Iron-Deficiency and Estrogen Are Associated With Ischemic Stroke by Up-Regulating Transferrin to Induce Hypercoagulability

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RATIONALE: Epidemiological studies have identified an association between iron deficiency (ID) and the use of oral contraceptives (CC) and ischemic stroke (IS). To date, however, the underlying mechanism remains poorly understood. Both ID and CC have been demonstrated to upregulate the level and iron-binding ability of Tf (transferrin), with our recent study showing that this upregulation can induce hypercoagulability by potentiating FXIIa/thrombin and blocking antithrombin-coagulation proteases interactions.

OBJECTIVE: To investigate whether Tf mediates IS associated with ID or CC and the underlying mechanisms.

METHODS AND RESULTS: Tf levels were assayed in the plasma of IS patients with a history of ID anemia, ID anemia patients, venous thromboembolism patients using CC, and ID mice, and in the cerebrospinal fluid of some IS patients. Effects of ID and estrogen administration on Tf expression and coagulability and the underlying mechanisms were studied in vivo and in vitro. High levels of Tf and Tf-thrombin/FXIIa complexes were found in patients and ID mice. Both ID and estrogen upregulated Tf through hypoxia and estrogen response elements located in the Tf gene enhancer and promoter regions, respectively. In addition, ID, administration of exogenous Tf or estrogen, and Tf overexpression promoted platelet-based thrombin generation and hypercoagulability and thus aggravated IS. In contrast, anti-Tf antibodies, Tf knockdown, and peptide inhibitors of Tf-thrombin/FXIIa interaction exerted anti-IS effects in vivo.

CONCLUSIONS: Our findings revealed that certain factors (ie, ID and CC) upregulating Tf are risk factors of thromboembolic diseases decipher a previously unrecognized mechanistic association among ID, CC, and IS and provide a novel strategy for the development of anti-IS medicine by interfering with Tf-thrombin/FXIIa interactions.

GRAPHICAL ABSTRACT: A graphical abstract is available for this article.

Key Words: blood◼ contraceptives◼ estrogens◼ thrombosis◼ transferrin

Ischemic stroke (IS) is typically caused by blood vessel blockage1 and accounts for ≈87% of strokes.2 In brief, IS occurs following severe reduction or interruption of cerebral blood flow (ischemia) induced by vascular occlusion.4–6 Currently, IS is the second-leading cause of death and the most important cause of permanent disability worldwide.7 Two acquired conditions or environment factors including iron deficiency (ID) and the use of combined oral contraceptives (CC) are associated with increased risk of IS8–20 and venous thromboembolism (VTE).14,21–26 In particular, ID is associated with IS and cerebral sinus thrombosis, but not other VTE, mostly in children,16,26 whereas the use of CC is associated with VTE and IS in adolescents and adults.27 Affecting >2 billion people globally, ID is the most common nutritional disorder, and ID anemia (IDA) remains the...
What Is Known?
- Iron deficiency anemia and the use of oral contraceptives are associated with increased risk of ischemic stroke.
- Tf (Transferrin) induces hypercoagulability by interacting with coagulation factors.

What New Information Does This Article Contribute?
- Both iron deficiency anemia and the use of oral contraceptives upregulate Tf level through hypoxia and estrogen response elements, respectively.
- Tf aggravates ischemic stroke, which is alleviated by Tf interferences in iron deficiency and estrogen administration mice model.

Two factors, including iron deficiency and the use of combined oral contraceptives, are associated with ischemic stroke, but the underlying mechanisms are not known. We found that Tf levels are elevated by iron deficiency and estrogen administration, suggesting an association between Tf-upregulation, iron deficiency anemia, and the use of oral contraceptives. Exogenous Tf, iron deficiency, estrogen administration, or Tf overexpression promoted hypercoagulability and aggravated ischemic stroke, while anti-Tf antibody or Tf knockdown and designed peptide inhibitors suppressed the symptoms. These data reveal that factors upregulating Tf are risk factors of thromboembolic diseases and a mechanistic association between iron deficiency anemia, the use of oral contraceptives and thrombosis, and further confirm the central role of Tf in maintaining coagulation balance. Our study provides a novel understanding as to why oral contraceptives may induce clinical thrombosis as well as an avenue for the development of antithromboembolic drugs associated with situations including iron deficiency anemia.

Nonstandard Abbreviations and Acronyms

| Abbreviation | Definition |
|--------------|------------|
| APTT         | activated partial thromboplastin time |
| C/EBP-α      | CCAAT/enhancer-binding protein α |
| CC           | use of oral contraceptives |
| ELISA        | enzyme-linked immunosorbent assay |
| GSK-3β       | glycogen synthase kinase 3β |
| HI           | high iron |
| HIF-1        | hypoxia-inducible factor 1 |
| ID           | iron deficiency |
| IDA          | iron deficiency anemia |
| IgG          | immunoglobulin G |
| IS           | ischemic stroke |
| LI           | low iron |
| PI3K         | phosphatidylinositol 3-kinase |
| PRT          | plasma recalcification time |
| PT           | prothrombin time |
| Tf           | transferrin |
| VTE          | venous thromboembolism |

foremost cause of anemia worldwide. Over 3 decades ago, IDA was found to be associated with hemiparesis and aphasia, thus highlighting the correlation between IDA and thrombotic complications. Over the last few years, the associations between ID/IDA and thrombophilia have been increasingly recognized. Various thromboembolic diseases, including central retinal vein occlusion, cerebral venous sinus thrombosis, and carotid artery thrombus, are associated with IDA, as are numbers of embolic stroke cases and IS. Population-based studies have also found that IDA is more frequent in cerebral venous thrombosis cases than in healthy controls. Previous meta-analysis has also indicated that the risks of myocardial infarction and IS are significantly increased in women using CC. The use of CC also correlated with an increased risk of VTE. However, although the above evidence suggests that ID and CC are associated with thrombotic diseases and IS, the underlying mechanisms are not fully understood. Platelets are reported to play key roles in IS and VTE and thrombocytosis or enhanced platelet aggregation is found in populations with IDA or using CC. Many platelet aggregation-associated factors, such as fibrinogen, von Willebrand factor, and fibronectin, regulate thrombosis. In addition, the use of CC is accompanied by elevated fibrinogen and FVIII. Both ID and CC can also upregulate the level or iron-binding ability of Tf (transferrin), a known iron carrier. Given that Tf interacts with and potentiates FXIIa/thrombin and blocks the inactivation of coagulation proteases by antithrombin to induce hypercoagulability, as reported in our recent study, we investigated whether Tf mediates the associations among ID, CC, and thrombosis.

METHODS
A detailed description of materials and methods are included in the Data Supplement. Please also see the Major Resource Table in the Data Supplement. The authors declare that all supporting data are available within the article and in the Data Supplement.

All the other data and materials of this study are available from the corresponding author upon reasonable request.
Collection of Human Samples

All the human samples were collected according to the clinical protocols approved by the Institutional Review Board of the Kunming Institute of Zoology and the Third People’s Hospital of Kunming (Protocol No. KMSYLL-20150101). All the specimens were collected in 2016 to 2018 with the informed consent of patients. The IS patients were diagnosed by head computed tomography and magnetic resonance imaging after exhibiting several clinical features (such as inability to move or feel, problems of understanding and speaking, blurring of vision or vision loss; Table I in the Data Supplement). All recruited IS patients had a history of IDA before IS attack (classified as IS-IDA patients). The IDA patients were diagnosed by primary clinical features, including low red blood cell, hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin values and high red blood cell distribution width value (Table I in the Data Supplement). The control group consisted of prospectively recruited healthy volunteers, who were age-matched whenever possible. Human plasma from the IDA group (n=454), IS-IDA group (n=453), and healthy volunteers (n=541) were collected. Human cerebrospinal fluid from the IS-IDA (n=40) and healthy group (n=40), as well as thrombus samples from acute IS-IDA patients undergoing mechanical thrombectomy, were also collected (n=5; Table II in the Data Supplement). Human plasma from VTE patients using CC (classified as VTE-CC patients; n=30, diagnosed as deep venous thrombosis by combined computed tomography venography and computed tomography pulmonary angiography) was also collected (Table III in the Data Supplement). Immediately following blood drawing (using 1.5% EDTA-Na2 as anticoagulant agent), plasma was obtained by centrifugation at 3000 rpm for 20 minutes at 4°C and then stored at −80°C after being divided into aliquots.

Animals and Ethics Statement

All animal experiments were approved by the Animal Care and Use Committee at the Kunming Institute of Zoology (SMXK-2016013) and conformed to the US National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (National Academies Press, Eighth Edition, 2011). C57BL/6J mice (female or male, 8 weeks old) were purchased from the Vitalriver Experiment Animal Company (Beijing, China) and housed in a pathogen-free environment. Mice were maintained in sterile isolators with autoclaved food and water under 12-hour light-12-hour dark cycle at 24°C. Animals were randomly assigned to the operators by independent persons not involved in data acquisition and analysis. Surgeries and evaluation of all readout parameters were performed blinded to the experimental groups.

Tf Concentration Determination

Tf concentrations in plasma and cerebrospinal fluid were determined by enzyme-linked immunosorbent assay (ELISA) using a human Tf ELISA kit (EK12012-96T, Multi Sciences, China) according to the manufacturer’s instruction. Western blot analysis was also used to determine Tf levels in plasma and cerebrospinal fluid. The plasma and cerebrospinal fluid were first separated by 12% SDS-PAGE and then transferred to polyvinylidene difluoride membrane. The membrane was first blocked with TBST (Tris-buffered saline and Tween-20) buffer (50 mmol/L Tris, 150 mmol/L NaCl, and 0.1% Tween-20) containing 5% BSA at room temperature for 2 hours, and then incubated with a primary antibody against Tf (1:2000, 11019-RP02, Sino Biological Inc, China) at 4°C overnight, followed by incubation with a secondary antibody at room temperature for 1 hour. After washing with TBST buffer, the membrane was developed with an enhanced chemiluminescence kit (PA112, Tiangen, China) using ImageQuant LAS 4000 mini (GE Healthcare). To control plasma or cerebrospinal fluid loading and transfer, the membranes were stained by Red Ponceau.

Anti-Tf Polyclonal Antibody Preparation

Mouse Tf was used to immunize rabbits to produce anti-Tf polyclonal antibodies as the method described in our previous work.45

IDA Mouse Induction

C57BL/6J mice (male, 8 weeks old) were randomly assigned to low-iron (LI; Fe<10 mg/kg) and high-iron (HI) diet (Fe>90 mg/kg) groups. Some mice fed a LI diet were further injected with a Tf-depleting antibody or an isotype control immunoglobulin G (IgG; 2 times/wk through the tail vein from the beginning of induction by Li forage, 50 μg per injection). After 4 weeks of induction, blood was collected (with 1.5% EDTA-Na2 used as anticoagulant agent) and then subjected to routine blood testing including red blood cell, hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin, using a blood routine test machine (BC-2800Vet, Mindray, China). Blood was also collected (with 3.8% sodium citrate used as anticoagulant agent), and plasma was obtained by centrifugation at 3000 rpm for 30 minutes at 4°C for plasma recalcification time (PRT), activated partial thromboplastin time (APTT), and prothrombin time (PT) assays. The tested mice were also subjected to test the tail-bleeding time and FeCl3-induced carotid artery injury model assays.

Estrogen Administration in Mice

C57BL/6J mice (female or male, 8 weeks old) were randomly assigned to groups. Some mice were first administered estrogen (0.2, 1, and 5 mg/kg, dissolved in 0.9% NaCl) by oral gavage twice a week for 1 week. After 1-week induction, the blood was collected, and the concentrations of Tf and estrogen in plasma were tested using a mouse Tf ELISA kit (ab157724, Abcam) and an estrogen ELISA kit (LH-E10064MU, Liuhe, China), respectively.

Female C57BL/6J mice (8 weeks old) were administered estrogen (1 mg/kg, dissolved in 0.9% NaCl twice a week) by oral gavage (long-term administration), together with receiving an anti-Tf antibody or an isotype control antibody treatment (twice a week though tail vein, 50 μg per injection). One week after gavage administration, the mice were used for PRT, APTT, PT, tail-bleeding time, and FeCl3-induced carotid artery injury model assays. Mice were intravenously injected with estrogen (6 ng/kg, dissolved by 0.01% DMSO, short-term estrogen administration) for 10 minutes, and the effects of estrogen on coagulation were also evaluated by APTT, PT, tail-bleeding time, and FeCl3-induced carotid artery injury model assays as the method described.
RESULTS

Elevated Tf and Tf-thrombin/FXIIa complexes in plasma and cerebrospinal fluid of IS, IDA, and VTE patients.

As illustrated in Figure 1A, elevated Tf levels were detected in the plasma of IS-IDA, IDA, and VTE-CC patients by ELISA. The average Tf concentrations in the plasma of IS-IDA (n=453, male 262; female 191; age: 18–90 years), IDA (n=454, male 228; female 226; age: 16–87 years), and VTE-CC patients (n=30, female; age: 31–56 years) were 4.497±1.57, 4.146±2.06, and 5.270±0.94 mg/mL, respectively, while that in healthy individuals (n=341, male 140; female 201; age: 16–84 years) was 2.820±0.55 mg/mL. Western blot analysis also showed elevated Tf levels in the plasma of these patients (Figure 1B and 1C). In addition, the Tf concentration in the cerebrospinal fluid of IS-IDA patients (n=40, male 22; female 18; age: 48–90 years) was 20.11±5.41 mg/L, which was about 0.5-fold greater than that (13.70±2.70 mg/L) in the healthy controls (n=40, male 22; female 18; age: 31–56 years). As illustrated in Figure 2E and 2F, mutations of the HIF-1 binding sites in the Tf gene enhancer region were mutated (Figure 2G). Notably, coimmunoprecipitation analysis revealed that amounts of Tf-prothrombin/FXIIa complexes in these patients’ plasma were higher than that in normal controls (Figure 1G). To determine whether the Tf-thrombin/FXIIa complexes were present in thrombi, colocalization of Tf with thrombin or FXIIa in thrombi was detected by confocal microscopy. As shown in Figure 1H (top), most Tf-positive deposits (green) were associated with thrombin (red), indicating the formation of the Tf-thrombin complex in vivo. Colocalization of Tf (red) and FXIIa (green) by immunofluorescence (Figure 1J, bottom) also confirmed the formation of the Tf-FXIIa complex.

ID Induces Tf Upregulation to Promote Hypercoagulability In Vivo

To further elucidate the relationship between Tf and ID, C57BL/6J mice were used and subjected to a LI or HI diet. After a 4-week induction, a substantial increase in Tf plasma level was observed in the LI-induced mice compared with the normal controls or HI-induced mice (Figure 3A). The elevated Tf was associated with typical characteristics of microcytic hypochromic anemia, such as decreased mean corpuscular volume and mean corpuscular hemoglobin (Table IV in the Data Supplement). Furthermore, significant reductions in APTT (Figure 3B), PT (Figure 3C), and PRT (Figure 3D and 3E) were observed in LI-induced mice compared with the control or HI-induced mice. Moreover, an obvious decrease in tail-bleeding time was observed in the LI-induced mice (Figure 3F). As illustrated in Figure 1A and 1B in the Data Supplement, platelet counts and platelet-based thrombin generation in LI-induced were also elevated. The increased platelet counts in LI-induced mice were consistent with that of IS-IDA and IDA patients (Figure IC in the Data Supplement). In addition, decreased red blood cells were also found in LI-induced mice (Table IV in the Data Supplement). Importantly, anti-Tf antibody treatment significantly reversed the reductions in APTT, PT, PRT, and tail-bleeding time and the elevation of platelet-based thrombin generation in LI-induced were also elevated. The increased platelet counts in LI-induced mice were consistent with that of IS-IDA and IDA patients (Figure IC in the Data Supplement). In addition, decreased red blood cells were also found in LI-induced mice (Table IV in the Data Supplement). Importantly, anti-Tf antibody treatment significantly reversed the reductions in APTT, PT, PRT, and tail-bleeding time and the elevation of platelet-based thrombin generation in LI-induced were also elevated. The increased platelet counts in LI-induced mice were consistent with that of IS-IDA and IDA patients (Figure IC in the Data Supplement). In addition, decreased red blood cells were also found in LI-induced mice (Table IV in the Data Supplement). Importantly, anti-Tf antibody treatment significantly reversed the reductions in APTT, PT, PRT, and tail-bleeding time and the elevation of platelet-based thrombin generation in LI-induced were also elevated. The increased platelet counts in LI-induced mice were consistent with that of IS-IDA and IDA patients (Figure IC in the Data Supplement). In addition, decreased red blood cells were also found in LI-induced mice (Table IV in the Data Supplement).

Estrogen Upregulated Tf Expression In Vitro

We next investigated the effects of estrogen on Tf expression using BNL CL.2. The Tf protein was upregulated by estrogen in a dose-dependent manner, as confirmed by both ELISA (Figure 4A) and Western blot (Figure 4B and 4C) analysis. A previous study has
indicated that estrogen response elements located at the human Tf gene promoter region are associated with the GGACA(N)3TGGCC motif, which binds estrogen receptor α.47 We found a similar motif (GGAGAGGATGGCC) at the 5′ promoter of the mouse Tf gene between −1021 and −1009. As illustrated in Figure 4D, estrogen-induced Tf upregulation in BNL CL.2 cells was significantly inhibited when the corresponding estrogen response element in the promoter region of Tf gene was mutated.

C/EBP-α (CCAAT/enhancer-binding protein α) is a key transcription factor involved in the expression of various genes by interacting with the regulatory sequences presented in the promoter and enhancer regions of target genes.48 AKT-dependent phosphorylation of GSK-3β (glycogen synthase kinase 3β) is reported to upregulate C/EBP-α activity.49 As illustrated in Figure 4E and 4F, AKT was activated by estrogen in a dose-dependent manner. Activated AKT, in turn, phosphorylated and inactivated GSK-3β (Figure 4G). GSK-3β phosphorylation was increased by ≈30%, 115% and 146% after 2-hour treatment with 0.1, 1, and 10 ng/mL of estrogen, respectively.

Figure 1. Elevated Tf (transferrin) and Tf-thrombin/FXIIa complexes in plasma and cerebrospinal fluid of ischemic stroke (IS-IDA), iron deficiency anemia (IDA), and venous thromboembolism (VTE-CC) patients.

A. Concentrations of Tf in the plasma from IS-IDA (IS), IDA, VTE-CC (CC) patients and healthy volunteers (normal) were determined by ELISA. Western blot analysis (B) and quantification (C) of Tf in plasma samples from IS-IDA, IDA, and VTE-CC patients and healthy volunteers. D. Level of Tf in the cerebrospinal fluid of IS-IDA patients and controls (normal). Western blot analysis (E) and quantification (F) of Tf in the cerebrospinal fluid samples of IS-IDA patients and controls. G. Coimmunoprecipitation of Tf and prothrombin or FXII in the plasma from IS-IDA, IDA, and VTE-CC patients and normal controls. H. Clots from acute IS-IDA patients were labeled with either anti-Tf (green) or antithrombin antibody (red) to detect the presence of Tf-thrombin complex (top) or labeled with either anti-Tf (red) or anti-FXIIa antibody (green) to detect the presence of Tf-FXIIa complex (bottom). Cell nuclei were labeled by DAPI. Arrows indicate Tf-thrombin- or Tf-FXIIa-positive structures. Scale bar represents 30 μm. Images were representative of at least 3 independent experiments. B and E. Red Ponceau (RP)-stained blot (below) is the loading control. Data represent mean±SD. A and D, **P<0.01, Mann-Whitney U test. C, **P<0.01, unpaired t test. F, **P<0.01, Kruskal-Wallis test followed by Bonferroni adjustment. Western blots (B, E, and G) were from different membranes.
Figure 2. Tf (Transferrin) is upregulated by iron deficiency in vitro.

After the treatment by iron chelator desferrioxamine (DFO), Tf RNA expression in BNL CL.2 cells was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR; A). Tf protein levels in BNL CL.2 cells were analyzed by ELISA (B) and Western blot (C), respectively. D, Quantitative analysis of Western blot in C. BNL CL.2 cells were treated by different concentrations of DFO, HIF-1α (hypoxia-inducible factor 1α) was analyzed by Western blot (E), and the quantification of E is shown (F). G, Tf levels in the supernatant of HepG2 cells transfected by Tf expression plasmid of wild-type hypoxia response elements (HRE; WT-HRE) or mutated HRE (MT-HRE) were analyzed by ELISA after the treatment by DFO. Data represent mean±SD of 6 independent experiments. A–F, **P<0.01, 1-way ANOVA with Dunnett post hoc test compared with control (normal control [NC]). G, **P<0.01, 2-way ANOVA with Bonferroni post hoc test compared with control (MT-HRE). Western blots (C and E) were from different membranes.

(Figure 4G). A 45%, 80%, and 95% elevation in C/EBP-α phosphorylation was also observed after the treatment with 0.1, 1, and 10 ng/mL of estrogen, respectively (Figure 4E and 4H). Furthermore, C/EBP-α expression was elevated after 24-hour treatment with estrogen (Figure 4I and 4J). The phosphorylation of AKT, GSK-3β, and C/EBP-α and the expression of C/EBPα induced by estrogen were significantly abrogated by the PI3K (phosphatidylinositol 3-kinase) inhibitor wortmannin (10 nmol/L; Figure 4E through 4J), suggesting that the PI3K/AKT signaling pathway is involved in Tf expression induced by estrogen. The above results thus indicate that estrogen upregulates Tf expression via estrogen response element and AKT activation.

Estrogen Induces Tf Upregulation to Promote Hypercoagulability In Vivo

As illustrated in Figure 5B, Tf levels in plasma of both male and female mice were upregulated by oral gavage administration of estrogen, which was in accordance with the elevation of estrogen in the mouse plasma (Figure 5A). Compared with the concentration in the plasma of male control mice (2.4 mg/mL), the Tf concentrations were increased to 3.0, 3.6, and 4.2 mg/mL in male mice following estrogen administration of 0.2, 1, and 5 mg/kg, respectively (Figure 5B). A similar situation was observed in female mice (Figure 5B). We then determined whether the association of estrogen and hypercoagulability was dependent on Tf. After oral gavage administration of
estrogen (1 mg/kg) for 1 week (long-term administration), the concentration of Tf in the plasma of treated mice was ≈3.9 mg/mL, while that in the controls was ≈2.3 mg/mL (Figure 5C). Furthermore, after 1-week estrogen administration, APTT, and PT were determined to elucidate whether estrogen treatment-induced hypercoagulability. As illustrated in Figure 5D, APTT in the control mice was ≈29 seconds, whereas that in mice treated with estrogen, estrogen combined with control IgG and anti-Tf antibody was ≈23, 20, and 26 seconds, respectively. The same tendency was also observed for PT (Figure 5E). Furthermore, PRT was also accelerated, consistent with the elevated concentration of Tf (Figure 5F and 5G). As illustrated in Figure 5D and 5E, the clotting time was calculated by measuring the time to half maximal increase in absorbance, and tail-bleeding time (F) were determined using mice fed a normal diet (NC), low-iron diet (LI induced), or high-iron diet (HI induced) alone, or from mice treated with anti-Tf antibody (LI-induced + Tf AB) or control IgG (LI-induced + IgG) when fed a low-iron diet. G, Representative images of carotid artery blood flow (left) in FeCl3-treated groups of mice by laser speckle perfusion imaging, with region of interest (rectangle in green) placed in carotid artery to quantify blood flow change. Red: blood flow; blue and black area: background; the color bar at the bottom indicates the perfusion unit scale (0–302). Relative blood flow in region of interest is shown (right). Data represent mean±SD (n=5–6). A–F, **P<0.01, 1-way ANOVA with Dunnett post hoc test compared with controls (NC or LI induced). G, Two-way ANOVA followed by Dunnett post hoc test compared with controls (NC or LI induced). NS indicates no significance.
in the Data Supplement, platelet counts and platelet-based thrombin generation were also elevated in mice treated with estrogen. The increased platelet counts in estrogen-treated mice were consistent with increased platelet counts in VTE-CC patients (Figure IF in the Data Supplement). As illustrated in Table IV in the Data Supplement, decreased red blood cells were also found in estrogen-treated mice. A shortened tail-bleeding time was observed in mice treated with estrogen (Figure 5H). As illustrated in Figure 5I, decreased blood flow was observed in the carotid artery of estrogen-treated mice compared with the control mice. Anti-Tf antibody treatment reversed the reduction in blood flow, whereas the control IgG showed no effect on the reduction (Figure 5I). Estrogen alone showed no effects on coagulant factors (Figure IIA and IIB in the Data Supplement) or coagulation (Figure IIC and IID in the Data Supplement).

Direct intravenous administration of estrogen (6 ng/kg; short-term administration) significantly increased the mouse plasma estrogen concentration (Figure IIIA in the Data Supplement), whereas the Tf concentration (Figure IIIIB in the Data Supplement), blood coagulation time (APTT and PT; Figure IIIC in the Data Supplement), tail-bleeding time (Figure IIIID in the Data Supplement), and carotid artery blood flow induced by FeCl₃ (Figure IV in the Data Supplement) showed no significant differences. Together, our results indicate that estrogen does not directly affect blood coagulation, and its effect to induce hypercoagulability is dependent on Tf upregulation.

**ID, Estrogen Administration, and Tf Overexpression Aggravate IS, Which Can Be Alleviated by Tf Interferences**

To further investigate the role of Tf in vivo, a transient middle cerebral artery occlusion model was used to assess...
Figure 5. Estrogen induces hypercoagulability and thrombus formation by upregulating Tf (transferrin) in mice. Concentration of estrogen (A) and Tf (B) in the plasma of male or female mice after oral gavage administration of different concentrations of estrogen for 1 wk was determined by ELISA. C, Plasma Tf level (NC: negative control, ES: 1-wk estrogen administration, ES+Tf AB: 1-wk estrogen administration in presence of Tf antibody, ES + IgG: 1-wk estrogen administration in the presence of control IgG), activated partial thromboplastin time (APTT; D), prothrombin time (PT; E), plasma recalification time (PRT; F and G), and tail-bleeding time (H) in mice from 4 groups are shown. I, Representative images of carotid artery blood flow (left) in FeCl₃-treated groups of mice by laser speckle perfusion imaging, with region of interest (rectangle in green) placed in the carotid artery to quantify blood flow change. Red: blood flow; blue and black area: background; the color bar at the bottom indicates the perfusion unit scale (0–302). Relative blood flow in region of interest is shown (right). Data represent mean±SD (n=5–6). A and B, "P<0.01, 2-way ANOVA with Dunnett post hoc test compared with control (NC). C–H, ""P<0.01, 1-way ANOVA with Dunnett post hoc test compared with control (NC or ES). NS indicates no significance.
the effects of Tf on IS. As illustrated in Figure 6A and 6B, a significant increase in infarct volume was observed in the LI-induced mice compared with normal controls and HI-induced mice. Furthermore, the bigger infarct volumes in LI-induced mice also translated into worse functional outcomes (Figure 6C and 6D). Estrogen administration induced similar tendency in infarct volume and functional outcomes (Figure 6E through 6H). Importantly, anti-Tf antibody treatment reversed the symptoms, whereas the control IgG showed no effects (Figure 6A through 6H). Furthermore, IS was not aggravated by direct intravenous administration of estrogen (short-term administration) in mice (Figure V in the Data Supplement).

The sequelae of Tf overexpression and knockdown was also evaluated. The Tf expression levels were validated by both quantitative real-time polymerase chain reaction and Western blot as presented in our previous study.45 The levels of Tf in mouse plasma were upregulated or downregulated by 96 hours of lentivirus (with Tf overexpression plasmid [PLP-Tf]) or retrovirus (with Tf knockdown plasmid [RNR-Tf]) infection compared with the levels in the controls (normal control; Figure VI in the Data Supplement).

**Figure 6.** Iron deficiency, estrogen administration, and Tf (transferrin) overexpression aggravate ischemic stroke. Representative images of 2,3,5-triphenyltetrazolium chloride (TTC)-stained coronal brain sections (A) and quantitative analysis of stained area (B) from mice in 5 groups, as mentioned in Figure 3, on day 1 after transient middle cerebral artery occlusion (MCAO). Ischemic infarctions appear white and brain infarct volumes were measured by planimetry (% of whole volume). Bederson score (C) and grip test score (D) in respective groups were also measured. Representative images of TTC-stained coronal brain sections (E) and quantitative analysis of stained area (F) from mice in 4 groups, as mentioned in Figure 5, on day 1 after tMCAO. Bederson score (G) and grip test score (H) in respective groups were measured. I, Representative TTC-stained corresponding coronal brain sections from sham-operated (sham), saline (NC), Tf overexpressed (PLP-Tf), Tf injected (MTf, 6 and 30 mg/kg), Tf knockdown (RNR-Tf), Tf antibody-treated (Tf AB), and control IgG-treated (IgG) mice on day 1 after tMCAO. M, Seven representative TTC-stained corresponding coronal brain sections from saline- (0.9% sodium chloride, NC), TH16-, and FX18-treated (0.2, 1, 5 mg/kg, respectively) mice on day 1 after tMCAO. Two peptides and saline were injected 10 min before reperfusion. Brain infarct volumes (N), Bederson (O), and grip test score (P) in the respective group on day 1 after tMCAO. Animal experiments were repeated 3 independent times. Scale bar represents 0.5 cm. Data represent mean±SD (n=6–10). A–D, **P<0.01, 1-way ANOVA with Dunnett post hoc test compared with control (NC or LI induced). E–H, **P<0.01, 1-way ANOVA with Dunnett post hoc test compared with control (NC or ES). I–P, **P<0.01, 1-way ANOVA with Dunnett post hoc test compared with control (NC). NS indicates no significance.
Data Supplement). Tf overexpression or administration induced a significant increase in infarct volume (Figure 6I and 6J). Furthermore, the increased infarct volume in Tf overexpressed and Tf-injected mice also translated into serious functional outcomes (Figure 6K and 6L). Conversely, Tf knockdown or anti-Tf antibody treatment evidently resulted in smaller infarct volume (Figure 6I and 6J) and better Bederson (Figure 6K) and grip test scores (Figure 6L). The IgG-administered mice showed similar IS symptoms as the control mice.

The anti-IS functions of the designed peptides, TH16 and FX18, which interfere with the interaction between Tf and thrombin/FXIIa, were also tested. Administration of TH16 and FX18 induced a dose-dependent, progressive decrease in infarct volume in the mouse IS model (Figure 6M and 6N). Furthermore, the peptides-treated mice likewise developed improved Bederson (Figure 6O) and grip test scores (Figure 6P). Thus, these results indicate that Tf aggravates IS, which is, conversely, alleviated by Tf interference.

**DISCUSSION**

Our previous work has demonstrated that Tf plays a central role in maintaining coagulation balance by interacting with coagulation and anticoagulation factors and further suggests that factors upregulating Tf cause hypercoagulability and thromboembolic diseases. Although recent decades have witnessed a growing awareness of the association between ID/IDA or CC and thrombosis, the underlying mechanisms remain unclear. Here, we showed that ID and CC upregulate Tf to induce hypercoagulability and thromboembolic diseases.

The mechanisms of adaptation to ID are associated with suppression of the hepatic hormone hepcidin, which leads to decreased levels of serum ferritin and Tf saturation. Decreased Tf saturation is partly due to the increased levels of Tf, as reduced levels of iron also trigger increases in synthesis of Tf. The rate of Tf synthesis in liver significantly increased (2- to 4-fold) in ID rat. Two adjacent hypoxia response elements (GAAATACGTGCAGCTGTGTGTACGTGCA) containing 2 critical (G/A)CGTG core sequences have been identified within the Tf gene enhancer, as binding sites of HIF-1. HIF-1 confers low oxygen upregulation of Tf gene expression by binding to the 2 HIF-1 sites present in the Tf enhancer. Previous research has also demonstrated the requirement for HIF-1 in the activation of ceruloplasmin transcription by ID. Thus, it is likely that ID upregulates Tf through HIF-1. Indeed, our results showed that the plasma level of Tf was significantly increased in IDA and IS-IDA patients, as well as in LI-induced mice (Figure 1A through 1C and Figure 3A). This adaptive mechanism may facilitate the absorption of iron or maintenance of homeostasis. However, an enhanced thrombosis potential may be induced because Tf has the ability to potentiate thrombin and FXIIa and to block the inactivation of coagulation proteases by antithrombin. Indeed, accompanied by upregulated Tf, the mice induced by LI diet, but not HI diet, displayed hypercoagulability represented by decreased APTT, PT, PRT, bleeding time, and blood flow (Figure 3A through 3G). However, a previous report showed that APTT and PT values were not significantly changed in IDA patients with ages of 2 to 14, although decreased clot formation time value and coagulation tendency were observed in these patients. The observed APTT and PT difference between the IDA patients and the mice may be as result of species difference.

In chickens, estrogen is reported to promote the expression of Tf in both liver and oviduct. Here, treated with estrogen for 1 week and in female VTE-CC patients (Figures 1A through 1C and 5A and 5B). We found that estrogen upregulated Tf expression via estrogen response elements located in the Tf gene promoter region and by AKT activation (Figure 4) and further induced hypercoagulability (Figure 5C through 5H). The ability of estrogen to induce a procoagulant state was confirmed by 2 in vivo thrombotic models (Figures 5I and 6E through 6H). The 2 diseases were aggravated by estrogen administration, whereas anti-Tf antibody administration reversed the disease development. In addition, increased platelet counts and platelet-based thrombin generation and decreased red blood cells were observed in LI-induced and estrogen-administered mice (Figure 1 and Table IV in the Data Supplement). As suggested in previous reports, the possible mechanisms related to the switch between increased platelets and decreased red blood cells include: (1) accelerated megakaryocyte differentiation promoting platelet generation; (2) iron depletion resulting in cell cycle arrest in tissues attenuating red blood cell production; and (3) decreased assembly and production of iron-containing proteins, such as hemoglobin, possibly inhibiting red blood cell generation.

Two designed peptides (TH16 and FX18), which block Tf-thrombin/FXIIa interaction to inhibit Tf's potential on enzymatic activity of thrombin and FXIIa, inhibited carotid artery thrombosis and IS induced by either LI diet or estrogen application (Figure 6M through 6P), further confirming that the 2 acquired conditions (ID and CC) exhibit the same pathological mechanism to induce thromboembolic development. Collectively, this study reveals a mechanistic association between ID, CC, Tf, and thrombosis, and the factors upregulating Tf are risk factors of thromboembolic diseases. The central role of Tf in the maintenance of coagulation balance was further confirmed. This study provides a novel avenue for development of antithromboembolic medicine associated with...
acquired conditions such as ID and CC by targeting Tf or interfering with TF-thrombin/FXIIa interaction.

ARTICLE INFORMATION
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Disclosures
None.

Supplementary materials
Expanded Materials and Methods
Online Figures I–VI
Online Tables I–IV
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