid and macromolecules into the renal interstitium. This is what progressively leads to renal failure.

So three main key events occur within the kidneys in AKI during sepsis: alteration in hemodynamics, inflammation and vascular permeability. It is possible that by treating and modulating these three, the number of AKI patients in sepsis could decrease and so could the mortality in septic patients.

The Ang/Tie2 system and endothelial integrity and permeability

The endothelium was once thought to be an inert vascular lining to all organs, but it is crucial for the regulation of vascular circulation because of its many functions: receiving and giving signals, regulating the passage of multiple elements such as fluids and cells, and storing active substances [van Meurs *et al*, 2009]. It does not have one unique phenotype; it depends on which organ the endothelium is situated on, and at what specific time. Also, ECs react to various stimuli in different ways, depending on their context.

Three main functions are attributed to ECs: First, they are involved in vasculogenesis and angiogenesis, the formation of new blood vessels either *de novo* or from pre-existing vessels (this can either be physiologic in embryogenesis and wound healing, but pathologic in the context of tumor development and diabetes). Second, ECs contribute to maintain homeostasis in adult organisms by regulating transport of fluids, proteins and electrolytes, cell migration and blood flow. And third, ECs respond when homeostasis is altered (*i.e.* inflammation). The Ang/Tie2 signaling system is involved in all three functions of ECs, making it a crucial system in the properties and functioning of the endothelium.

Angiopoietins are 70-kDa (kilodalton) glycoproteins, basically growth factors that promote new blood vessel formation. 4 types have been identified in humans: Angiopoietins 1 to 4. The two most studied and described glycoproteins of these four are Ang-1 and Ang-2.

The Ang/Tie ligand-receptor system consists also in two receptors: Tie1 and Tie2, both mostly expressed by ECs and hematopoietic stem cells. They are tyrosine kinase receptors that share a similar structure, homologous to immunoglobulin and epidermal growth factor. There is no identified specific ligand that binds to Tie1 (although high concentrations of Ang-1 do bind to it through integrins). Angiopoietins are specific ligands for Tie2-receptor binding: Ang-1 and Ang-4 act as agonists for Tie2 by phosphorylation of tyrosine, while Ang-2 and Ang-3 are antagonists by

inhibiting Ang-1. However, as I will discuss later, the role of Ang-2 on Tie2 is much more complex than by defining it as antagonist [Fiedler *et al*, 2006] [Kranidioti *et al*, 2009].

Ang-1 production originates in pericytes, smooth muscle cells, and glomerular podocytes (since the glomerulus lacks pericytes) [van Meurs *et al*, 2009]. It initiates signaling when it binds to Tie2 receptors, inducing autophosphorylation. Once Tie2 is activated, the downstream pathway can vary depending on the cell type, localization and whether there is a cell-cell or a cell-matrix interaction present.

So when it comes to Ang-1, binding and activating Tie2 leads to a subsequent Akt phosphorylation and activation via PI3K when there is a cell-cell interaction, while a cell-matrix interaction leads to ERK activation. After Ang-1 binding (and also Ang-2 binding), Tie2 is internalized and degraded, with Ang-1 becoming reusable later on. The effect of Ang-1 on ECs is dependent on the location of cells and the biological and biomechanical processes that surround it.

Regarding angiogenesis, Ang-1 is in charge of stimulating it in the presence of hypoxia or other stimuli that might alter ECs. Under normal conditions, Ang-1 maintains the architecture of blood vessels by inhibiting apoptosis of ECs (as said before, through PI3K/Akt signaling): the main function of Ang-1 is the maintenance of a ‘quiescent’ endothelium. The Ang/Tie2 system helps stabilize vascular walls by maintaining EC survival. Also, endothelial inflammation is suppressed by Ang-1 by restricting the release of several inflammatory and adhesion molecules (tissue factor induced by vascular endothelial growth factor –VEGF-, TNF- α , vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and E-selectin). With all of these attributes, Ang-1 avoids vascular leakage.

Ang-2 is produced within ECs, and stored in Weibel-Palade bodies (WPBs). The release of Ang-2 through exocytosis is regulated independently from other proteins stored in the WPBs. Ang-2 mRNA expression can be upregulated by several stimuli, like VEGF, fibroblast growth factor-2 and hypoxia. While Ang-2 mRNA expression can be downregulated either by Ang-1 or Ang-2 itself (*figure 2*).

Ang-2 was first found to have an antagonist effect on Ang-1/Tie2 signaling. In 1997, Maisoinpierre *et al* reported crucial findings with respect to Ang-2 and its role in angiogenesis. Physiologically, angiogenesis is involved with embryogenesis and organogenesis during development, and with wound repair, the menstrual cycle and pregnancy during adult life. However, it can arise under pathological conditions, such as tumor formation and diabetes, aiding to establish and spread the disease. Angiogenesis is regulated both by positive (VEGF) and negative regulatory molecules. To identify further agents, they first unraveled Ang-1, observing several vascular abnormalities in mouse embryos when either this angiopoietin or their receptors were missing. In 1997 they continued to isolate ligands of orphan receptors involved in angiogenesis and began describing

Ang-2 [Maisonpierre et al, 1997]. After homology screening and identifying this angiopoietin, they proceeded to clone it and create transgenic mice embryos overexpressing Ang-2 in their blood vessels, to evaluate the interaction with Tie2 *in vivo*. They reported that all transgenic embryos exhibited an abnormal appearance of the endothelium lining of several blood vessels and the heart. The appearance in the ECs seemed to be like “eaten by moths”, given that there were multiple openings in vessel walls. What was also relevant was that these effects were similar (and also, worse) than what was observed with transgenic mice embryos lacking either Ang-1 or Tie2 receptors. Overall, these observations led to the conclusion that Ang-2 is a natural antagonist for Ang-1 binding to the Tie2 receptor, and that both of these angiopoietins are positive and negative regulators for angiogenesis.

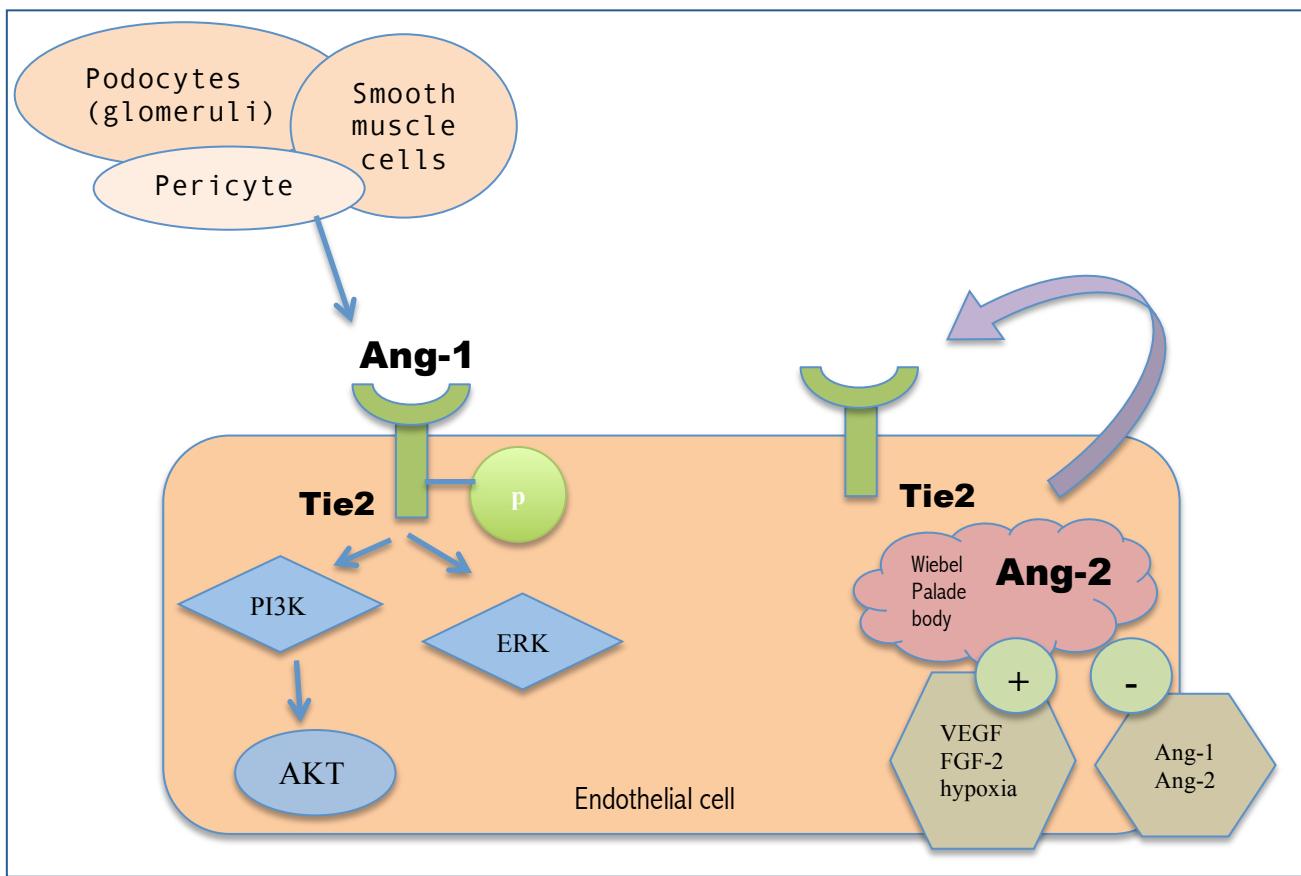


Figure 2- Angiopoietins 1 and 2 in the endothelial cell: Angiopoietin 1 is produced in various cells: pericytes, smooth muscle cells and podocytes in the glomeruli. Tie2 receptors in the endothelial cell are activated by this angiopoietin, leading to an autophosphorylation, and depending on the context it can result in an activation of the PI3K pathway or activation of the ERK protein. Angiopoietin 2 is produced in endothelial cells and stored in Wiebel Palade bodies along with other elements such as Von Willebrand factor. It acts in an autocrine way on Tie2 receptors. mRNA expression of angiopoietin 2 can be upregulated by VEGF, FGF-2 or hypoxia and downregulated by either angiopoietin 1 or angiopoietin 2. The role of angiopoietin 2 on Tie2 receptors will be elaborated on this essay.

This antagonistic proposal of Ang-1/Ang-2 on Tie 2 receptors was later developed in a model that showed the mechanistic effects. It was performed by Scharpfenecker *et al* in the year 2004, using a three-dimensional (3D) spheroidal co-culture model of ECs and smooth muscle cells that attempted

to mimic the structure of real vessel walls. The smooth muscle cells express Ang-1, which maintains the quiescent state of the ECs in this model [Scharpfenecker *et al*, 2004]. One of the first discoveries was that the stability of the ECs wall in this model was mediated by Tie2 receptors, given that with administration of a Tie2 inhibitor several individual ECs detached from the spheroid. Another disturber of the EC-monolayer integrity was the administration of exogenous Ang-2, with ECs detaching very quickly from the 3D spheroidal model. Exogenous Ang-1 and Tie2 could reverse this effect, as Maisonpierre reported. ECs in 3D models that constitutively overexpressed Ang-2 also showed detachment from the walls, but these effects could not be inhibited by exogenous soluble Tie2, proving that endogenous Ang-2 acts in an autocrine manner. These findings could explain the previous Maisonpierre's study, since Ang-2 mediated EC detachment in all models studied.

In 2000, Kim *et al* studied Ang-2 compared to Ang-1, based on the knowledge that several cancers that are highly vascularized, like glioblastoma and hepatocellular carcinoma, exhibited high levels of Ang-2 mRNA. This Ang-2 could possibly induce ECs survival for later mitosis and further proliferation of blood vessels, contributing to tumor formation. Kim et al evaluated cultured human umbilical vein endothelial cells (HUVECs) with different concentrations of Ang-2, and studied whether this could induce EC survival. Serum deprivation induces apoptosis in ECs, but this changed with Ang-2: high concentrations (800 ng/ml) of this angiopoietin saved ECs from going into apoptosis (this was not the case with a low concentration -50-400 ng/ml-) [Kim *et al*, 2000]. Ang-1 was used as a positive control, and it also induced an anti-apoptotic effect that was dose-dependent. Later on, the same experiment was repeated with an excessive concentration of soluble Tie2 receptor and PI3K inhibitors, which further blocked the antiapoptotic effects of Ang-2 in high concentrations. This last experiment suggests that Ang-2 is related to Tie2 receptor binding and involves the PI3K signaling pathway. This is analogous to Ang-1, so it makes it somewhat clear that they are not entirely antagonists, it is far more complex than that.

Yuan *et al* intended to further unveil the actions of Ang-2 on ECs, wondering whether Ang-2 was an antagonist of Ang-1 as past work stated, or an agonist. This study showed that exogenous Ang-2 at different concentrations (200 or 400 ng/ml) increased phosphorylation of soluble Tie2 receptors, but this phosphorylation was even higher when administering exogenous Ang-1 alone (200 ng/ml) as a positive control [Yuan *et al*, 2009]. Ang-2 seems to be a less potent activator of Tie2 than Ang-1 and apparently, this comes along with an Ang-2 affinity to bind to Tie2 that is lower than Ang-1: competition binding assays were performed with Ang-1 and Ang-2 related to Tie2, showing that with 100 ng/ml Ang-1 (1/5 of the Ang-2 concentration) the binding of 500 ng/ml of Ang2 to Tie2 was reduced compared with the absence of Ang-1. Other studies by this group also showed that endogenous Ang-2 acts toward Tie2 in an autocrine way (similar to the Scharpfenecker study). Two

other findings of this publication were similar to Kim's study mentioned in the previous paragraph: the downstream pathway for Ang-2 to act on Tie2 is the PI3K/Akt pathway, and both Ang-1 and Ang-2 can have anti-apoptotic effects on serum-deprived ECs (with Ang-1 having a stronger effect). Thus, the main conclusion of this study by Yuan is that cultured ECs go under anti-apoptotic effects with endogenous Ang-2, but the presence of Ang-1 can actually diminish all of the Ang-2 mediated effects. This means that Ang-2 is a partial agonist and also antagonist of Tie2.

Finally, a study by Roviezzo *et al* was done about Ang-2 and the phenomenon that is crucial to this paper essay: vascular permeability. This work was done on two *in vivo* mouse models of acute inflammation to evaluate whether Ang-2 can affect vascular leakage. The area used for acute inflammation was the mouse hind paw. Maisonneuve earlier stated that overexpression of Ang-2 leads to leaky vessels, and the same findings were evident with subplantar injections of Ang-2 with a dose- and time-dependent increase in paw volume and edema formation. This change did not occur when performing the same study with Ang-1 or Ang-4 [Roviezzo *et al*, 2005]. Edema formation was Ang-2 specific, since injections of both Ang-2 and Ang-1 only lead to Ang-1 abolishing the increase in volume. Also, measuring NO and PGE2 (mediators produced by ECs that modulate permeability) showed that edema formation by Ang-2 did not depend on these two molecules, and the edema was mainly fluid passage since measuring for myeloperoxidase (MPO, that helps estimate the presence of neutrophils in tissues) activity showed a low activity, indicating there was no major cell migration and passage. This indicates that Ang-2 has a behavior dependent on its context, since it has been proven that it facilitates EC migration. The overall conclusion of Roviezzo and his group on the endothelial barrier function was that Ang-2 can modulate an inflammatory response by facilitating vascular leakage, but this was not a typical inflammatory response since it mainly consisted of fluid passage but did not promote leukocyte migration.

Ang-2 in septic shock

Jumping off from Roviezzo's work on Ang-2 and inflammatory responses with edema formation in the mouse paw, there is plenty of evidence to sustain that this expression of this glycoprotein is increased with proinflammatory stimuli (Ang-2 levels are also increased in other diseases that have either inflammation or vascular leakage, such as congestive heart failure). Orfanos *et al* linked this statement with the fact that sepsis is a systemic inflammatory response, and postulated that serum levels of Ang-2 (sAng-2) would be high and correlate with inflammatory mechanisms and disease severity [Orfanos *et al*, 2007]. 61 patients were enrolled and categorized according to their sepsis stage as follows: patients with systemic inflammatory response syndrome (SIRS) or no SIRS, sepsis, severe sepsis and septic shock. When measuring sAng-2 in patients with no SIRS, sepsis and severe sepsis, the angiopoietin had its higher levels in the latter. Also, sAng-2 correlated to disease

severity scores in sepsis (Acute Physiology and Chronic Health Evaluation, *APACHE II* and Sequential Organ Failure Assessment, *SOFA*) but had an inverse correlation to serum albumin levels in patients. Moreover, the levels of the inflammatory mediators TNF- α and IL-6 showed a positive linear relationship with sAng-2 levels. Based on these results, Orfanos stated that high sAng-2 could contribute to the processes that take place in severe sepsis such as vascular leakage (also taking into consideration the inverse relation between sAng-2 and serum albumin in patients), edema formation and organ dysfunction, given that it was correlated with two important mediators that are key in the pathophysiology of sepsis, TNF- α and IL-6. Ang-2 certainly related to the severity of disease, maybe representing a prognostic factor for sepsis. This study further established the link between Ang-2 and the inflammatory systemic response in sepsis.

Multiple studies correlated levels of Ang-2 with the severity of septic shock, showing higher levels of patients with worse outcomes and non-survivors. One example was the study done by Kranidioti et al, where out of 110 patients enrolled, sAng-2 was higher in patients with septic shock than sepsis, severe sepsis and healthy volunteers. After a follow-up period of 28 days, sAng-2 levels proved to be higher in non-survivors compared with survivors [Kranidioti *et al*, 2009].

Ang-2 in AKI

Not many studies have been performed with Ang-2 in AKI, but previous work describing vascular stability and leakage can be translated into the renal environment. This is because septicemia does induce endothelial activation in the kidneys, along with vascular permeability and glomerular barrier dysfunction.

Given that angiopoietins exert their effect by acting on Tie2 receptors, van Meurs *et al* proposed that alterations in these receptors might be the underlying cause for the loss of vascular stability in kidneys after shock, either hemorrhagic or septic [van Meurs *et al*, 2009]. Their study was performed to evaluate Tie2 receptor expression and changes in the glomerular barrier function, in the context of acute shock. In mouse models of AKI during hemorrhagic and septic shock, alteration in Tie2 expression were investigated and correlated with the development of proteinuria as a measure of glomerular barrier loss. And later, human studies were performed with a volunteer endotoxemia model and also kidney slices that had been exposed to mediators present in sepsis. In the septic shock model (which is the one relevant to this paper essay), Tie2 expression was downregulated, on both the mRNA and protein expression level, although levels of both increased to be almost normal 24 hours after LPS administration. This drop in mRNA and protein expression of Tie2 could not be explained with neither an *in vitro* experiment (glomerular ECs incubated with LPS and TNF- α , to try to unveil a molecular mechanism) or in human kidney slices to find a

possible cell participating in this phenomenon. Along with reduced Tie2 levels, LPS administration also resulted in an inflammatory response (with inflammatory proteins such as E-selectin), and alterations in the glomerular barrier (with an increase in urinary albumin/creatinine ratio). The majority of white cells in this LPS-induced response were neutrophils, and the interaction between leukocytes and ECs can activate and damage the endothelium. This is why the next step was to abolish neutrophils, to find a link between neutrophil-EC interaction and the loss of Tie2 with glomerular alterations. Neutrophils were depleted prior to the administration of LPS, and later Tie2 levels and presence of proteinuria were examined. Depletion of neutrophils did not rescue Tie2 downregulation at the mRNA and protein levels, but it did decrease the occurrence of proteinuria (figure 3).

Overall, these data demonstrate that under conditions of shock Tie2 expression levels in the renal microvasculature are downregulated, however this effect may not be the cause for altered glomerular function. Acute shock with damage in the glomerular barrier is accompanied by the influx of neutrophils, and when these cells were depleted no change was observed in Tie2, suggesting no relation between Tie2 expression and the control of glomerular filtration.

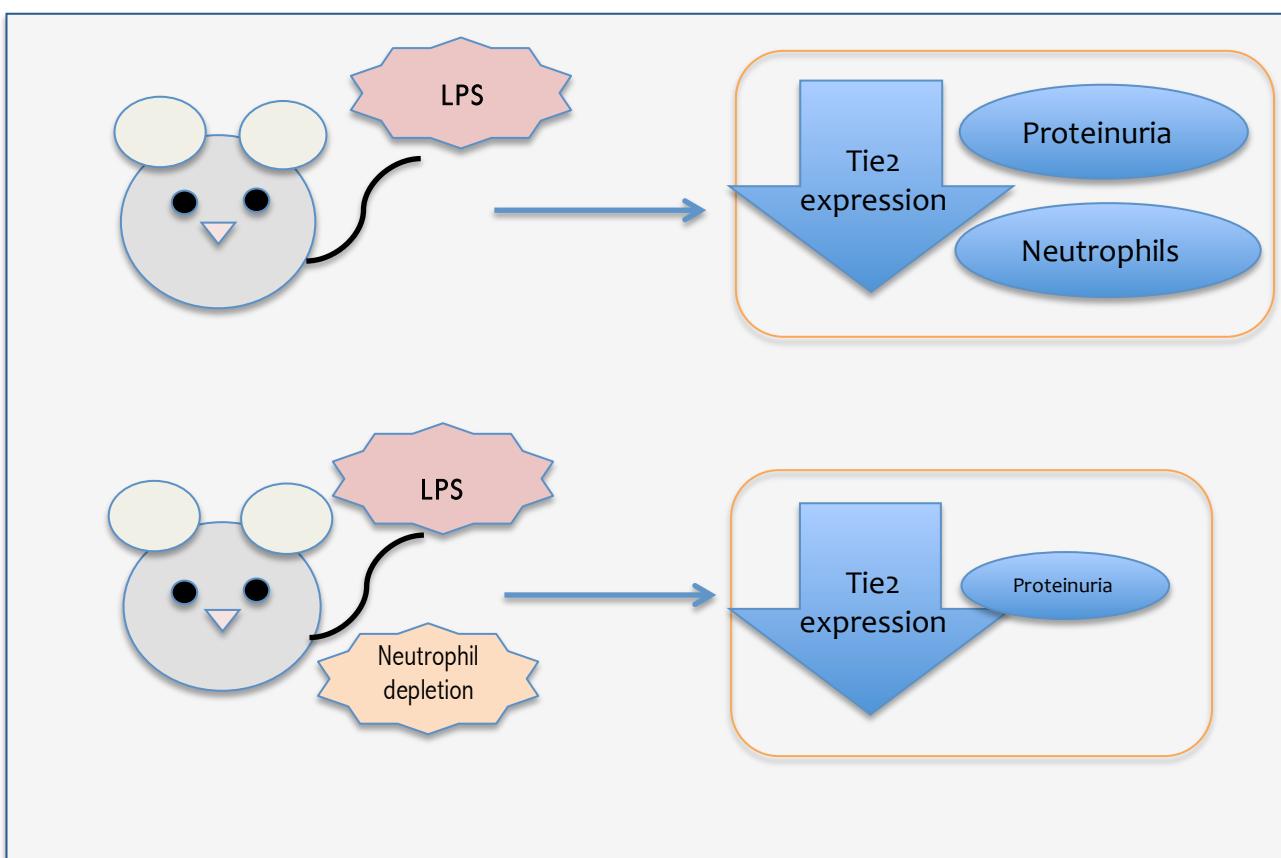


Figure 3: On two mouse models, LPS injections induced acute shock. On the first experiment, while analyzing the glomeruli, a downregulation of Tie2 receptor expression (on mRNA and protein levels) was reported, along with proteinuria and an inflammatory response. The predominant white cells on this inflammatory response were neutrophils. The second experiment depleted neutrophils before treatment with LPS, resulting in the same response on Tie2 expression, but a decrease in the occurrence of proteinuria [van Meurs *et al.*, 2009].

In mice, there is expression of Ang-2 during development, whereas it decreases in adult kidneys. Ang-2 levels in glomeruli are very low or even absent, but an increase in Ang-2 expression has been reported in diseases such as diabetic nephropathy and glomerulonephritis. Davis *et al* performed a study to analyze whether Ang-2 has a significant role in the pathophysiology of glomerular disease, by generating transgenic mice where Ang-2 could be induced and overexpressed in the glomeruli only. Overexpression of Ang-2 in mice showed a significant but modest increase in albumin excretion, but no edema formation, alterations in creatinine clearance or drastic changes in blood pressure. This increased albumin excretion was maintained for several weeks: when measures were done 5 weeks after administration of doxycycline to induce transgenic overexpression on Ang-2 and also after 10 weeks [Davis *et al*, 2007]. Histologically, it was reported that the glomeruli structure was neither swollen nor contracted, but there was an increase in cell apoptosis, more particularly in ECs. This study established a direct correlation between the abnormal presence of Ang-2 in the glomerular cells and an alteration in barrier function with proteinuria.

Why target Ang-2 to restore integrity of the endothelium?

The studies described above suggest that the Ang/Tie2 system can represent a good target to treat several conditions, specifically the disruption of the endothelium with consequent permeability in AKI during septic shock.

Overexpression of Ang-2 was shown to disrupt the endothelial quiescence that Ang-1 acting on Tie2 can produce, and also aid vascular cancer cells to have anti-apoptotic effects and further proliferate and form new vessels (also in diabetes and ophthalmologic diseases). The Ang/Tie 2 system has advantages and disadvantages when it comes to targeting it to treat diseases. Side effects are a concern, especially since both angiopoietins involve the PI3K/Akt pathway, and this is a signaling pathway common to many other diseases (such as endometrial cancer, to name one example). Special attention must be drawn to develop drug-targeted pathways that are specific to a certain disease. Since the functions of the Ang/Tie2 system are not performed by other system, the desired therapeutical benefit can become uncontrolled and not reversible. Sometimes that is an undesired effect of targeted therapy that should not arise. However, the Ang/Tie2 system lacks another system that could replace its functions, it is very specific, so this could represent a benefit since its effects could be inhibited or exacerbated for therapy without a “bypass” effect of the cell [van Meurs *et al*, 2009].

Another possible confounder that should be taken into account is to assume that Ang-1 is always a positive agent (it has been linked to the development of pulmonary hypertension), and Ang-2 always a negative agent to the ECs. This is the case in sepsis and septic shock, with high levels of

sAng-2 associated with severely ill patients and high mortality, but at the same time Ang-2 is a key mediator of the inflammatory response, needed for host defense. Maybe abolishing Ang-2 entirely would not be suitable, since it would make the cell lose other vital functions, like taking part in the menstrual cycle for example. Also, two other important characteristics to consider about Ang-2 before defining it as a negative agent: it can be an agonist for Ang-1, which is described to have positive quiescent effects on ECs, and it differs depending the cell where it is located and the context. Ang-2 is necessary for vessel regression and remodeling, since Gale *et al* demonstrated that Ang-2 knock-out mice died around the age of 2 weeks because of abnormal vascular remodeling [Gale *et al*, 2002]. It should also be present in women during their menstrual cycles and pregnancy. Targeting Ang-2 should be specific to the area desired (in the case of this paper essay, the ECs in kidneys during or after septic shock), since this glycoprotein is located in the ECs of multiple organs that are vital, such as the heart and brain.

Despite all the negative aspects, the Ang-2/Tie2 system has great potential to be targeted to treat disease, since many other cytokines and proteins that act alongside this system are not that good to target because they may lose other vital functions (for example, targeting TNF- α to reduce inflammation responses may lead to an unwanted immunosuppression).

“Ang-2 does not act alone”: VEGF and integrins

An important agent that is related to many effects of Ang-2 is VEGF. VEGFs are endothelium-specific cytokines and crucial regulators of microvascular permeability and angiogenesis. They are synthesized and released by smooth muscle cells, lung epithelium, platelets, leukocytes and macrophages. They go through upregulation in response to the following pathologic conditions: wound repair, myocardial ischemia, tumor growth and also, endotoxemia. Pickkers *et al* reported a contributing role of VEGF to vascular permeability during sepsis by studying children with invasive meningococcal infections [Pickkers *et al*, 2005]. VEGF interacted with other cytokines and complement to modulate vascular permeability reported in this study.

The study already mentioned by Roviezzo *et al* measuring paw volume in mice while administrating Ang-2, also measured VEGF during this phenomenon [Roviezzo *et al*, 2005]. By itself, VEGF induced edema formation in the hind paw of mice, with similar kinetics as Ang-2 and being time- and dose-dependent. Next, injections of both VEGF and Ang-2 were administered to evaluate whether they had a synergistic effect. When given at a submaximal dose (30 ng of Ang-2 and 3 ng of VEGF) this did result in an additive effect, but when given at maximal doses the total edema formation was not greater than the effect obtained with each molecule by its own. This could mean that VEGF and Ang-2 act through similar mechanisms to enhance vascular permeability and

edema formation.

In the study by Davis *et al* overexpression of induced Ang-2 in glomeruli developed albuminuria and apoptosis of glomerular ECs. In these experiments, a decrease in VEGF-A expression was observed. Since VEGF-A is crucial for the maintenance of endothelial integrity, this indicates that Ang-2 does not act alone, so targeting therapy towards this angiopoietin does not guarantee complete success. VEGF-A is also a potential target to restore integrity in the endothelium, but the focus should be solely on the glomeruli, since this protein is present in all endothelial cells. Since it is also involved with cell migration and inhibiting apoptosis, these functions should not be dismissed.

Integrins are heterodimers that are in charge of connecting the extracellular matrix with the cytoskeletal proteins. They consist of two subunits: α and β . The $\alpha v \beta 3$ integrin is related with ECs and their activation, migration and adhesion. Also, antibodies that block $\alpha v \beta 3$ integrins inhibit angiogenesis. Thomas et al proposed to examine whether these integrins are related to the Ang/Tie2 system, by evaluating the stimulation of Ang-2 on $\alpha v \beta 3$ integrin cell surface trafficking. HUVECs were cultured and treated with myc-tagged Ang-1 and Ang-2, antibodies anti- $\alpha v \beta 3$, anti-Tie2, and anti-FAK [Thomas *et al*, 2010]. Both angiopoietins induced translocation of the Tie2 receptor and $\beta 3$ integrins to cell-cell junctions, but only Ang-2 was observed to induce complex formation between Tie2 receptors and $\alpha v \beta 3$ integrins, and this effect was dose-dependent (*figure 4*).

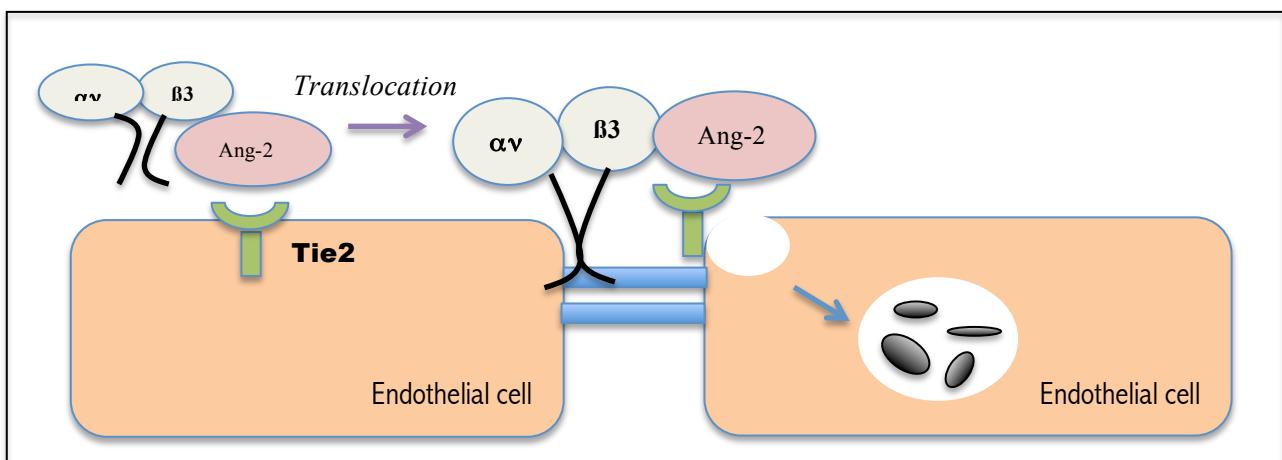


Figure 4: Angiopoietin 2 induced formation of a complex: $\alpha v \beta 3$ integrin with Tie2 receptor, and translocation of these to cell-cell junctions. Later, these integrins were reported to go through internalization and degradation in lysosomes, suggesting that angiopoietin 2 is crucial for $\alpha v \beta 3$ integrin turnover [Thomas *et al*, 2010].

Along with complex formation by Ang-2, several mechanisms occurred that were inherent to this event: the recruitment of FAK, which is responsible for enhancing signal transduction of growth factor receptors; dissociation of the adaptor proteins Talin and p130cas from the $\alpha v \beta 3$ complex (this adaptor proteins usually connect integrins with cytoskeleton proteins) and $\alpha v \beta 3$ integrin ubiquitinylation, internalization and lysosomal degradation. Overall, Ang-2 stimulates $\alpha v \beta 3$

turnover and according to Thomas *et al* this may be a possible mechanism behind Ang-2 induced endothelial dysfunction.

The future: Possible perspectives

As said previously, Ang-2 can be modulated, but not in a way that it has to be abolished completely. All the previous studies discussed in this essay prove that Ang-2 has a crucial role in endothelial integrity with increased permeability and edema formation. Also, it arises with high levels during sepsis in its many stages (*i.e.* septic shock) and is associated with an adverse prognosis. The interest in this essay is AKI, and studies on Ang-2 upregulation have shown glomerular injury and proteinuria. This angiopoietin could represent a fundamental target to restore integrity in glomerular ECs and improve disease in a septic patient.

In the area of targeted therapy, several agents have the potential to possibly modulate Ang-2 in a safe and effective way.

One example would be to study an animal model (murine if possible), induce sepsis with LPS, and try using a secretagogue (a substance that induces the secretion of another) that can modulate secretions from WPBs. This is the compartment in the endothelium where Ang-2 is stored, so to control it means regulating how much Ang-2 would be liberated and at what precise time. Something to take into account in this approach is that WPBs store other molecules besides Ang-2, for example von Willebrand Factor (glycoprotein in charge to regulate hemostasis), so this should be minded in order not to unleash an unwanted effect.

Another possible agent to target therapeutically is the transcription factor Kruppel-like factor 2 (KLF-2), which normally downregulates Ang-2 expression on ECs while upregulating Tie2 expression [Augustin *et al*, 2009]. This would also be beneficial since the overexpression of Tie2 would later on interact with Ang-1 and enhance the quiescent non-activated state of ECs. The expression of KLF-2 is regulated by shear stress, so a model could mimic this phenomenon and in that way, regulate levels of KLF-2.

A clear concept that is key to working with Ang-2 is that it is not a merely antagonist of Ang-1. However, the balance of both angiopoietins is delicate, and the actions of Ang-2 are context-dependent, so if targeted therapy were to be developed, it should take into account levels of other angiopoietins and most importantly, Tie2 levels. Also, both angiopoietins act through the PI3K pathway, so this could represent a common link that could be modulated to have a real impact on glomerular integrity.

In my opinion, more studies should be performed on Ang-2 acting on the glomerular barrier during AKI but also integrating other molecules, watching closely if there is a synergy between them. Also, other molecules could act through similar mechanisms as Ang-2 (*i.e.* VEGF-A), so maybe

studying this fact a bit deeper could lead to another molecule to be modulated, an intermediary. Maybe it is not as simple as only regulating Ang-2 expression, and co-factors that interact with it could help in targeted therapy. Several antibodies exist against VEGFs, and maybe this could easily modulate Ang-2 effects, given that some studies have shown either that VEGF can rescue the damage done by Ang-2 [Scharpfenecker *et al*, 2004] or aid to the effects of this angiopoietin [Davis *et al*, 2007].

Integrins also present a possible target, with the possibility of trying to find a way to “deconstruct” the complexes formed by Ang-2 in order to avoid the turnover of integrins. By avoiding the turnover of $\alpha\beta 3$ integrins, endothelial destabilization may be prevented.

Another option instead of modulating Ang-2 could be the exogenous administration of a certain agent that could revert the damage already done. Ang-1, sTie2 and VEGF already proved to do that in Scharpfenecker’s study, and it could be a possibility to look into it if the exogenous administration is not harmful to a human subject.

Conclusion

Ang-2 is a glycoprotein that acts on the Tie2 receptor expressed by ECs, but not always in the same way. Many researchers believe that Ang-1 is the “good” agent to modulate endothelium and maintain and restore its quiescent state, while Ang-2 is the “bad” agent which only activates ECs to make them become disrupted and causing vascular leakage. Although this is true, and relevant to the topic of this paper essay, that is not always the case, and it should be taken into consideration when targeting Ang-2 as a possible effective therapy. Septic shock and its subsequent AKI are complex systemic diseases, with multiple agents causing damage, so it is not as simple as just targeting one glycoprotein and expecting systemic integrity of endothelial barriers. However, Ang-2 may represent a potential element for target therapy when taking into account other molecules that act along or against it.

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