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NATURAL AND ACQUIRED INHIBITORS OF HAEMOSTASIS IN SELECTED SYMPTOMATIC OUTPATIENTS WITH VENOUS THROMBOEMBOLIC DISEASE

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ABSTRACT

Background and Objective. Deficiencies of natural inhibitors and the presence of lupus anticoagulant are important risk factors leading to venous thromboembolic events. Before of resistance to activated protein C (APC-R) was identified, the overall prevalence of inherited abnormalities of hemostasis in non-selected outpatients with venous thromboembolic disease was under 10%. This cast doubts on the of cost effectiveness and clinical significance of assaying hemostasis inhibitors in all such patients. The goal of this study is to evaluate the prevalence of inherited and acquired abnormalities of hemostasis in younger symptomatic outpatients with objectively diagnosed venous thromboembolic disease (VTD).

Methods. From October 1994 to October 1996, we diagnosed, treated and followed191 consecutive outpatients with an objective diagnosis of venous thromboembolic disease, and assayed natural and acquired hemostasis inhibitors in 81 of them aged less than 50; in addition, 129 relatives of patients with inherited deficiencies were evaluated.

Results. Twenty-six of the patients under age 50 showed inherited deficiencies of natural inhibitors (3 antithrombin, 5 protein C, 3 protein S and 14 APC-R, 1 dysfibrinogenemia) and 8 patients had lupus anticoagulant (LA): abnormalities of hemostasis were found in 41.9% (95% confidence interval 31.1-53.5). In older selected patients, 60% (95% confidence interval 40.6-77.3) of the subjects showed abnormalities. Seventy-two of the relatives displayed natural inhibitor deficiencies. 88.5% of the families studied had at least one relative with the same defect as the propositus.

Interpretation and Conclusions. A simple selection based on age, clinical and family history shows the existence of a high prevalence and the important clinical significance of abnormalities of hemostasis in symptomatic outpatients with venous thromboembolic disease.

Key words: hemostasis, thrombosis, thromboembolism, APC-R

Venous thromboembolic disease (VTD) has two clinical manifestations: deep vein thrombosis (DVT) and the more serious pulmonary embolism (PE). VTD can result from congenital or acquired factors, or more frequently from a combination of the two.[1] Congenital factors include defects of natural inhibitors of hemostasis,[2-5] such as antithrombin, proteins C and S, heparin cofactor II (HC-II), resistance to activated protein C (APC-R), hyperhomocysteinemia, dysfibrinogenemia and dysplasminogenemia. The largest study in patients with instrumentally diagnosed DVT, in 1990,[6] showed a prevalence of natural hemostasis inhibitors less than 10%.

The mechanism of resistance to activated protein C (factor V Leiden mutation of Arg 506→Gln) was not known at that time. The prevalence of this defect in the heterozygotic form in the healthy population is about 3-6%, while the prevalence of APC-R in patients with VTD ranges from 10 to 60%.[7-9] Thrombophilia due to hereditary abnormalities of hemostasis carries a life-long risk of thrombosis. When this condition is identified, anticoagulant treatment of adequate duration and intensity must be provided for patients with a prior thrombotic event, prophylactic protection must be given at times of risk, and their families must be evaluated to find asymptomatic carriers. A frequent acquired abnormality of hemostasis causing VTD is the presence of antiphospholipid antibodies (APLA);[10] a strong association between lupus anticoagulant (LA) and DVT has recently been found[11] and high intensity anticoagulant treatment has been suggested in patients with APLA and DVT.[12] Since the prevalence of hemostatic abnormalities in patients with VTD in earlier studies was low (under 10%),[6] the current issue is whether natural inibitors should be assayed in all VTD patients. Congenital hemostatic abnormalities are known to cause thrombosis, particularly at a young age,[13,14] so we assayed natural inhibitors and the LA in patients younger than 50. The goal of this study was to evaluate the

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prevalence of inherited and acquired abnormalities of hemostasis in younger symptomatic outpatients with objectively diagnosed VTD.

Patients and Methods

Patients

Between October 1994 and October 1996, 191 consecutive outpatients with symptomatic objectively confirmed VTD (97 younger and 94 older than 50 years) were followed in our Unit for thrombotic and hemorrhagic diseases: 97 showed DVT, 87 PE with DVT and 7 PE without evidence of DVT. At the end of anticoagulant treatment (at least 7 days after its completion; for diagnosis and treatment flowchart, see Table 1), natural and acquired inhibitors of hemostasis were studied in 81 patients younger than 50; 16 were not evaluated because 5 had dropped out and 11 were receiving ongoing anticoagulant treatment. Furthermore, 30 patients over 50 considered *at risk* because they had either a personal history of thrombotic recurrences (at least twice in the last 3 years) or a family history of thrombosis (at least one first-degree relative younger than 50 with an acute thrombotic event without any triggering cause) were studied. We also investgated the 26 families (129 individuals) of patients with deficiencies of natural inhibitors.

VTD diagnosis

DVT was diagnosed by means of an echo-color Doppler Ultramark 9 HDI (ATL) operating with linear electronic (5 MHz) and convex probes (2-4 Mhz). The diagnostic criteria[15,16] were: resistance of the vein to compression by the echographic probe and direct visualization of the thrombus. For patients with venous thrombosis extending above the inguinal ligament and not evaluable by ultrasound because of intestinal gas or the constitution of the patient, we used a contrast medium CT scan. Each patient underwent a pulmonary scintigraphy with perfused macroaggregates of Tc99-labelled albumin. Patients with perfusion abnormalities were studied using ventilatory pulmonary scintigraphy with microcolloidal Tc99. We used the PIOPED criteria to confirm diagnosis.[17]

Laboratory methods

Blood samples for protein C and protein S, HC-II, antithrombin, APC-R, fibrinogen and LA assays were collected in vacutainer tubes containing sodium citrate (1:10). Platelet-poor plasma was obtained by centrifugation at 4,000 rpm for 30 minutes at 4°C, then stored at –80°C. Genomic DNA for the factor V mutation analysis was obtained from whole blood collected in tubes containing EDTA.

LA, protein C, protein S, antithrombin, HC-II and fibrinogen assays. LA was diagnosed according to *Standardization of Lupus Anticoagulant/Antiphospholipid Antibody Subcommittee* guidelines.[18] The presence of LA was detected by using the APTT (Actin-FSL, Baxter, Miami-USA) and kaolin clotting time (KCT) screening tests. The two tests were performed either on the patient's plasma or on a 1:1 mixture of the patient's plasma and normal pool plasma (obtained from 20 healthy donors). The platelet neutralization procedure (Stago)[19] was used as a confirmation test. The antithrombin plasma level was assayed by a chromogenic method (Immunochrom AT-III, Immuno); protein C and total protein S were determined by using the coagulative assays from Stago (protein C and protein S clotting tests). HC-II was measured by an amidolytic method using a commercial kit (Stachrom HC-II Stago). Fibrinogen was quantified according to Clauss. Normal ranges were: from 80 to 120% for antithrombin, from 70 to 140% for protein C, from 60 to 140% for protein S, from 65 to 145% for HC-II.

The normal ranges were obtained from an analysis of one hundred healthy blood donors. Diagnosis of a deficiency was established only if the plasma level of a protein was below the lower limit of its normal range in at least two samples.

APC-R test. Plasma was incubated with an APTT reagent for a standard period. Clotting was triggered by the addition of $CaCl_2$ in the absence or presence of activated protein C (APC). The time for clot formation was recorded. A commercial kit (Cromogenix-Coatest APC Resistance-Ortho Diagnostic System) was used. Clotting times were determined with a KJ16S semi-automated coagulometer (Ortho). Results were expressed as an APC sensitivity ratio, obtained by dividing APTT plus APC by APTT minus APC. When APC-SR was lower than 1.8, it was considered to be positive. This value was obtained by ROC curve analysis: this cutoff best discriminated between normal subjects and patients with defects (sensitivity = 58%, specificity = 100%, efficiency = 91.6, positive predictive value = 100%, negative predictive value = 90.5%). Each sample found to be positive or borderline for the functional test was then confirmed by molecular biology assay (factor V gene mutation test).

Factor V gene mutation test. The factor V 506 Arg to Gln mutation was determined as described elsewhere.[20,21] The region in exon 10 encoding for the APC cleavage site in Factor V was PCR™ amplified using the primers 506.5 (5' TGTTATCACACTGGTGCT 3') and 506.9 (5'TGTTATCACACTGGTGCT 3'). PCR conditions consisted of 37 cycles at 95°C for 45 sec, 55°C for 90 sec, 72°C for 180 sec, preceded by 5 min at 65°C. The presence of the factor V mutation was detected by digestion with MnlI).

Statistical analysis

The factor V mutation was used as the reference standard to establish the presence of the APC-R. Sensitivity and specificity of APC-R clotting assays were calculated by ROC analysis. The diagnostic efficiency of functional assays was calculated by Reverse Galen and Gambino analysis. The prevalences are shown as percentage and 95% confidence intervals (95% CI).

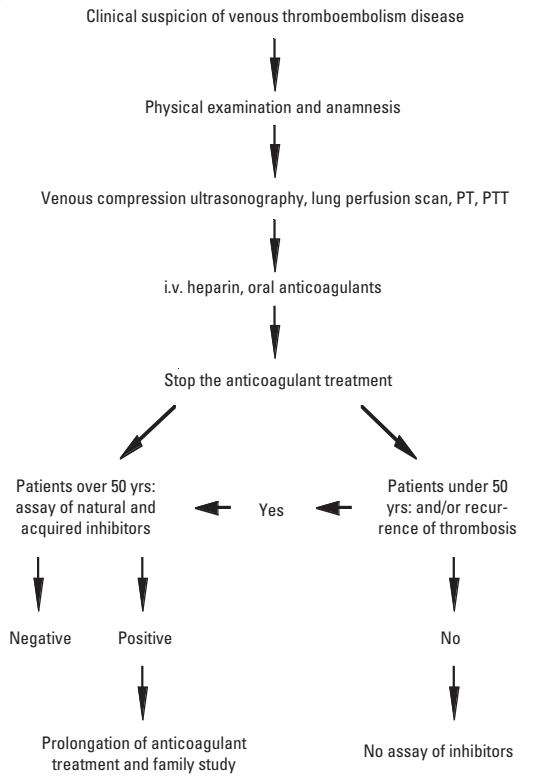

Table 1. Diagnosis and treatment flowsheet in patient with venous thromboembolic disease.

Table 2. Percentage and 95% confidence intervals (95% CI) of hemostasis abnormalities in selected symptomatic patients with venous thromboembolic disease. Group A: patients under 50 yrs. Group B: patients over 50 yrs., with recurrences of thrombotic events and/or a family history of thrombophilia.

	Group A % (95% CI)	*Group B % (95% CI)*
Antithrombin	3.7 (0.78-10.4)	10 (2.1-26.5)
Protein C	6.1 (2.03-13.8)	10 (2.1-26.5)
Protein S	3.7 (0.78-10.4)	3.3 (0.8-17.2)
APC resistance	17.3 (9.8-27.4)	20 (7.7-38.6)
Dysfibrinogenemia	1.2 (0.03-6.7)	0 (0-11.6)
Lupus anticoagulant	9.8 (4.4-18.5)	16.6 (5.6-34.7)
Total abnormalities	41.9 (31.1-53.5)	60 (40.6-77.3)

Results

Natural and acquired inhibitors of hemostasis were studied in 81 patients under 50 years old; of these, 26 (32.1%; 95% CI 22.1-43.4) showed inherited deficiencies of natural inhibitors and 8 (9.9%; 95% CI 4.4-18.5) patients had LA: 41.9% (95% CI 31.1-53.5) of patients were found to have hemostasis abnormalities. In addition, 30 patients over 50 were selected and studied: 13 (43%; 95% CI 25.5-62.6) displayed deficiencies of natural inhibitors and 5 (16.6%; 95% CI 5.6-34.7) had LA: 60% (95% CI 40.6-77.3) presented hemostasis abnormalities. Frequencies (% and 95% CI) of each abnormality are shown in Table 2.

Relatives (129 individuals) of the 26 patients with inherited hemostasis abnormalities were also studied: 72 carriers of inhibitor deficiencies were found and 88.5% of these families had at least one carrier of the defect.

Discussion

The prevalence of hemostasis abnormalities in VTD patients is extremely variable. The issue is if and when inherited and acquired inhibitors should be assayed in patients presenting with VTD. Up to 1990 the answer was probably negative because the largest study on non-selected patients with VTD published showed a prevalence of abnormalities lower than 10%. To our knowledge, no recent study assaying both acquired and inherited hemostatic abnormalities in VTD outpatients is available.

Our data show that in symptomatic outpatients with VTD diagnosed by objective methods and selected on the basis of age and clinical history, there is a very high prevalence of hemostatic abnormalities: about 42% in patients under 50 and 60% in those over 50 with recurrences of thromboembolic disease or a family history of VTD. This study, because of the relatively low number of patients enrolled, cannot answer the above question; nevertheless, the high prevalence of hemostasis abnormalities observed suggests the clinical relevance of the assay at least in similarly selected patients. Intensity and duration studies are needed to clarify the role of oral anticoagulant treatment in these patients. Even more important is the diagnosis of inherited abnormalities in symptomatic and asymptomatic relatives[22] in order to the prevent the first thrombotic event or reduce recurrences.

References

1. Girolami A, Prandoni P, Simioni P, Girolami B, Scarano L, Zanon E. Pathogenesis of venous thromboembolism. Haematologica 1995; 80(suppl to no. 2):25-35.
2. Finazzi G, Barbui T. Different incidence of venous thrombosis in patients with inherited deficiencies of antithrombin III, protein C and S. Thromb Haemost 1994; 71:15-8.
3. Milatich PJ, Prescott SM, White R, Majerus PW, Bowill EG. Inherited predisposition to thrombosis. Cell 1993; 72:477-80.
4. Pabinger I, Kyrle PA, Heistinger M, Eichinger S, Wittman E, Lechner K. The risk of thromboembolism in asymptomatic patients with protein C and protein S deficiency: a prospective cohort study. Thromb Haemost 1994; 71:441-5.
5. De Stefano V, Finazzi G, Mannucci PM. Inherited thrombophilia: pathogenesis, clinical syndromes, and management. Blood 1994; 97:3531-44.
6. Svensson PJ, Dahlbäck B. Resistance to activated protein C as a basis for venous thrombosis. N Engl J Med 1994; 330:517-22.
7. Dahlbäck B. Inherited resistance to activated protein C, a major basis of venous thrombosis, is caused by deficient anticoagulant cofactor function of factor V. Haematologica 1995; 80(suppl to no. 2):102-13.
8. Heijboer H, Brandjes DPM, Buller HR, Sturk A, ten Cate JW. Deficiencies of coagulation-inhibiting and fibrinolytic proteins in outpatients with deep-vein thrombosis. N Engl J Med 1990; 323: 1512-6.
9. Bazzan M, Aluffi E, Vaccarino A, Schinco P, Montaruli B. Resistance to activated protein C (APC-r) in selected outpatients with pulmonary embolism. Lancet 1996; 347:1842.
10. Triplett DA. Antiphospholipid antibodies, lupus anticoagulants and thromboembolic disease. Haematologica 1995; 80(suppl to no. 2): 122-6.
11. Ginsberg JS, Wells PS, Brill-Edwards P, et al. Antiphospholipid antibodies and venous thromboembolism. Blood 1995; 86:3685-91.
12. Khamashta MA, Cuadrado MJ, Mujic F, Taub NA, Unt BJ, Hughes GRV. The management of thrombosis in the antiphospholipid-antobody syndrome. N Engl J Med 1995; 332:993-7.
13. Briet E, Van Der Meer FJ, Rosendaal FR, Houwing-Dustermaat JJ, Van Houwelingen HC. The family history and inherited thrombophilia. Br J Haematol 1994; 87:348-52.
14. Tosetto A, Frezzato M, Rodeghiero F. Family history and inherited thrombophilia. Br J Haematol 1995; 89:227-8.
15. Lensing AWA, Prandoni P, Branjies D, et al. Detection of deep vein thrombosis by real-time B-mode ultrasonography. N Engl J Med 1989; 320:342-5.
16. Heijboer H, Buller HR, Lensing AWA, Turpie AGG, Colly LP, Ten Cate JW. A comparison of real-time compression ultrasonography with impedance plethysmography for the diagnosis of deep-vein thrombosis. N Engl J Med 1993; 329:1365-9.
17. Value of the ventilation/perfusion scan in acute pulmonary embolism: results of the Prospective Investigation of Pulmonary Embolism Diagnosis (PIOPED). JAMA 1990; 263:2753-9.
18. Brandt JT, Triplett DA, Alving B, Scahrrer I. Criteria for diagnosis of lupus anticoagulant: an update. On behalf of the Subcommittee on lupus anticoagulant/antiphospholipid antibody of the Scientific and Standardization Committee of the ISTH. Thromb Haemost 1995; 74:1185-90.
19. Triplett DA, Brandt JT, Kaczor D, Schaffer J. Laboratory diagnosis of lupus inhibitors: a comparison of the tissue thromboplastin inhibition procedure with a new platelet neutralization procedure. Am J Clin Pathol 1983; 79:678-82.
20. Bertina RM, Koelemann BPC, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 62:654-60.
21. Voorberg J, Roelse J, Koopmann R, et al. Association of idiopathic venous thromboembolism with single point mutation at Arg506 of factor V. Lancet 1994; 343:1535-6.
22. De Stefano V, Leone G. Resistance to activated protein C due to mutated factor V as a novel cause of inherited thrombophilia. Haematologica 1995; 80:344-56.