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Circulating Heme Oxygenase-1 and Complement Activation in Transplant-Associated Thrombotic Microangiopathy

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A B S T R A C T

Transplant-associated thrombotic microangiopathy (TA-TMA) is a severe complication in patients after hematopoietic stem cell transplantation. The pathogenesis of TA-TMA is still unclear. Previous studies showed that complement activation plays an important role in the development of TA-TMA. However, no data showed which kind of complement component triggers this process. In this study we found that heme oxygenase-1, which could induce decay-accelerating factor (DAF) and inhibit the membrane-attack complex, was significantly decreased in patients with TA-TMA. DAF levels in the TA-TMA group were in line with the levels in the myocardial infarction group but were lower than levels in the healthy, noncomplication, infection, and graft-versus-host disease groups ($P < .05$). Human umbilical vein endothelial cells (HUVECs) incubated with TA-TMA plasma showed lower DAF levels compared with that incubated with normal human plasma. Notably, treatment with N-acetylcysteine (NAC), a drug against oxidation, increased the level of DAF. NAC could also inhibit complement activation in HUVECs incubated with TA-TMA plasma. Taken together, we propose that NAC represents a new potential therapy for patients facing TA-TMA.

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INTRODUCTION

Transplant-associated thrombotic microangiopathy (TA-TMA) is a severe complication in patients after hematopoietic stem cell transplantation (HSCT). Previous investigations suggest that multiple factors resulting in endothelial cell injury lead to the occurrence of TA-TMA. Specifically, the abnormity of the complement system contributes to the development of TA-TMA. TA-TMA shares a similar clinical manifestation with thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (aHUS), which is characteristic with microangiopathic hemolytic anemia, platelet consumption, fibrin deposition in the microcirculation, and, ultimately, end-organ injury [1]. However, the diagnostic criteria of TA-TMA are far from accurate. Although several diagnostic criteria have been proposed, no criterion has been rigorously evaluated clinically because of a lack of pathologic diagnosis. Moreover, the pathogenesis of TA-TMA is still unclear. Conventional treatment management is less efficient [2]. Plasma infusions or therapeutic plasma exchange is considered to have limited efficacy, with relatively low response rates (20% to 50%) in comparison with idiopathic TTP (75%) generally reported [3]. Studies showed that activation of the complement system may be the key to TA-TMA [4,5]. Our previous investigations demonstrated that plasma levels of complement component C5b-9 and C3b were significantly increased in the patients with TA-TMA [6]. In addition, eculizumab, a synthetic anti-C5 monoclonal antibody, inhibits complement-mediated thrombotic microangiopathy in aHUS [7]. Jodele et al. [8] found that 61% of patients achieved complete resolution of TA-TMA with the complement blocker eculizumab. Future studies are important to understand TA-TMA further and may figure out the role of complement activation in TA-TMA, enhance diagnostic strategy, and determine therapeutic approaches and strategies [9]. It is still unclear, however, how the complements are activated.

Heme oxygenase-1 (HO-1) is a stress-inducible cytosolic cytoprotective protein that degrades heme into carbon

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monoxide (CO), ferrous iron, and biliverdin [10]. In oxidative stress response, it protects endothelial cells from apoptosis [11,12]. HO-1 attenuates cellular injury by its antioxidative, antiapoptotic, and anti-inflammatory effects [13,14]. The dysfunction of HO-1 has been implied in a variety of pathologic conditions, including hypoxic lung disease [15], vascular injury [16], and cardiac transplant rejection [17], especially in acute myocardial infarction [18]. In addition, it also regulates the complement pathway by activating complement regulatory proteins [19]. HO-1 increases the level of the complement inhibitor decay-accelerating factor (DAF), a membrane-bound complement regulatory protein that inhibits the synthesis of complement 3 (C3) and C5 convertases. HO-1 may also exert potent cytoprotective and anti-inflammatory effects by minimizing the deposition of C3 and the membrane-attack complex [20,21].

N-acetylcysteine (NAC) has been suggested as a potential treatment for patients with TTP. Li et al. [22] reported a case of a TTP patient relieved with NAC, providing the first clinical evidence for the efficacy of NAC in treating TTP. Furthermore, Cai et al. [23] reported that NAC contributes to a significant increase in the expression of HO-1 mRNA in liver injured by carbon tetrachloride (CCl_4). In our study we explored whether TA-TMA patients might benefit from NAC via targeting HO-1. We hypothesized a strong correlation between lack of HO-1 and TA-TMA. Therefore, HO-1, as a physiologic regulator of DAF, minimizes complement component C5b-9. HO-1 may play a protective role in the pathogenesis of TA-TMA.

METHODS

Study Population

Fifteen patients diagnosed with TA-TMA at the First Affiliated Hospital of Soochow University from June 2011 through December 2016 were consecutively enrolled in our study. The diagnostic criteria [24] included schistocytes $\geq$ 2 per high-power field in peripheral blood, increased lactate dehydrogenase, thrombocytopenia with $<50 \times 10^9$/L or a 50% decrease in platelet count, decreased hemoglobin, negative Coombs test, decreased haptoglobin, and no coagulopathy. We also enrolled 75 age- and sex-matched participants, including 15 healthy subjects, 15 patients with myocardial infarction, 15 patients with severe infections after HSCT, 15 patients with grades III to IV acute graft-versus-host disease (GVHD), and 15 patients with no complications (cardiovascular, nephrologic, metabolic, and autoimmune disorders) after HSCT. The clinical characteristics of the patients are summarized in Supplementary Table S1.

The study protocol conforms to the ethical guidelines of the Declaration of Helsinki and has been approved by the institutional review boards and ethics committees at the First Affiliated Hospital of Soochow University. Written informed consent was obtained from all study participants.

Sample Collection

Peripheral venous blood was collected at the onset of complications and anticoagulated with EDTA-K_2. Samples were centrifuged at 3000 rpm for 10 minutes. Plasma and pelleted cells were preserved at −80°C.

Enzyme-Linked Immunosorbent Assay

The concentrations of plasma HO-1 were determined using commercial human HO-1 ELISA kits (Ameko, Hu, China) according to the manufacturer's instructions. Freezing–thawing cycles were avoided, and each sample was measured in triplicate. The concentrations of HO-1 were determined regarding a standard curve built for the same test set. Laboratory technicians performing the measurements were blind to patients and control subjects.

Reagents

Zinc protoporphyrin IX (ZnPP; Cayman, USA), an inhibitor of HO-1, was dissolved in DMSO. ZnPP was stored in darkness and protected from light. NAC (Beyotime, China) was dissolved in deionized water. All reagents were prepared freshly for each experiment.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and cultured in DMEM medium (Sangon Biotech, China) supplemented with 10% FBS and 1% penicillin-streptomycin (Solarbio, China). Cells were maintained at 37°C in a standard cell culture incubator with 95% air and 5% CO_2. HUVECs were grown to confluence in Petri dishes for 12 hours before indicated treatments.

Flow Cytometry

Cell surface expression of DAF (CD55) and C3 was measured by flow cytometry. After treatments, HUVECs were double-stained with an APC-conjugated antihuman CD55 antibody (BioLegend, USA) and an FITC-conjugated anti-C3c antibody (Abcam, UK). Intracellular reactive oxygen species levels were determined by measuring the oxidative conversion of cell with a reactive oxygen species detection kit (Beyotime, China). A FACS Calibur (BD Biosciences) flow cytometer was used for the measurements. Data analysis was performed using the WinMDI or CFlow Plus (Becton Dickinson) programs. Three replicate repeats were quantified for every experiment.

HO-1 Detection by Real-Time Quantitative PCR

RNA was isolated from up to 10 million cells using the RNAsimple Total RNA Kit (Tiange) after the HUVECs were cultured in indicated conditions. cDNA was prepared from 1 μg of RNA using Reverse Transcriptase MasterMix Kit (Abm). Quantitative PCR for targeted genes was performed using SYBR green (Biosharp). For the quantification of HO-1, a pair of target primers (forward, 5'- TGTGGCCTTGGTCTAACTTTT -3'; reverse, 5'- GAAAAGGTCAGTTCCCCAAGA -3') and the endogenous control GAPDH (forward, 5'-GAGAAGGCTGGGGCTCATTT -3'; reverse, 5'-ATGACGAACATGGGGGCATC -3') were used. Each sample was assayed in duplicate. The amount of target HO-1 gene, normalized to an endogenous reference gene relative to calibrator, was calculated by using the ΔΔCt method.

Statistical Analysis

Data were presented as means ± standard errors of the mean. For comparison between the 2 groups, the Wilcoxon rank sum test was used, and a 2-tailed $P \leq .05$ was considered as statistically significant. Statistical analyses were performed using SPSS software (version 22.0; IBM, Armonk, NY).

RESULTS

Plasma HO-1 Level Is Decreased in Patients with TA-TMA

To explore the link between HO-1 and TA-TMA, we compared plasma levels of HO-1 in TA-TMA patients and control subjects using ELISA kits. As shown in Figure 1, plasma levels of HO-1 in the TA-TMA group were significantly lower than the control subjects after HSCT ($P < .05$). No statistical significance in HO-1 levels was observed among HSCT patients with GVHD, with severe infection, and with no complications. Level of HO-1 in MI patients was provided as a positive control [18].

DAF Expression Is Decreased in Patients with TA-TMA

To evaluate the potential involvement of DAF in TA-TMA, HUVECs were incubated with normal human plasma

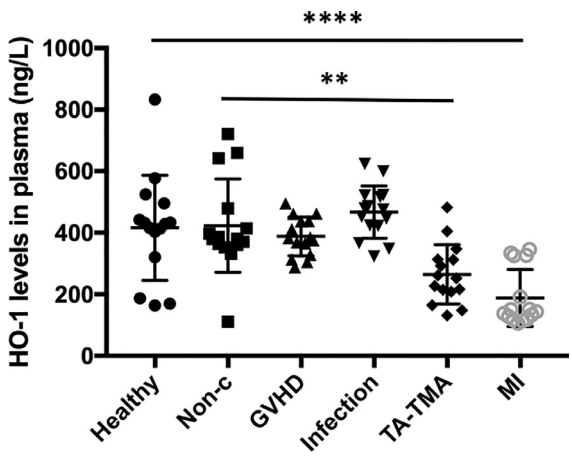

Figure 1. Plasma levels of HO-1 in different groups were measured by ELISA, including healthy control subjects (Healthy), patients with noncomplications after HSCT (Non-c), GVHD, infection, TA-TMA, and acute myocardial infarction patients (MI). $^{**}P < .002$; $^{****}P < .0001$.

or plasma from patients with noncomplications or TA-TMA patients (TA-TMA plasma) for 12 hours. Cell surface expression of DAF (CD55) and C3b was measured by flow cytometry (Figure 2A,B). As shown in Figure 2E, the DAF level was decreased in TA-TMA plasma-treated cells. Moreover, cell surface C3 deposition was increased in the HUVECs incubated with the TA-TMA plasma (Figure 2F). We found no statistical significance between normal human plasma and plasma from patients with noncomplications (Supplementary Figure S1).

HO-1 Regulates Endothelial DAF Expression and C3 Deposition

To test the role of HO-1 in DAF regulation, HUVECs were incubated with normal human plasma in the presence of 10 μM HO-1 inhibitor, ZnPP. We proved the activity of its inhibitory role of ZnPP on HO-1 by Western blot (Supplementary Figure S2). As shown in Figure 3, ZnPP, the inhibitor of HO-1, significantly suppressed DAF expression in HUVECs. The deposition of C3 on HUVECs was slightly enhanced, but no significant difference was observed.

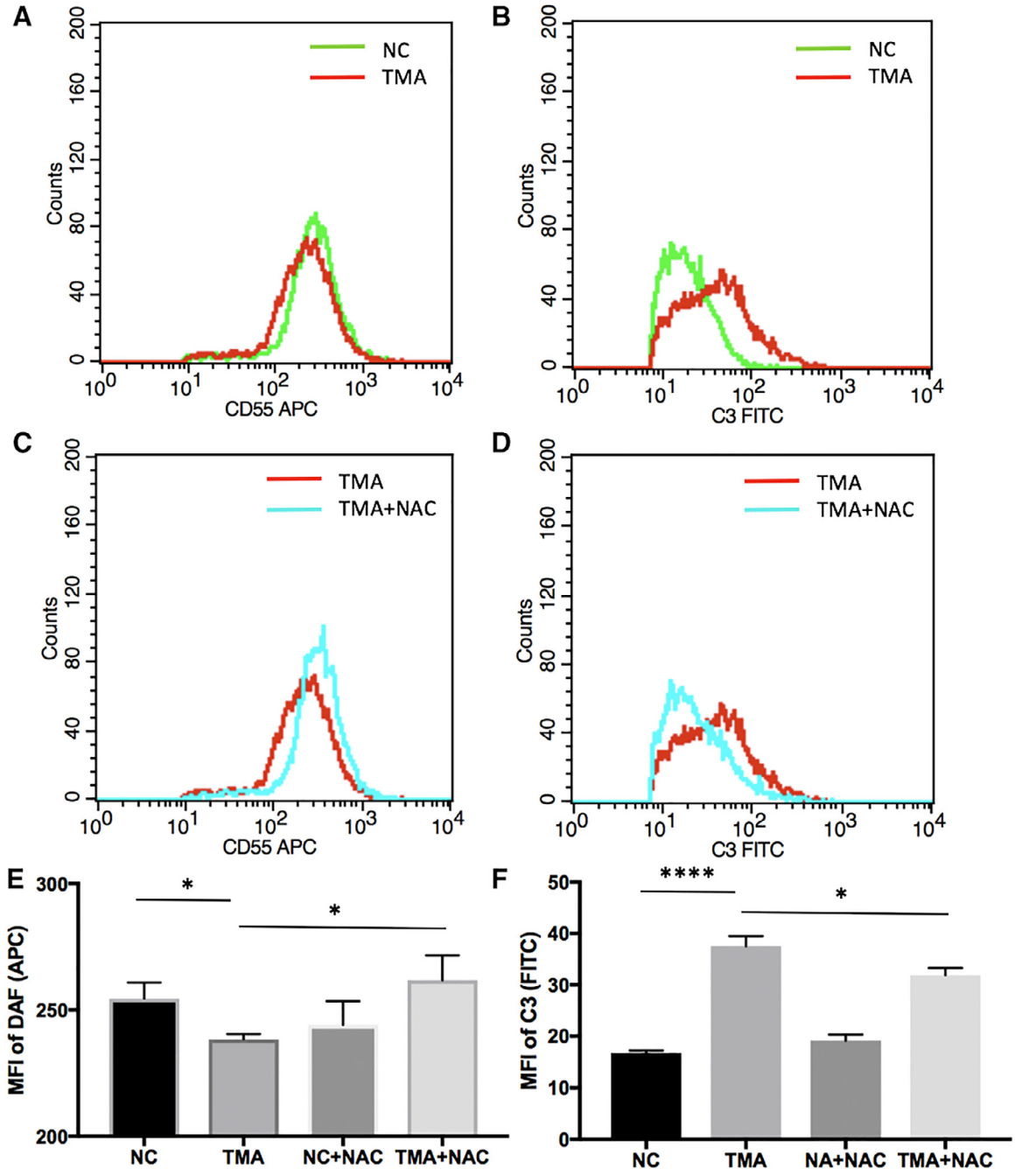

Figure 2. Expression of DAF and C3 deposition on HUVECs were measured by flow cytometry. HUVECs were incubated with different plasma, including normal control (NC) plasma, TA-TMA plasma, normal control (NC) plasma with 10 mM NAC, and TA-TMA plasma with 10 mM NAC. Data from flow cytometry display expression of DAF (A) and C3 (B) of HUVECs incubated with NC and TA-TMA plasma. The expression of DAF (C) and C3 (D) of HUVECs incubated with TA-TMA and TA-TMA plasma with 10 mM NAC were also measured by flow cytometry. Bar graph shows median fluorescence intensity (MFI) DAF (E) and complement 3 (F). Three experiments. $^{*}P < .05$; $^{****}P < .0001$.

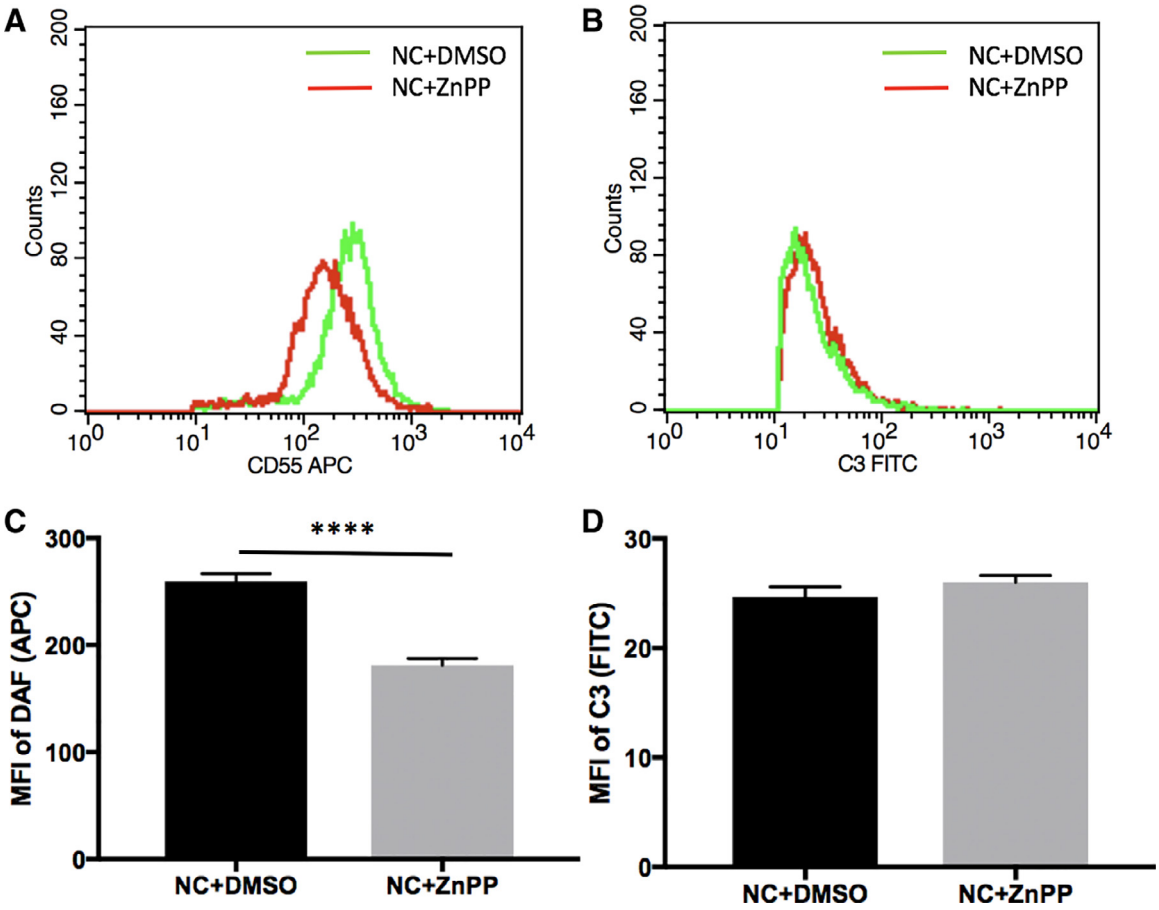

Figure 3. The expression of DAF (A) and C3 (B) of HUVECs incubated with normal control (NC) plasma with solvent DMSO, normal control with inhibitor of HO-1, and ZnPP (NC + ZnPP) were measured by flow cytometry. Bar graph shows fluorescence intensity of DAF (C) and complement 3 (D). Three experiments. $^{****}P < .0001$.

NAC Upregulates DAF and Protects Endothelial Cells from Complement-Mediated Cytotoxicity

Here, we confirmed the antioxidation of NAC by detecting intracellular reactive oxygen species levels in HUVECs (Supplementary Figure S3). A relatively low level of reactive oxygen species was detected in HUVECs incubated with 10 mM NAC in TMA plasma. NAC has been reported to ameliorate TTP, a kind of thrombotic microangiopathies. Therefore, we measured DAF levels in HUVECs treated with NAC. As shown in Figure 2C,D, treatment of 10 mM NAC in TMA plasma-challenged HUVECs increased the endothelial surface DAF expression and attenuated complement deposition as compared with the vehicle (Figure 2E,F).

HO-1 mRNA Level Is Decreased in HUVECs Incubated with Plasma of TA-TMA

In the current study HUVECs were incubated with normal human plasma or plasma from TA-TMA patients for 12 hours. We examined the gene expression of HUVECs by real-time quantitative PCR. As shown in Figure 4, the HO-1 mRNA level was decreased in HUVECs incubated with plasma of TA-TMA compared with normal human plasma.

HO-1 Promoter $(GT)_n$ Repeat-Length Polymorphism

We extracted the DNA from peripheral blood samples in 5 patients with TA-TMA and appointed the Shanghai Genechem Company to detect the nucleotide partial sequence of HO-1 promoter, as shown in Figure 5. The result demonstrated the presence of >32 $(GT)_n$ repeat lengths in 3 of 5 TA-TMA patients. Because of the small size we cannot conclude that HO-1 promoter $(GT)_n$ repeat-length polymorphism is associated with TA-TMA.

DISCUSSION

TA-TMA is a severe complication for patients after HSCT. The pathogenesis of TA-TMA is unclear. Complement activation and endothelial cell injury may be involved in the pathogenesis of TA-TMA. Our previous investigations had demonstrated that plasma levels of C5b-9 and C3b were

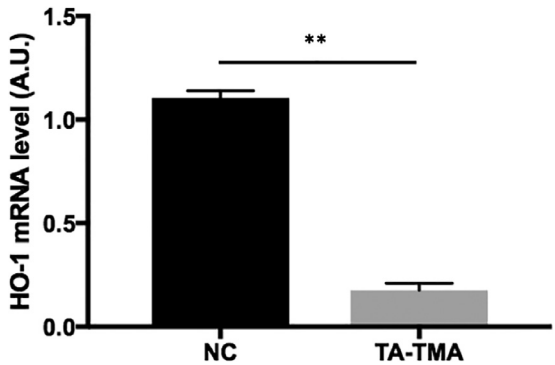

Figure 4. HO-1 mRNA level decreased in HUVECs co-cultured with TA-TMA plasma. Three experiments. $^{**}P < .01$.

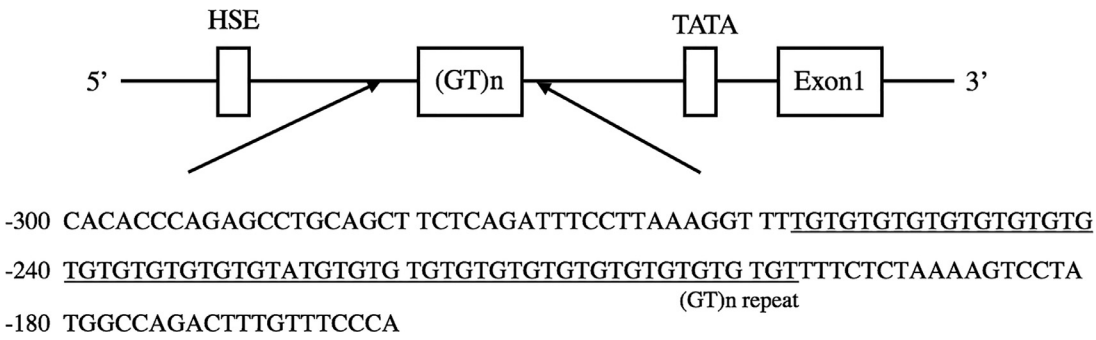

Figure 5. The partial nucleotide sequence of the 5'-flanking region and exon of human HO-1. Heat-shock element (HSE) and TATA box are significant components in the HO-1 gene promoter.

significantly increased in patients with TA-TMA [6]. An early rise in the C5b-9 level indicates poor prognosis of TA-TMA [25]. In these patients, complement factor autoantibodies and gene variations or mutations were found to be associated with TA-TMA [1,8], which is similar to the complement-mediated aHUS. A mutation or autoantibody leads to overactivation of the alternative pathway and predisposes to the development of aHUS [26]. Case reports [27,28] and clinical trials [7] proved that complement inhibition was a promising therapy in aHUS, a disease caused by complement dysregulation. Accordingly, Jodele et al. [8] found that 61% of TA-TMA patients achieved complete resolution of TMA from the complement blocker eculizumab. Therefore, complement activation and endothelial cell injury may be related to the pathogenesis of TA-TMA. It is still unclear, however, how the complement system was activated.

In our study we found that plasma levels of HO-1 were significantly decreased in patients with TA-TMA. HO-1 is normally expressed at low levels in most tissues but is highly inducible by a variety of stimuli. Therefore, HO-1 may be involved in the process of cytoprotection. This process was activated upon cellular stresses such as inflammation, ischemia, hypoxia, hyperoxia, hyperthermia, and radiation [29]. In fact, infection, radiation, and hypertension are potential mechanisms underlying endothelial damage in TA-TMA [30]. In addition, neutrophil extracellular traps may serve as a mechanistic link between endothelial injury and complement activation in TA-TMA [31]. It is suggested that elevated serum levels of HO-1 are predictive of adverse events after acute myocardial infarction in patients with coronary artery disease [18]. HO-1 plays a key role in maintaining antioxidant/oxidant homeostasis and the prevention of vascular injury [32]. TA-TMA is a disease with histologic evidence of microangiopathy and endothelial injury. Here we proposed that HO-1 deficiency is associated with the vascular injury. This notion was supported by reduced HO-1 mRNA levels in HUVECs treated with TA-TMA plasma (Figure 4).

A meta-analysis indicated that the HO-1$(GT)_n$ repeat-length polymorphism is associated with decreased risk of coronary heart disease or restenosis after percutaneous coronary intervention [33]. This polymorphism was located in the HO-1 promoter region [34]. The nucleotide partial sequence of the 5'-flanking region and exon of human HO-1 is shown in Figure 5. Previous studies suggested a long $(GT)_n$ sequence in the HO-1 promoter leads to a lower antioxidative activity of this protein [35,36]. To date, several HO-1 single nucleotide polymorphisms have been identified: class S (<27 $(GT)_n$ repeats), class M (27 to 32 $(GT)_n$ repeats), and class L (>32 $(GT)_n$ repeats) [36]. Interestingly, in our study sequencing of the HO-1 promoter revealed the presence of class L $(GT)_n$ repeat length in 3 of 5 TA-TMA patients. Regarding the limited number of TA-TMA patients, it is still too early to conclude that the HO-1 $(GT)_n$ repeat-length polymorphism is associated with the pathogenesis of TA-TMA.

On the other hand, HO-1 also works in regulating the complement pathway. HO-1 was identified as a regulator of DAF, and the protection by HO-1 in complement-dependent glomerular injury involves DAF upregulation [21]. In this light, TA-TMA may be a disease caused by aberrant complement activation. We showed that HO-1 mediates TA-TMA via regulating the DAF-dependent pathway. In addition, in the group of HUVECs incubated with plasma of TA-TMA patients, DAF expression was significantly decreased. Mason et al. [37] showed that DAF expression on endothelial cells is regulated by cytokines, suggesting that induction of DAF may be important for maintenance of vascular integrity during inflammation. In our investigation we found that HO-1–inhibited HUVECs are susceptible to complement-mediated injury via the DAF pathway. We further demonstrated that DAF expression was suppressed after HO-1 inhibition by ZnPP. However, ZnPP only moderately enhanced complement deposition on HUVECs. On the other hand, Kinderlerer et al. [20] showed that in addition to DAF induction, HO-1 may inactivate alternative pathways to enhancing protection against complement activation.

Our investigation also showed NAC increased the level of DAF and might protect HUVECs from complement activation. NAC, an antioxidant/mucous modifier, has displayed benefits in chronic obstructive pulmonary disease. Martinez de Lizarrondo et al [38] provided evidence that the molecular target underlying the thrombolytic effects of NAC is principally von Willebramd Factor (VWF) that cross-link platelets in arterial thrombi. They also found that NAC did not worsen hemorrhagic stroke outcomes, suggesting that it exerts thrombolytic effects without significantly impairing normal hemostasis. It was reported that NAC inhibits platelet adherence to endothelial cell–anchored soluble ultralarge VWF multimers by reducing their size [39,40]. It has been reported that NAC resulted in a significant increase in the expression of HO-1 mRNA in liver injured by carbon tetrachloride (CCl_4) [23]. In our study we found that NAC might enhance DAF expression on the cell surface, which protects endothelial cells from complement-mediated cytotoxicity. NAC may play a promising role in TA-TMA treatment in the future.

The levels of HO-1 in plasma in these cases were analyzed retrospectively. Although the time of sample collection is matched, we did not consider the variety of the use of immune suppression, such as the wide range of drugs and different medications, because it was too difficult to analysis. It also introduced inaccuracy, particularly in the setting of a complex

with acute myocardial infarction. In addition, the number of patients was relatively small. Further prospective studies and those that investigate exact mechanisms are needed.

In summary, our results showed that plasma HO-1 is significantly decreased in patients with TA-TMA. DAF, which protects endothelial cells from complement-mediated cytotoxicity, is downregulated on the endothelial surface in patients with TA-TMA. NAC may be a potential treatment for TA-TMA via upregulating the HO-1–DAF axis, whereas the microsatellite polymorphism in the HO-1 gene promoter may be associated with the vulnerability to develop TA-TMA.

We detected the nucleotide partial sequence of HO-1 promoter, and found the presence of >32 $(GT)_n$ repeat length in three TA-TMA patients among five. Owing to the small size, we cannot conclude that HO-1 promoter (GT)n repeat length polymorphism is associated with TA-TMA.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.bbmt.2019.03.002.

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