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Molecular regulation of acute Tie2 suppression in sepsis

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Abstract

Objective—Tie2 is a tyrosine kinase receptor expressed by endothelial cells (ECs) that maintains vascular barrier function. We recently reported that diverse critical illnesses acutely decrease Tie2 expression and that experimental Tie2 reduction suffices to recapitulate cardinal features of the septic vasculature. Here we investigated molecular mechanisms driving Tie2 suppression in settings of critical illness.

Design—Laboratory and animal research, post-mortem kidney biopsies from acute kidney injury (AKI) patients and serum from septic shock patients

Setting—Research laboratories and ICU of Hannover Medical School, Harvard Medical School & University of Groningen.

Patients—Deceased septic AKI patients (n=16) and controls (n=12) & septic shock patients (n=57) and controls (n=22)

Interventions—Molecular biology assays (western blot, qPCR) + flow and transendothelial electrical resistance (TER) experiments in human umbilical vein endothelial cells (HUVECs); murine cecal ligation & puncture (CLP) and LPS administration

Measurements and main results—We observed rapid reduction of both Tie2 mRNA and protein in mice following CLP. In cultured ECs exposed to TNFα, suppression of Tie2 protein was

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more severe than Tie2 mRNA, suggesting distinct regulatory mechanisms. Evidence of protein-level regulation was found in TNF α -treated ECs, septic mice, and septic humans, all three of which displayed elevation of the soluble N-terminal fragment of Tie2. The matrix metalloprotease MMP14 was both necessary and sufficient for N-terminal Tie2 shedding.

Since clinical settings of Tie2 suppression are often characterized by shock, we next investigated the effects of laminar flow on Tie2 expression. Compared to absence of flow, laminar flow induced both Tie2 mRNA and the expression of GATA3. Conversely, septic lungs exhibited reduced GATA3, and knockdown of GATA3 in flow-exposed ECs reduced Tie2 mRNA. Post-mortem tissue from septic patients showed a trend toward reduced GATA3 expression that was associated with Tie2 mRNA levels ($p < 0.005$).

Conclusions—Tie2 suppression is a pivotal event in sepsis that may be regulated both by MMP14-driven Tie2 protein cleavage and GATA3-driven flow regulation of Tie2 transcript.

Keywords

Tie2; Angiopoietin; Endothelium; flow; shedding; GATA3; MMP14

INTRODUCTION

Pathological host responses to infection drive the syndrome of sepsis (1). Mortality in its most severe form - septic shock - can exceed 60%, and its incidence is rising (2). So far, specific therapeutic strategies that target the host response have focused on the modulation of the immune response (e.g. TNF α inhibition etc. (3)). However, the individual immune status of a given patient is highly variable, can change quickly and so far, no reliable monitoring tools are available. In response to pathogen products and early-response cytokines, the microvasculature undergoes a dramatic transformation during sepsis. (4). The septic host vascular response is characterized by a pro-adhesive, pro-apoptotic, pro-inflammatory endothelial surface and increased permeability (5-7). These changes collaborate to reduce tissue perfusion and oxygenation, thereby contributing to multiple organ dysfunction (MOD) and death.

The Tie2 tyrosine kinase has been increasingly recognized as a molecular switch that shifts the endothelium from quiescence to promote the vascular phenotypes of sepsis (8). Reports from many laboratories now show that numerous manipulations of Tie2's endogenous ligands - the Angiopoietins (Angpts) - that favour receptor activation lead to improvement of survival and organ function in rodent sepsis (9-12). Comparatively less attention has focused on regulation of the receptor itself, whose expression is markedly reduced in sepsis (14-16). We recently showed that diverse severe infections share a common reduction of Tie2 expression, and that loss of Tie2 even in the absence of infection is sufficient to destabilize the vasculature (13). Together, these studies implicate a model in which sustained or increased Tie2 signalling may combat adverse outcomes in sepsis whereas the pathogenic host response of sepsis may be intimately linked to Tie2 suppression.

In this study, we sought to understand mechanisms by which diverse severe infections could converge on the suppression of Tie2. We therefore focused on board pathophysiological

processes common to such settings, namely severe inflammation and reduced flow. We found that inflammation drives a protein-level, i.e., post-translational, suppression of Tie2 and that reduced flow suppresses Tie2 transcripts.

MATERIAL & METHODS

Antibodies and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Antibodies against Tie2 (C-20) (Santa Cruz Biotechnology, Inc.), Tie2 (clone Ab33) (Merck Millipore), GATA3 (D13C9) (Cell Signaling), MMP14/MT1-MMP (D1E4) (Cell Signaling), pAKT (Ser473, D9E) (Cell Signaling), AKT (11E7) (Cell Signaling) and ubiquitin (Cell Signaling) β -tubulin and GAPDH (Santa Cruz Biotechnology, Inc.) were used. Soluble Tie2 levels were determined using the human Tie-2 DuoSet ELISA and the mouse Tie-2 Quantikine Kit. HUVECs were stimulated with TNF α , Angpt-1 and Angpt-2 (all from R&D systems, Minneapolis, MN).

Clinical observational trial

From the medical ICU at Hannover Medical School, Germany, 57 septic patients were enrolled at the time of ICU admission and studied prospectively. Per definition from 2003 only patients with severe sepsis or septic shock were included (SCCM/ESICM/ACCP/ATS/SIS definitions) (17). Local ethics committee approved the study (No. 4373). Only patients between 18 and 75 years were included. Patients' characteristics, including demographic, clinical and laboratory parameters were obtained at the time of enrollment and are shown in Table 1. Twenty-two age-/and gender-matched healthy volunteers served as controls (9 males, 13 females; age 58 (25 to 73 years)). Serum samples for quantification of sTie2 and Angpt-2 were obtained at the time of enrollment. Angpt-2 and sTie2 levels were measured with commercial ELISAs.

Mouse studies and experimental design

All experiments were approved by the local authorities and conducted in accordance with institutional and governmental guidelines. Eight-week-old male C57Bl/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Sepsis was induced *via* cecal ligation & puncture (CLP) as described before (18, 19). Sham mice underwent the same procedure except for ligation & puncture. For induction of endotoxemia, mice received 17.5 mg/kg BW LPS i.p. from Escherichia coli (serotype O111:B4) or vehicle. ECs were isolated from murine lungs using a dissociation kit (MACS Miltenyi, Bergisch Gladbach) and CD146 microbeads together with the MACS magnetic separation system. The purity was 91.3 ± 0.7 %.

In vitro cell culture studies

Human umbilical vein endothelial cells (HUVECs) (passage 3-5) were cultured in EGM-2 medium (Lonza, Basel, Switzerland). For stimulation experiments, HUVECs were starved overnight (0.5% FBS, without supplements) and stimulated with TNF α . Transfection of HUVECs with siRNA was accomplished using Silencer[®] siRNAs (Thermo Fisher Scientific Inc.). For flow experiments, 1.2×10^5 HUVECs were seeded on 0,4 luer slides (Ibidi,

Martinsried, Germany). After 24 hrs cells were put under flow (20 dyne/cm²) for indicated times using a fluidic unit and pump system from Ibidi (Martinsried, Germany).

Real-time quantitative (RT-q)PCR

Total RNA was extracted using the RNeasy Mini/Micro Kit (Qiagen, Hilden, Germany) and then reverse transcribed using Transcriptor First Strand cDNA Synthesis (Roche Diagnostics). RTqPCR was performed by a LightCycler 480 II (Roche). Triplicate RT-qPCR analyses were performed for each sample. Gene expression was normalized to the expression of the housekeeping gene, yielding the CT value.

Immunoblotting

Protein was extracted by using RIPA buffer, resolved with a 10% polyacrylamide gel, followed by blotting on a PVDF (polyvinylidene fluoride) membrane (Merck Millipore, Darmstadt, Germany), blocked with 3% bovine serum albumin (BSA) and incubated with a primary antibody overnight (4°C). Incubation with the 2nd antibody was performed for 1 h at room temperature. Bands were visualized with SuperSignal™ West Pico Chemiluminescent Substrate (Life Technologies) and Versa Doc Imaging System Modell 3000 (BioRad). Quantification of immunoblots was accomplished using Quantity One 4.6.6 (BioRad).

GATA3 plasmid transfection and cell sorting

HUVECs were transfected with a GATA3-overexpressing (GATA3 pEZ-M61) or control plasmid (hLuc pReceiver-M61) using Xtreme Gene HP reagent (Roche) 3 h after seeding to 70% confluency. Both plasmids express GFP under the same promoter to mark transfected cells. After 48 hrs, sorting of GFP⁺ cells was accomplished with a FACS Calibur (BD Biosciences) for further analysis.

Transendothelial Electrical Resistance (TER)

TER was measured using an electric cell-substrate impedance sensing system (ECIS) (Applied BioPhysics Inc.) (20). Values were pooled at discrete time points and plotted versus time. Each conditions' end point resistance was divided by its starting resistance to give the normalized TER.

Human Kidney biopsies

We re-evaluated post-mortem kidney biopsies (21) from 16 patients aged 18 years or older, who died of sepsis. Patients with pre-existing chronic kidney disease, active auto-immune disorder with renal involvement, and immune-suppressive treatment were excluded. Normal parts of kidneys from 12 patients who underwent a total nephrectomy for renal tumors, served as controls. Families were given oral and written information about the postmortem study. The postmortem biopsies were waived by the Medical Ethical Committee of the UMCG, Groningen, The Netherlands (METc 2011/372).

Statistical Analysis

Statistical significance was evaluated using Mann-Whitney test unless otherwise noted. For correlation statistics, Spearman test was performed. All experimental results are presented as

mean $\pm$ SEM and two-tailed p value of less than 0.05 was considered to indicate statistical significance. Analysis and graph generation were performed in GraphPad Prism 6.0 (La Jolla, CA).

RESULTS

Acute downregulation of Tie2 *in vivo* and *in vitro*

Applying the CLP surgical model in mice, we confirmed that sepsis profoundly lowers Tie2 expression both at the level of protein and mRNA (Supplemental Digital Content - SFigure 1AC). In order to elucidate the underlying mechanisms, we focused on two central pathophysiological responses associated with sepsis: inflammation and reduced flow. Testing different inflammatory mediators of sepsis (IFN γ , IL1 β , LPS and TNF α) to ECs, we found TNF α was a potent and sustained suppressor of Tie2 protein. In contrast, the transcriptional response to TNF α was biphasic with an early drop in Tie2 mRNA followed by an increase up to two-fold after 24 hrs. TNF α was also able to replicate functional endothelial consequences of sepsis (Supplemental Digital Content - SFigure 1D-G). The discrepancy between protein and mRNA response suggested that TNF α may be provoking a distinct effect on Tie2 protein unrelated to previously described transcriptional regulation (22).

Regulation of Tie2 protein by posttranslational ectodomain shedding

To evaluate TNF α -dependent post-translational Tie2 regulation, we first sought to establish an effect on protein distinct from an effect on mRNA. We therefore compared Tie2 mRNA stability to protein expression. The *in vitro* half-life of Tie2 mRNA appears to be approximately 10 hrs (Supplemental Digital Content - SFigure 2), supporting a degradation mechanism as Tie2 protein was almost absent after 8 hrs of TNF α stimulation (SFigure 1E). Notably, we found no effect of ligand-driven Tie2 stimulation (Supplemental Digital Content - SFigure 3), and TNF α did not induce Tie2 protein ubiquitination (Supplemental Digital Content - SFigure 4). We then applied cellular fractionation to assess which compartment(s) exhibit the most reduction of Tie2 protein. TNF α more severely lowered membrane-bound Tie2 protein as compared to cytosolic Tie2, suggesting protein degradation at the cell surface (Supplemental Digital Content - SFigure 5). Using an ELISA technique to detect the extracellular N-terminal epitope of Tie2, we found that TNF α indeed increased the soluble ectodomain fraction of Tie2 (sTie2) in HUVEC supernatants (Figure 1A & Supplemental Digital Content - SFigure 6). Consistent with this *in vitro* finding, we also observed an increase of the soluble N-terminal extracellular sTie2 fragment in the serum of septic C57/Bl6 mice (Figure 1A). We then measured the circulating sTie2 fragment in sera from 57 septic shock patients and 22 healthy controls (demographics summarized in Supplemental Digital Content - Table 1). Septic humans had significantly higher sTie2 levels compared to healthy controls (control: 12.29 ± 0.71 ng/mL vs. septic humans 18.77 ± 0.48 ng/mL, $p < 0.0001$, Figure 1A). Moreover, levels of sTie2 were associated with circulating CRP, a marker of systemic inflammation ($r = 0.3$, $p = 0.008$), with the Tie2 antagonist Angpt-2 ($r = 0.4$, $p = 0.0025$) and with the complement factor C3 ($r = 0.3$, $p = 0.009$, Supplemental Digital Content - SFigure 7). Together, these data suggested that inflamed ECs, septic mice, and septic humans may share a common mechanism of Tie2 shedding.

Mechanistic role of matrix metalloproteinase (MMP) 14 in septic Tie2 shedding

During angiogenesis, Tie2 is proteolytically cleaved by the matrix metalloprotease MMP14 to generate a soluble ectodomain fragment (22). MMP14 was increased in septic murine lungs (Figure 1B & Supplemental Digital Content - SFigure 8), proposing a putative mechanism. We therefore conducted RNAi against MMP14 in TNF α -stimulated HUVECs (Supplemental Digital Content - SFigure 9). This manoeuvre abrogated the TNF α -driven appearance of sTie2 (Figure 2A) in EC supernatants. The same dependence on MMP14 was evident when LPS was applied to ECs (Supplemental Digital Content - SFigure 10). A pharmacological blocker of MMPs with a compound termed GM6001 (Supplemental Digital Content - SFigure 11) not only affected TNF α -driven shedding but also strongly inhibited baseline Tie2 shedding (Figure 2A, *comparison bar 1 & 3*). Next, we added human (rh) MMP14 to resting ECs to confirm that MMP14 was sufficient to cleave Tie2 ($p < 0.01$) (Figure 2B).

To investigate the signaling consequences of Tie2 proteolytic cleavage, we measured phosphorylation of Akt, a canonical kinase downstream of Tie2. Knockdown of MMP14 enhanced both basal and ligand-induced Akt phosphorylation in HUVECs (Figure 2C, representative immunoblot is shown in the Supplemental Digital Content – SFigure 12A). Since intact Tie2 signaling helps defend EC barrier function, we next tested the effect of MMP14 inhibition in a quantitative assay of transendothelial electrical resistance (TER). In response to TNF α , TER readings declined progressively. Either Angpt-1 application or MMP4 knockdown significantly increased TER in this setting; and the combination of both was better than either alone (Supplemental Digital Content – SFigure 12B). Together these data indicate that proteolytic Tie2 shedding may reduce Tie2 protein during inflammation, which in turn, is driven by induction of MMP14 in sepsis.

Transcriptional Tie2 regulation in sepsis is flow-responsive

The above data demonstrated that MMP14 induction is necessary and sufficient for Tie2 proteolytic cleavage during inflammation but did not explain why sepsis yields a sustained reduction in Tie2 transcripts. Based on an earlier observation from our group showing flowdependency of Tie2 transcription (13) and on the fact that reduced perfusion of the microvasculature is a common shared phenomenon in different critical illnesses that all lead to Tie2 suppression (14), we further analyzed flow-responsive genes in this context. First, we confirmed that Tie2 mRNA was indeed flow-regulated in ECs. Under no flow condition, Tie2 mRNA was modestly, but significantly, reduced as compared to a 20 dyne/cm² flow. To further analyze the loss of mRNA, we screened for flow-responsiveness of several transcription factors that have been linked to Tie2 in previous reports [4]. Among others (Supplemental Digital Content - SFigure 13), we found that the transcription factors GATA binding protein (GATA) 3 and Krüppel-like factor (KLF) 2 were most responsive to flow (Figure 3A). However, only GATA3 reduction (Figure 3B, black bars), but not KLF2 reduction (white bars), was sufficient to reduce Tie2 transcript levels. Furthermore, knockdown of GATA3 reduced both basal barrier function and Angpt-1 induced barrier strengthening (Figure 3C), revealing the functional impact of GATA3 on the Angpt-Tie2 pathway.

The reverse approach (i.e. overexpression) was applied to test if GATA3 was sufficient to induce Tie2 transcription in the absence of flow. Using a GFP-tagged GATA3 plasmid and sorting for GFP+ cells, we observed an immense increase in GATA3 mRNA (10.000 fold) but no significant change in Tie2 (Supplemental Digital Content - SFigure 14).

In comparison to the several other transcription factors previously linked to Tie2, only GATA3 expression was suppressed by sepsis (Figure 4A, black bars, Supplemental Digital Content - SFigure 15). Since GATA3 is also expressed by non-ECs, we isolated CD146⁺ ECs from septic and control murine lungs to confirm that sepsis suppresses endothelial GATA3 in vivo (Figure 4A, white bars).

Returning to the concept of shear stress that led us to explore GATA3 among a larger set of flow-responsive genes, we tested whether flow-dependent induction of Tie2 expression requires GATA3. ECs in which GATA3 had been silenced by siRNA (Supplemental Digital Content - SFigure 16) were markedly impaired in the Tie2 transcriptional response to flow compared to non-silenced ECs exposed to the same flow (Figure 4B & Supplemental Digital Content - SFigure 17).

Further, we tested whether there was a graded association between the degree of GATA3 expression and the degree of Tie2 expression in several model systems: (1) whole lung homogenates from LPS-challenged mice; (2) whole lung homogenates from CLP mice; (3) isolated pulmonary ECs from LPS-challenged mice; and (4) *in vitro* from ECs under flow and static conditions. In all four of these settings, we found a highly significant correlation between Tie2 and GATA3 expression (Supplemental Digital Content - SFigure 18).

GATA3 is associated with Tie2 expression in human septic kidneys

Despite a large body of evidence implicating imbalanced circulating Tie2 ligands in septic humans (8), data on tissue expression or even the activation status of Tie2 in septic humans have been challenging to obtain. Recently, changes in renal Tie2 mRNA expression were reported in human sepsis (23). Given the present findings, we tested whether the association between GATA3 and Tie2 expression observed in septic mice is reproducible in human samples. To do this we analyzed kidney biopsies from deceased septic patients for GATA3 mRNA expression. We observed a trend for reduced GATA3 expression among the septic tissues (Figure 5A). When we conducted the continuous comparison against Tie2 expression, we observed an inter-correlation that was statistically significant (Figure 5B). This result not only validated our findings in cultured ECs and septic mice, but also suggested that GATA3dependent regulation of Tie2 may be a cell-autonomous mechanism of vascular inflammation conserved across species.

DISCUSSION

Tie2 actively promotes vascular homeostasis during quiescence, and in the setting of sepsis, phosphorylation of Tie2 counteracts the development of a pro-adhesive, pro-coagulant, and leaky phenotype (11, 24). While the imbalance in Tie2 ligands that develops during sepsis has been appreciated for over a decade (25), only recently has attention focused on the regulation of Tie2 expression itself during sepsis and related kinds of severe illness (14).

Shared across these settings, reduced Tie2 expression has been shown to suffice to trigger vascular leakage and to enhance coagulation in vivo even in the absence of infection or inflammation. As such, reduction in Tie2 expression during critical illnesses may be a sentinel event in the switch from vascular quiescence to activation. By focusing on twin fundamental pathophysiological disturbances in sepsis—inflammation and reduced microvascular flow—the present results identified two distinct mechanisms by which Tie2 expression is downregulated during sepsis, advancing the field toward a more complete understanding of the host vascular response to severe infection.

We first identified a protein-level downregulation of Tie2 that was uncoupled from the Tie2 mRNA response to canonical inflammatory mediators. After ruling out ubiquitin-mediated Tie2 degradation and ligation-dependent receptor internalization, we focused on proteolytic cleavage that results in shedding of Tie2's extracellular ligand-binding domain and degradation of its membrane-associated endodomain. We found that MMP14 was both necessary and sufficient for Tie2 cleavage and that MMP14 status impacted both the basal and ligand-induced barrier function of confluent endothelium. These results strongly propose endothelial MMP14 as a key molecular and functional regulator of vascular quiescence during sepsis. Based on these findings, endothelial MMP14 may well be a promising target for clinical inhibition.

To understand the sustained reduction in Tie2 mRNA reported in vivo, we hypothesized a role for laminar flow and shear stress, which are largely lost at the apical surface of microvascular endothelium as sepsis evolves into shock. Analyzing a set of previously reported flow-responsive genes (26-28), we found that flow enhances Tie2 mRNA expression in a GATA3-dependent fashion. Targeted depletion of GATA3 reduced flow-dependent Tie2 induction. Moreover, we observed a quantitative relationship between GATA3 and Tie2 mRNA in septic mice, suggesting that gradations of flow impairment may “tune” the host vascular response across a spectrum of phenotypic severity. Integrating these results, we propose that Tie2 expression during sepsis is downregulated at a minimum of two different levels, which together, may synergize to rapidly switch the endothelium from quiescence to activation in response to severe infection (Supplemental Digital Content - SFigure 19).

The present results advance our fundamental understanding of Tie2 regulation in several ways. Proteolytic cleavage of cell surface receptors is a widely recognized mechanism for modulating signal transduction (29, 30). Tie2 ectodomain shedding has been reported in non-septic classical vascular pathologies such as atherosclerosis (31-33), and a link to MMP14 has also been proposed (34). Our results extend this body of work into sepsis, where our data suggest that MMP14 inhibition may enhance both basal barrier function and Tie2-dependent barrier strengthening. These results suggest that Tie2 cleavage by MMP14 is pathogenic in the context of sepsis. However, one study showed that MMP14^{-/-} mice have increased vascular leakage (35). Several possibilities could explain the contrast with our results: (1) developmental effects on MMP14 knockout; (2) MMP14 action in non-ECs; and (3) MMP14 action in ECs, but not on Tie2. For example, the orphan receptor Tie1 is itself rapidly cleaved during acute inflammation (36). Future studies could focus on conditional, endothelial-specific MMP14 disruption. With promising results in such a genetic model

subjected to sepsis, therapeutic endothelial MMP14 inhibition - e.g., by encapsulated RNAi (37) - could be developed for sepsis. Simultaneously, proteomic efforts could be undertaken to identify the set of endothelial targets for MMP14. TNF α surely can induce endothelial permeability and inflammation by a variety of additional mechanisms independent from Tie2 shedding. Finally, while the cleavage studies point to endothelial MMP14 as a compelling target for future study, the mechanisms by which TNF α induces vascular hyperpermeability are likely to be manifold in vivo (e.g. (38))

Whereas the proteolytic cleavage of Tie2 may facilitate a rapid initial response, the transcriptional down regulation of Tie2 may be important for sustaining the activated endothelial phenotype during severe infections that progress to shock. Diminished flow is mechano-sensed by ECs, resulting in flow-dependent gene regulation (39). Kurniati *et al.* showed that Tie2 is flow-regulated in endotoxemia (13). Here, we tested several known Tie2 transcription factors according to their flow responsiveness. Although some of them were flow responsive, only GATA3 was suppressed in sepsis. Our interest in GATA3 was further stimulated by a report that GATA3^{-/-} mice display a heavily abnormal endothelial phenotype (40). Future studies should also explore the mechanisms by which sepsis suppresses GATA3 expression. Nonetheless, our results suggest that GATA3 is important for normal expression of Tie2 when microvascular flow is intact, but that GATA3 alone cannot restore Tie2 expression to flow-associated levels in the absence of flow. One possibility is that GATA3 cooperates with other flow-responses transcriptional machinery to regulate Tie2 gene expression.

The kidney biopsies from deceased donors constitute an early pilot study to investigate Tie2 itself in human sepsis. While tissue was only available at a very late stage of sepsis, the promising trends furnish motivation to study septic tissue from humans obtained across earlier time points that would more closely match the septic mouse tissue studied. More broadly, tissue-based investigation of the host response to sepsis could generate novel hypotheses about disease mechanisms.

If regulation of the Tie2 receptor itself—whether at the level of protein or mRNA—is so critical for the vascular phenotypic switch observed in sepsis, then why have ligand-based interventions met with success in experimental models (12)? First, it is possible that Angiopoietins are acting through Tie2-independent alternate receptors such as integrins (41). However, at least in the context of sepsis, studies from several laboratories propose the importance of Tie2 signaling *per se* downstream of Angpts (18, 42). Second, ligand-based interventions - e.g., excess Angpt-1, Angpt-2 siRNA, Angpt-2 neutralizing antibody, Angpt-2 clustering antibody ((11, 18, 42, 43) - have enhanced survival in septic models, but not fully.

This raises the possibility that interventions to increase the available Tie2 protein for signalling Akt and downstream quiescence pathways could synergize with ligand-based interventions to increase the pool of phosphorylated Tie2 receptors. Conversely, the existence of several mechanisms to downregulate Tie2 suggests that mammals have evolved the ability to “finetune” Tie2 signaling both across time scales and in a quantitative fashion. Consistent with this notion of exquisite regulation are studies demonstrating how the

phosphorylation of Tie2 can be modulated by heteromeric interactions with an orphan receptor, Tie1 (36, 44), and with a transmembrane tyrosine phosphatase enriched in the endothelium, VE-PTP (45). Altogether, the present studies add significant new data regarding the multi-layered mechanisms of Tie2 gene control in the context of severe infection and shock.

CONCLUSIONS

Downregulation of Tie2 expression in sepsis may be a consequence of at least two distinct mechanisms. First, we found that TNF α , a canonical inflammatory mediator of sepsis, triggered MMP14-dependent proteolytic cleavage of Tie2. Endothelial MMP14 was necessary and sufficient for Tie2 cleavage in ECs. Second, we found evidence that reduced microvascular flow—a common phenomenon as sepsis progress to shock—suppresses Tie2 transcription by downregulating GATA3. Future studies are needed to investigate the mechanisms and therapeutic relevance of these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Posttranslational modification by Tie2 ectodomain cleavage in cells, mice and men
(A) sTie2 ELISA (detects N-terminal Tie2 ectodomain) of human umbilical vein endothelial cells (HUVEC) supernatants stimulated with 50 ng/mL $\text{TNF}\alpha$ or vehicle after 24 hrs (n=8, ***p<0.001) and serum from C57/B16 mice after sepsis induction via cecal ligation and puncture (CLP). Mice were sacrificed after 16 hrs. SHAM surgery served as control (n=7-9, **p<0.01). sTie2 ELISA of serum from septic human patients (n=57) and healthy controls (n=22) (healthy control: 12.29 ± 0.71 ng/ml vs. septic humans 18.77 ± 0.48 ng/ml, ****p < 0.0001). **(B)** Densitometric quantification of immunoblots for matrix metalloproteases (MMP) 14 and GAPDH from C57/B16 murine lungs harvested 16 h after cecal ligation & puncture (CLP) or sham surgery (n=9-11, ***p<0.001); qPCR for MMP14 mRNA from the same conditions (n=11-13, ****p<0.0001).

Figure 2. MMP14 expression is both required and sufficient for Tie2 shedding *in vitro* and affects Tie2 downstream signaling

(A) Human umbilical vein endothelial cells (HUVECs) transfected with control or MMP14 RNA(interference)-i were stimulated with 50 ng/mL TNF α for 24 hrs and sTie2 was quantified in supernatants by ELISA (n=6, **p<0.01). (B) sTie2 ELISA of supernatants from HUVECs stimulated with vehicle, 10 μ mol/L GM6001 (a global MMP blocker as positive control) or recombinant human (rh) MMP14 at 3 different doses (low: 40 μ g/mL, med: 100 μ g/mL and high: 300 μ g/mL) (n=6, **p<0.01, ***p<0.001). (C) Tie2 downstream signaling in control or MMP14 siRNA transfected HUVECs has been evaluated by immunoblot for pAKT, AKT and GAPDH after stimulation with 500 ng/mL Angiopoietin-1 (Angpt-1) for 10 minutes. Bar graphs show densitometric quantification of the above results for pAKT over AKT (n=7, **p<0.01, ***p<0.001). Exemplary immunoblot can be found in the Supplemental Digital Content – Figure 11)

Figure 3. Tie2, GATA3 and KLF2 are highly responsive to flow but only GATA3 is required for Tie2 transcription

(A) Tie2 mRNA, GATA3 mRNA and KLF2 mRNA was assessed by qPCR in human umbilical vein endothelial cells (HUVECs) under flow (20 dyne/cm²) and no flow conditions for 48h (n=6-8, **p<0.01, ***p<0.001). (B) Tie2 mRNA level assessed by qPCR in HUVECs transfected with control or either GATA3 siRNA (black bars n=4, **p<0.01) compared to HUVECs transfected with control or KLF2 siRNA (white bars, n=4, p=ns). (C) Transendothelial electrical resistance (TER) was measured using an electrical cellsubstrate impedance sensing system (ECIS) in HUVECs pretreated with control or GATA3 siRNA for 48hrs and then stimulated with 500 ng/mL Angiopoietin-1 (Angpt-1) or control (PBS) (n=4 per condition) TER was recorded over the course of 5h.

Figure 4. GATA3 is analogously downregulated in experimental sepsis and involved in the flow-response of Tie2

(A) Black bars indicate GATA3 mRNA (assessed by qPCR) from mice that underwent sham or CLP surgery and were sacrificed 16 h later (n=10-12, ****p<0.0001). White bars indicate GATA3 mRNA from isolated murine pulmonary endothelial cells. C57Bl/6 mice received 17.5 mg/kg bodyweight LPS or vehicle control for 12 hrs. Endothelial cells were isolated using CD146⁺ magnetic beads (n=5-6, p=0.05). (B) Tie2 mRNA levels in HUVECs transfected with control or GATA3 RNAi for 48h. HUVECs were set under flow (20 dyne/cm²) or no flow conditions for additional 48h followed by qPCR analysis. Bar graphs show the Δ Tie2 mRNA increase upon 20 dyne/cm² flow in control and GATA3 RNAi transfected cells (n=4, *p<0.05).

Figure 5. Regulation of GATA3 in human septic samples

(A) GATA3 mRNA expression in immediate post-mortem kidney biopsies from patients who died of sepsis (n=16) and healthy controls (n=12) (p=0.096) (B) Spearman correlation of Tie2 and GATA3 mRNA level from the same human biopsies ($r=0.519$, $p<0.005$). White dots indicate healthy control biopsies compared to the grey dots (septic shock AKI patients).