Investigation of the Etiology of Anemia in Thromboangiitis Obliterans

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Abstract

During a review of patients admitted with thromboangiitis obliterans (TAO), there was evidence of normochromic normocytic anemia and abrupt changes in hemoglobin (Hgb) levels in patients with several hospital admissions. Therefore, the evidence of hemolytic anemia was evaluated based on 37 banked plasma samples taken from Caucasian male TAO patients during disease exacerbation between 2012 and 2014. The patients' hospital records, including clinical manifestations and complete blood count, were evaluated. The following tests were performed on all samples: indirect antiglobulin test (IAT), C-reactive protein (CRP), high-sensitivity CRP (hsCRP), lactate dehydrogenase (LDH), haptoglobin, indirect bilirubin, D-aspartate aminotransferase (AST), and D-alanine aminotransferase (ALT).

The mean age of the patients was 40 ± 7 years. Two patients underwent below-knee amputation. The mean hospital-documented Hgb of the patients was 12.9 ± 2.6 g/dL. CRP and IAT were positive in 75.6 and 70.2% of the samples, respectively. The tests and corresponding results were as follows: hsCRP, 14.07 ± 2.37 µg/mL; LDH, $2,552 \pm 315$ u/L; haptoglobin, 2.27 ± 1.1 g/L; indirect bilirubin, 0.09 ± 0.04 mg/dL; AST, 67 ± 7 u/L; and ALT, 26 ± 3 u/L. There was a significant inverse correlation between hsCRP and hospital-documented Hgb level ($p = 0.03$).

Keywords

- thromboangiitis obliterans
- Buerger disease
- anemia
- hemolysis
- thrombosis

Anemia with the positive IAT in most of the samples, high LDH and AST, and normal ALT are suggestive of hemolytic anemia. Normal indirect bilirubin is consistent with intravascular hemolysis. The positive CRP and elevated haptoglobin levels could be due to systemic inflammation in TAO. However, it is not known if an autoantigen or an infectious antigen is responsible for TAO systemic inflammation and induction hemolytic anemia. As such, the underlying mechanism of anemia in TAO could be part of the footprint of its main etiology.

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Thromboangiitis obliterans (TAO) is an episodic, sharply segmental, inflammatory, and thrombotic-occlusive peripheral vascular disease. Its etiology is unknown.¹ In severe cases, it can result in gangrene and limb loss.² TAO is more prevalent in the Middle East, Far East, and Eastern Europe.¹ There are also some reports of TAO from South Europe and South America.^{3,4} At present, there is no consensus regarding the etiology of TAO, and its pathophysiology remains uncertain.⁵ The progression and prognosis of TAO has been shown to be closely related to cigarette smoking.^{1,5} No reasons have yet been proposed to explain why, of the many millions of smokers around the world, only a small number of people, mostly from low socioeconomic classes, develop the disease.⁶ Furthermore, there is still debate as to whether TAO is an autoimmune or autoinflammatory disorder.^{5,7}

A recent review of banked plasma from TAO patients revealed macroscopic hemolysis in some of the samples. We proceeded to evaluate the records of patients who were admitted to hospital in acute phases of TAO from 2005 to 2011. We found that 49 of the 125 patients (39%) had anemia (hemoglobin [Hgb] < 13.5 g/dL for male patients). The change in Hgb level in 24 patients who had more than two admissions was 3.56 g/dL.⁸ Unfortunately, no additional data about the patients' reticulocyte counts or other indices for evaluating the type of anemia, which could explain this finding, were available. Nevertheless, we concluded that hemolytic anemia was the most likely cause of the abrupt decline in Hgb. The aim of this study was therefore to evaluate this hypothesis based on 37 banked plasma samples taken from TAO patients between 2012 and 2014.

Materials and Methods

In this study, we analyzed 44 banked plasma samples stored in a -20°C freezer from patients diagnosed with TAO, who were admitted to hospital for disease exacerbation between 2012 and 2014 (ethical code: 900133).

The diagnosis was made based on Shionoya criteria, including age of disease onset before 50 years, a history of cigarette smoking, upper limb involvement or thrombophlebitis migrans, infrapopliteal arterial occlusion, and absence of atherosclerotic risk factors other than smoking.⁹ Seven samples which had macroscopic hemolysis were excluded, so the study proceeded with 37 plasma samples. The patients' hospital records, including clinical manifestations and complete blood count, were also evaluated.

To investigate the evidence of hemolytic anemia, the following investigations were performed on the banked plasma: indirect antiglobulin test (IAT); C-reactive protein (CRP); high-sensitivity CRP (hsCRP); level of haptoglobin which binds to free Hgb^{7,10}; lactate dehydrogenase (LDH), which is the dominant enzyme in red blood cells (RBCs) and is released upon lysis of RBCs,¹¹; indirect bilirubin; and liver function tests, including those for D-aspartate aminotransferase (AST) and D-alanine aminotransferase (ALT).

For the IAT, three EDTA-blood samples were obtained from patients with O+ blood. All samples were retested to

confirm blood group using monoclonal antibodies against A and B as well as Rh antigens. Samples were washed three times with normal saline (NaCl 9.8% gr/w) and dry drops were then mixed with NaCl 9.8% gr/w to create a 5% concentration w/w. Plasma from all samples was diluted to a 1/64 titer and 50 μL of O+ RBCs (washed and diluted) was added to each tube. All tubes were incubated at 37°C for 45 minutes. After incubation, the tubes were centrifuged at 2,500 g for 3 minutes and then one drop of antihuman globulin was added to each tube. Finally, this was mixed completely and centrifuged at 2,500 g for 1 minute. Each tube was checked for macroscopic agglutination of particles in front of a visible light source.

Visible agglutination in the plasma samples which were diluted up to a 1/32 and 1/64 titer was considered to be positive for IAT.¹²

The results of IAT were double-checked on two separate occasions during a 1-month period.

As a control, IAT was performed on five plasma samples that had been taken from healthy volunteers and banked in a -20°C freezer since 2010.

LDH was evaluated using the Pars Azmun Number KA140022 (Tehran, Iran) protocol with a normal range up to 480 u/L. This nonradioactive colorimetric LDH assay is based on the nicotinamide adenine dinucleotide (NADH)-coupled enzymatic reaction that creates NADH 2 from NAD during the LDH function that converts lactate to pyruvate. This reaction uses a maximum optical absorption in the range of 340 nm. LDH isoenzymes were not investigated due to lack of the related facilities.

A competitive enzyme-linked immunosorbent assay kit, Abcam catalog number ab108856, was used for detection of haptoglobin level in the plasma samples. Its normal range is 0.3 to 2 g/L.

An Aspartate Transaminase Assay Kit (Jhongli City, Taiwan) was used to quantify the amount of oxaloacetate produced by AST (for normal values up to 45 u/L). In this assay, oxaloacetate and NADH are converted to malate and NAD by the enzyme malate dehydrogenase. The decrease in NADH absorbance at 340 nm is proportionate to AST activity (Abnova catalog number 93KA1625).

ALT was investigated with In BioVision's ALT Assay Kit, catalog number: K752-100 (Milpitas, CA), with a normal range up to 41 u/L. ALT catalyzes the transfer of an amino group from alanine to ketoglutarate and the products of this reversible transamination reaction are pyruvate and glutamate. The pyruvate is detected in a reaction that concomitantly converts a nearly colorless probe to produce color ($\lambda_{\text{max}} = 570 \text{ nm}$).

Direct bilirubin was evaluated using the Diazyme group kit catalog number DZ151A-K that is based on a colorimetric assay in which a sample of serum or plasma is mixed with vanadate at a pH of 3. It oxidizes direct bilirubin to biliverdin. This causes the absorbance of yellow, specific to bilirubin, to decrease. Therefore, the direct bilirubin concentration in the sample can be obtained by measuring the absorbance before and after the vanadate oxidation. The normal value is in the range of 0 to 0.3 mg/dL.

CRP, a sensitive marker for acute phase reactions,¹³ was evaluated with the latex agglutination test. This test is deemed positive if macrolatex particles form and are seen under a direct light.

High-sensitivity CRP was evaluated using the Monobind kit catalog number 3125-300 (Lake forest, CA) that is based on a microplate immunoenzymometric assay. The normal value is considered less than 3 µg/mL.

Statistical Analysis

Statistical Package for the Social Sciences, version 11.5, was used for statistical analysis. The descriptive data are presented as the mean $\pm$ standard deviation (mean $\pm$ SD). Kolmogorov-Smirnov tests were used to determine whether the results followed a normal distribution.

Independent sample *t*-tests and Pearson correlations were then used to identify significant differences between samples and to evaluate correlations among quantitative variables, respectively. CRP and TAO signs and symptoms were compared in the positive and negative IAT groups using the chi-square test, and *p*-values less than 0.05 were considered significant, with a confidence interval (CI) of 95%. The acceptable study power was decided a priori to be greater than or equal to 80% (type-I error of ≤ 0.20). This was also used for sample size calculations.

Results

In total, 37 banked plasma samples were used in the study. All the samples were from Caucasian men who were experiencing an exacerbation of TAO without the history of using drug-induced immune hemolytic anemia. Based on the patient records associated with each sample, the mean age of the patients was 40 ± 7 years, with the youngest being 27 years old and the oldest being 49 years old. The mean cigarette consumption was 397 ± 77 packs per year (minimum 60 packs and maximum 1,110 packs per year). The frequencies of signs and symptoms of TAO were as follows: rest pain, 75%; nonhealing ulcer, 90%; toe gangrene, 55%; foot or calf claudication, 80%; upper limb ischemia, 75%; and absence of popliteal pulse, 57%.

Anemia, defined as Hgb < 13.5 g/dL, was present in 70.5% of the patients. The mean hospital-documented Hgb was 12.9 ± 2.6 g/dL, with a maximum of 16.6 g/dL and a minimum of 7.2 g/dL. The mean Hgb value for six of the patients who had a previous admission 1 to 3 months before the sample was collected was 13.2 ± 1.6 g/dL. The mean Hgb changes in these patients were 1.54 ± 1.2 g/dL, with a maximum of 3.2 g/dL and a minimum of 0.6 g/dL. The RBC indices were measured as follows: mean corpuscular volume, 85.7 ± 3.9 fL; mean corpuscular hemoglobin, 28.6 ± 1.8 pg; and mean corpuscular hemoglobin concentration, 33.2 ± 1.2 g/dL. A significant inverse correlation between Hgb level and the duration of smoking was observed ($p = 0.01$; CI = -0.82). However, no significant correlation between Hgb level and the duration of the disease was observed ($p = 0.38$).

IAT was positive in 26 samples (70.2%). None of the control samples was positive for IAT.

Significant correlations between IAT and claudication ($p = 0.025$) and IAT and upper limb ischemia ($p = 0.046$) were found. Notably, the number of cigarettes smoked daily in the patients positive for IAT was 30 ± 4 , which is significantly higher than in patients with negative IAT (16 ± 2) ($p = 0.02$). No significant difference was found between the Hgb level of the patients with positive and negative IAT ($p = 0.6$).

CRP was positive in 28 samples (75.6%). Significant correlations between CRP and rest pain ($p = 0.008$) and CRP and absence of popliteal pulse ($p = 0.014$) were found. However, no significant correlation between CRP and gangrene ($p = 0.102$) or CRP and nonhealing ulcer ($p = 1.34$) was found.

The mean hsCRP was 14.07 ± 2.37 µg/mL, with a maximum of 49 µg/mL and a minimum of 0.5 µg/mL. The results of hsCRP were quite compatible with latex agglutination CPR test. There was a significant inverse correlation between hsCRP and hospital-documented Hgb level ($p = 0.03$).

The plasma level of LDH was high in all of the samples. The mean LDH was $2,552 \pm 315$ u/L, with a maximum of 5,588 u/L and a minimum of 621 u/L. The mean LDH level in positive IAT samples ($3,219 \pm 339$ u/L) was significantly higher than in negative IAT samples ($1,632 \pm 404$ u/L) ($p = 0.02$).

The mean haptoglobin level was 2.27 ± 1.1 g/L, with a maximum of 4.7 g/L and a minimum of 0.9 g/L. The haptoglobin level was significantly higher in the CRP-positive group compared with the CRP-negative group ($p = 0.03$). However, no significant difference between haptoglobin levels was observed in the patients with positive and negative IAT results ($p = 0.22$).

The mean AST was 67 ± 7 u/L, with a maximum of 126 u/L and a minimum of 18 u/L. The mean ALT was 26 ± 3 u/L, with a maximum of 56 u/L and a minimum of 6 u/L.

The mean indirect bilirubin level was 0.095 ± 0.04 mg/dL, with a maximum of 0.25 mg/dL and a minimum of 0.04 mg/dL. In addition, a difference which was not quite significant was noticed between the levels of indirect bilirubin of the patients with positive and negative IAT values ($p = 0.06$).

The results are summarized in ► **Table 1**.

Discussion

Our previous study of the records of TAO patients who had hospital admissions found that 39% of these patients had anemia. It is notable that the Hgb levels of patients who were admitted for below-the-knee amputation were significantly lower than in patients admitted for sympathectomy or treatment with prostaglandin I2 analogues.⁸ Unfortunately, none of the patients had been worked up for their anemia.

In this study, evidence of normochromic normocytic anemia in TAO patients during acute exacerbations was identified based on their hospital records during admission. It was found that Hgb had a significant inverse correlation with duration of smoking. This was not anticipated because a high Hgb level would be expected in long-term, heavy smokers.¹⁴

Table 1 Summarized results of the current study comparing the results against those expected from the two main suspected mechanisms of anemia in TAO: the anemia of chronic disease and hemolytic anemia^{14–16}

	Normal range	TAO patients	Anemia of chronic disease	Hemolytic anemia
Hgb	13.5–17 g/dL	12.9 ± 2.6 g/dL ↓	↓	↓
MCV	80–100 fL	85.7 ± 3.9 fL N	N	Variable
MCH	27–34 pg	28.6 ± 1.8 pg N	N	Variable
MCHC	32–36 g/dL	33.2 ± 1.2 g/dL N	N	Variable
LDH	Up to 480 u/L	2,552 ± 315 u/L ↑	N	↑
Hpt	0.3–2 g/L	2.27 ± 1.1 g/L N↑	N	Hemolysis in patients with infections N↑ Intravascular hemolysis ↓
ALT	Up to 41 u/L	26 ± 3 u/L N	Variable	N
AST	Up to 45 u/L	67 ± 7 u/L ↑	Variable	↑
Indirect bilirubin	Up to 0.3 mg/dL	0.095 ± 0.04 mg/dL N	N	N↑
Total bilirubin	0.3–1.9 mg/dL	0.12 ± 0.04 mg/dL N	N	N↑
Positive CRP	–	75%	Inflammatory disease Positive	Variable
Positive IAT	–	69.4%	None	Positive

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; Hgb, hemoglobin; Hpt, haptoglobin; IAT, indirect antiglobulin test; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; N, normal; ↑, High; ↓, low.

Therefore, the underlying mechanisms of anemia in these patients were investigated based on their banked plasma samples, which had been collected at the time of their hospital admission.

In light of the positive CRP in 75.6% of patients, TAO seems to be a systemic inflammation and the anemia in TAO could be due to the effects of chronic inflammation on RBC production in the bone marrow.¹⁷ However, the positive IAT and high levels of LDH and AST with normal level of ALT in the banked samples, and abrupt changes in the Hgb levels of the patients between hospital admissions cannot be explained simply by the “anemia of chronic disease.”¹⁸ The high and normal level of haptoglobin and its significant correlation with the level of CRP could be explained by increased levels of haptoglobin as an acute phase reactant protein¹⁰ and the normal levels of indirect bilirubin can also be found in intravascular hemolysis.¹⁹ Notably, intravascular hemolysis correlates with activation of endothelial cells and expression of endothelial adhesion molecules such as vascular cell adhesion molecule (VCAM-1).¹¹ High expression of VCAM-1 on endothelium of both occluded and patent arteries in TAO, which resembles endothelial cell activation, has been reported.²⁰ In our recent study, we incubated human umbilical vein endothelial cells (HUVECs) with the sera of TAO patients in the acute phase of their disease. We found that gene expression of intercellular adhesion molecule (ICAM-1) and VCAM-1 were significantly higher in HUVECs after incubation with TAO sera compared with the smoking habit-matched control group.²¹ We found a significant correlation between VCAM-1 and duration of smoking.²⁰ In this study, we also noted a

significant inverse correlation between Hgb level and duration of smoking. There could, therefore, be an inverse correlation between Hgb level and endothelial cell activation, as Kato et al showed in their study.¹¹ This finding may explain why the Hgb of TAO patients who were admitted for below-knee amputation is significantly lower than that of saved limb patients.⁸

A limitation of our study was the lack of fresh blood for measuring direct antiglobulin test (DAT) and reticulocyte counts. However, since all of our patients were male and most of them had anemia, we can conclude that positive IAT samples should also be positive for DAT.¹²

Taken together, the findings of the current study suggest that hemolytic anemia, in particular the autoimmune type, was the main cause of the anemia in these patients. The negative IAT with high LDH and AST levels in some cases may represent another mechanism of hemolytic anemia, such as mechanical RBC hemolysis due to passing through the damaged endothelium vessels and fibrin strands of intravascular thrombosis.^{22,23} This may explain why there was no significant difference between the Hgb levels of positive and negative IAT patients.

On the other hand, anemia in TAO may have several underlying mechanisms, including suppression of bone marrow in anemia of chronic disease, autoimmune hemolysis due to inflammation and as a result of an unknown trigger in TAO, and intravascular hemolysis due to mechanical damage of RBCs from passing through the fibrin strands of segmental vascular thrombosis (–Fig. 1). The consequences of each of these mechanisms would be anemia and intravascular hemolysis, which will cause derangement in tissue

Fig. 1 A suggestive schematic figure for the pathophysiology of Buerger disease. An autoantigen or an infectious antigen, perhaps *Rickettsia*, induces immune response. During the immune response, proinflammatory cytokines including IL-1, IL-6, and TNF- α would release. Proinflammatory cytokines can activate endothelial cells by upregulation of vascular endothelium adhesion molecules including ICAM-1 and VCAM-1. In addition, proinflammatory cytokines such as IL-1 can induce the expression of very late antigen-4 (VLA-4) ligand on the surface of RBCs, enabling them to attach to the VCAM-1 and consequently can increase the risk of thrombotic events in arteries with low-velocity blood flow. Besides, during the immune response, autoantibodies against RBCs can develop, leading to intravascular hemolysis. Free hemoglobin can decrease the bioavailability of nitric oxide and also can activate endothelial cells by upregulation of ICAM-1 and VCAM-1 and therefore increase the risk of thrombus formation. Smoking and genetic background by influencing on nitric oxide bioavailability can augment this process for thrombus formation.^{15,27-31} Passing through activated endothelium and fibrin strands of the multiple thrombosis alongside a vessel can lead to mechanical damage of RBCs and intravascular hemolysis as a consequence, making a vicious circle.²³

oxygenation and increase the risk of thrombotic events due to platelet activation, vascular endothelial dysfunction, and activation by scavenging of nitric oxide.²⁴⁻²⁶

Finally, this study is devisable from two points of view. First, despite a general acceptance that TAO is not a systemic inflammation, we could demonstrate positive CRP and high level of hsCRP in more than 75% of the patients implying systemic inflammatory reaction in TAO patients, at least during their disease exacerbation—in particular, that positive CRP was not limited to patients with chief complaint of gangrene. Second, identifying the etiology and underlying mechanism of anemia in TAO may help to determine the etiology of the disease.

According to the limitations of our study including not being able to evaluate DAT and LDH isoenzymes, precise anemia work-up on a larger sample size of TAO patients is highly recommended for better understanding of TAO puzzle.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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