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Screening tests for abnormalities of the protein C anticoagulant pathway

Suchteste für Anomalien im antikoagulatorischen Protein C-System

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Abstract

There is an increasing demand for a rational diagnosis of patients with thrombophilia by clinical laboratories. The protein C pathway plays an important part in this system. To rationalize the diagnostics, methods have been introduced to test the anticoagulant capacity of the whole protein C pathway. These screening assays should reliably detect a factor V Leiden mutation, protein C and S deficiency. These assays differ in detection methods, interfering factors and even in their responsiveness to protein S. The methodological approach is based on the activation of endogenous plasma protein C by a snake venom extract. Clincial evaluations demonstrated the validity of these tests to screen for a factor V Leiden mutation and protein C deficiency. Protein S deficiency cannot be identified sufficiently. The assays, however, seem to have additional positive effects. They could be used to assess the risk for thrombosis in the future. Initial studies confirm this hypothesis. Hence, a positive test result in protein S-deficient patients correlated with a higher risk for thrombosis.

Keywords: thrombophilia; protein C pathway; screening method; venous thrombosis.

Zusammenfassung

Klinische Laboratorien stehen zunehmend vor der Aufgabe, eine Thrombophilie-Abklärung möglichst rational durchzuführen. Dazu gehört, die antikoagulatorische Kapazität des gesamten Protein C-Systems zu testen. Solche Suchteste sollten zuverlässig eine Faktor V Leiden-Mutation, einen Protein C- and S-Mangel erkennen. Der methodische Ansatz besteht in der Aktivierung von patienteneigenem Protein C durch ein Schlangengiftextrakt. Die Assays unterscheiden sich in Nachweismethode, Einflußgrößen und auch in ihrer Empfindlichkeit für Protein S. Klinische Studien bestätigen, daß diese Tests geeignet sind, eine Faktor V Leiden-Mutation und einen Protein C-Mangel zu identifizieren. Ein Protein S-Mangel wird aber nur ungenügend erkannt. Doch scheinen diese Screeningmethoden noch einen anderen Nutzen zu versprechen. Sie könnten dazu dienen, das künftige Thromboserisiko einschätzen zu helfen. Erste Studien bestätigen diese Ansicht. So deutete ein positives Testergebnis bei Protein S-defizienten Patienten auf ein erhöhtes Thromboserisiko hin.

Schlüsselwörter: Thrombophilie; Protein C-System; Suchtest; Venöse Thrombosen.

The protein C (PC) system is a major physiologic inhibitory mechanism that regulates blood coagulation. After PC is activated by thrombin, it cleaves, forming activated protein C (APC), the activated factors (F) V and VIII. Thereby, protein S (PS) serves as an important cofactor. Another cofactor, namely unactivated FV, supports the cleavage of activated FVIII. In the PC pathway, the known genetic risk factors for venous thromboembolism are PC deficiency, PS deficiency, and resistance to APC, which is strongly associated with a single point mutation in the FV gene that leads to the replacement of Arg 506 by Gln (FV R506Q) [1, 2]. Since APC resistance together with PC and PS deficiency is responsible for most cases of inherited thrombophilia, the identification of these defects is an important task for the management of thrombotic patients. However, detection of PC pathway defects requires the use of several specific assays. In the last years, screening assays have been developed to assess the total capacity of the PC pathway. They should detect all the known defects in the PC pathway at once.

Test principles

The main principle of PC pathway tests is the initial activation of endogenous PC. This is in contrast to functional APC resistance tests, where exogenous APC is added to

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the sample. Protein C activation is accomplished by the snake venom from Agkistrodon contortrix contortrix [3]. This results in reduced thrombin generation that can be measured by a prolonged clotting time or a reduced conversion of chromogenic substrates. Usually, the test is carried out with and without PC activation. The ratio is calculated by dividing the result obtained after PC activation with the one obtained with non activated PC.

A few manufacturers offer PC pathway tests. Their tests differ mainly in the detection procedures. The most commonly used and "classical" assay is the ProC® Global assay (Dade Behring, Marburg, Germany). One volume of the patient's plasma is incubated with one volume of an activated partial thromboplastin time (APTT) reagent (Pathrombin SL, Dade Behring) and one volume of either the PC activator or buffer for 3 minutes. Two clotting times are recorded after adding one volume of 0.025 M calcium chloride. In order to obtain comparable results and to exclude influences of instrumentation and/or reagents, the results are expressed as normalized ratio (NR). This is calculated by dividing the raw ratio of the patient's plasma by the ratio of a lyophilized standard human plasma, and multiplying by the sensitivity value defined by the manufacturer for each batch of standard plasma. Each batch is calibrated against an internal reference pool obtained from selected subjects with no known coagulation defect. According to the manufacturer's recommendations, a NR below 0.80 should be considered as pathological. A similar assay is the Diagen PCA test (Diagnostic Reagents, Thame, Oxon, UK).

The GradiThrom PCP test (Gradipore, North Ryde, Australia) uses a phospholipid-rich diluted Russell's viper venom reagent (PRVV) to initiate the clotting process. First, equal volumes of plasma and PC activator (or buffer) are preincubated together for several minutes at 37°C. Second, one volume of PRVV reagent containing calcium chloride is added, and the time until clotting occurred is accurately determined. NRs can be derived by dividing the patient's clotting times with and without PC activator by the results obtained with a pooled normal plasma.

A new assay for screening the PC pathway will be introduced in the near future. The HemosIL PC Pathway (PCP) assay (Instrumentation Laboratory, Orangeburg, USA) is the first chromogenic test for screening the PC pathway. Plasma and PC activator (or buffer) are preincubated together for 2 minutes at 37°C. Thromboplastin is added, thereby inducing the clotting process mediated by tissue factor. After incubation for 2 minutes at 37°C, the substrate is added and the ratio is calculated.

Modifications of the original assays have also been reported. An increase in specificity for the FV Leiden mutation can be achieved by using a 1:5 predilution of the patient's plasma in FV-depleted plasma. This procedure may allow an accurate detection of all mutation carriers while the character of a screening test for the whole PC system is lost [4].

Similar, non-commercially available tests, e.g., based on the prothrombin time (PT) [5, 6], have also been developed.

Method characteristics

Preanalytical conditions need special attention. Storage of whole blood at room temperature is allowed up to 8 hours. Once frozen at −20°C or −70°C and thawn, a significant change of ratio is observed [7, 8]. The imprecision of all available tests is acceptable. The within-run imprecision did not normally exceed 6%. For PT-based assays, slightly higher CV were reported. The between-run imprecision for all tests ranged mostly between 5% and 10% [5–14].

Clearly, the application of screening assays for the anticoagulant PC pathway are not suitable for orally anticoagulated patients [6, 7, 10]. Nevertheless, the laboratory can screen for FV Leiden by dilution with factor V-depleted plasma [4]. The reagents often contain heparin neutralizer like polybrene so that screening may be possible under heparin treatment (<1U Heparin /ml plasma) [15].

APTT-based assays are subject to many influences since a large number of clotting factors are involved in this test system. High FVIII levels, which can occur during inflammatory processes, pregnancy, or estrogen substitution, lower the ratio [3, 16]. However, high FVIII levels not only affect the test result but have also emerged as a new risk factor for venous thromboembolism [17, 18]. Therefore, inclusion of high FVIII levels in the screening strategy should be considered. The sensitivity for high FVIII levels, however, does not seem to be satisfactory. Choosing a cutoff NR of 0.8, Siegemund et al. calculated a sensitivity of 48% for levels >150% [19]. Lupus anticoagulants are further interfering factors [7, 16, 19]. Manufacturers have tried to minimize these effects. For instance, the GradiThrom PCP test is insensitive for FVIII or other factors of the intrinsic system. Only the coagulation factors X, V and II are involved. Excess phospholipids should invalidate lupus anticoagulants, but one study group found lupus anticoagulants to interfere with the GradiThrom PCP test [14].

One remarkable feature of at least APTT-based assays is the minor response to PS. If plasma is increasingly diluted with PS-deficient plasma, the ratio becomes slightly lower. Compared to PS, the response to PC is much more enhanced [9, 13]. In contrast, the HemosIL PC Pathway (PCP) assay seems to be equally sensitive to PS and PC [20, 21]. This could be a major advantage over the other available test systems.

Clinical studies

Originally, these tests were proposed to be used as first-step screening assays for the functionality of the PC

Table 1 Identification of congenital defects by APTT-based screening assays for the protein C anticoagulant pathway. Sensitivity is only indicated if subgroups of >10 patients were investigated. Mainly, data on the ProC® Global test are indicated. Evaluations on other APTT-based assays are quoted in italics. Usually, the cutoff is given as NR (normalized ratio). Ratios are listed in brackets.

First author/year	Cutoff	Specificity (%)	Sensitivity (%)		
			Factor V Leiden	protein C	protein S
Robert 1996 [9]	*(3.7)*	*97*	*100*	*100*	*100*
Dati 1997 [15]	0.8	96	100	90	63
Ruzicka 1997 [7]	(2.0)	95	100	100	79
Hafner 1997 [22]	0.8	95	100	95	69
Engelhardt 1997 [23]	0.8	72	100	91	84
Tripodi 1998 [11]	(2.8)	100	100	96	30
Gould 1999 [24]	0.8		100	94	73
Siegemund 1999 [19]	0.9		100	97	75
Rosendaal 1999 [25]	0.6	97	100	57	
Toulon 2000 [26]	0.8		100	91	69
Zotz 2000 [27]	0.8		100	82	71
Toulon 2001 [28]	0.75	62	100	100	66
Dragoni 2001 [29]	0.8	100	100	89	74
Gemmati 2001 [30]	0.84	95	100	95	76
Sarig 2002 [31]	0.8	95	100		100
Gardiner 2002 [14]	0.86/0.85*	95	100	55	35
	(6.5/11.2)*	*95*	*100*	*55*	*47*

*female/male

pathway. Specific assays should be performed only in the case of a decreased response. For this purpose, screening assays have to be highly sensitive. The specificity should be in an acceptable range. Many studies have shown the value of this kind of screening assays. Most of the investigators used the ProC® Global test or other APTT-based assays (Table 1). The manufacturer of the ProC® Global assay recommends a cutoff value of NR = 0.8. Many investigators used this cutoff and calculated the sensitivity for their patient groups. By this procedure, not one FV Leiden patient remained undetected and nearly all patients with PC deficiency could be found. However, PS deficiency was only insufficiently identified. The sensitivity for this inhibitor deficiency averaged at about 70%, thereby reflecting the minor response of APTT-based assays to PS. One gets another picture if all studies are taken together independently from the recommended cutoff: Except for FV Leiden, sensitivities for PC deficiency ranged widely from 55% to 100%, and those for PS deficiency from 35% to 100%. This is a major point of all these evaluations. As a reference group, most authors chose healthy blood donors. Other groups, like thrombosis patients without PC pathway abnormalities, were also used for calculation of specificity. The composition of the patients groups is also not a matter of course. Patients after venous thromboembolism or such patients as well as asymptomatic persons, each with PC pathway abnormalities, were grouped. This difference can bias the sensitivity of the test as we will see later in regard to PS deficiency. Moreover, the definition for inhibitor deficiency differs from study to study. The methods for measurement of the inhibitors were regularly indicated, but the normal ranges had often been adopted and not especially determined. Certainly, the use of in-house or instrument-specific reference ranges influences the performance of the assay.

A closer look at the methodologically best studies could help to define the appropiate use of the assay. Toulon et al. had no healthy controls. They compared patients who shared the history of venous thrombosis but differed in presence of PC pathway abnormalities. That way, the common thrombophilia work-up where usually thrombosis patients are included is simulated. It is not clear whether the authors used in-house reference ranges. As Table 1 shows, all patients with FV Leiden mutation or protein C deficiency could be detected with an acceptable specificity [28]. They also compared the economic impact of a strategy based on the ProC® Global test (thereby excluding screening for PS deficiency) with their standard strategy. Only 0.9% of all tests could be saved by this screening strategy. This would mean a cost reduction of 3.4% (in France) [32]. There are two studies with own reference ranges for the inhibitors. One study used a not commercially available APTT-based assay and yielded a very optimistic specificity-sensitivity-profile for their screening test [9]. The other study evaluated different assays, among them the ProC® Global test. This assay showed a 100% sensitivity for FV Leiden and PC deficiency at a specificity of 33%. The test seemed to be worthless for the identification of PS deficiency [14].

The clinical data are similar for the GradiThrom PCP test. Three groups reported a 100% sensitivity for FV Leiden (95% or 98% specificity). The detection rate for PC deficiency was 96%, 100%, and 76% [8, 12, 14]. Regarding PS deficiency, there was no advantage over the APTT-based assays. Haas et al. and Gardiner et al. calculated a sensitivity of 56% and 53%, respectively

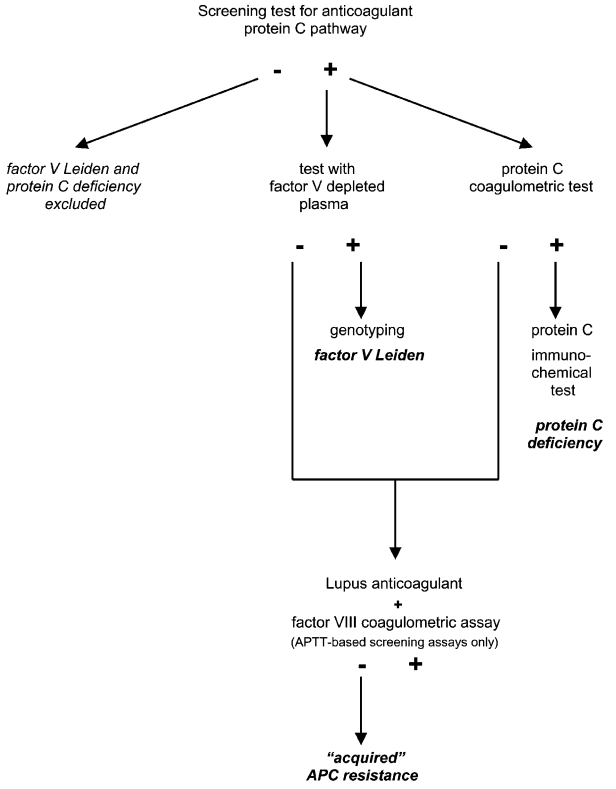

Figure 1 Proposal for a diagnostic strategy on the basis of screening tests for the anticoagulant protein C pathway. Valid only for not orally anticoagulated patients.

[12, 14]. Only preliminary data on the performance of the HemosIL PC Pathway (PCP) assay are available so far. But it is obvious that PC-, PS-deficient and APC-resistant patients had very similar ratios [20, 21].

Not only a screening test

"The real potency of this screening assay", as Kraus argued 5 years ago, "lies in its application as a measure of the PC system potential itself" [16]. From this point of view the application is no longer limited to a diagnostic strategy that aims to detect congenital defects effectively. The test result can even be interpretated to express the PC pathway capacity and to estimate the future thrombosis risk. A few studies support this idea. Zotz et al. investigated the ProC® Global ratio of 731 patients with venous thromboembolism and 512 healthy controls. The data were adjusted for the inhibitors, FV Leiden, FVIII and other factors. An odds ratio of 2.3 (95% CI 1.2–4.3) could be calculated so that ProC® Global ratio seems to be an independent risk [25, 27]. The ProC® Global could especially be a tool for risk assessment in PS-deficient persons. There was significant difference between symptomatic and asymptomatic members of families with PS deficiency (OR = 12.1; 95% CI 2.0–72.9). Additional unknown acquired or inherited situations were discussed to affect the ProC® Global test result in thrombotic patients with such a defect. This reflects the different penetrance of thrombosis among PS-deficient subjects [30]. A similar result was expected for PC deficiency and FV Leiden, but this was not the case. It could be hypothesized that the described effect may be hidden by a stronger dependence of clotting times from PC or FV. The data are consistent with studies which showed APC resistance to be a risk factor of its own, independently from the FV Leiden mutation [33, 34]. Prospective studies will show the real potential of the ProC® Global test or similar assays to predict the risk for thrombosis.

Taken together, these assays can currently be used to screen for FV Leiden and PC deficiency as it is shown in Figure 1. This procedure might be cost-effective and therefore reasonable from an economic point of view. Special attention should be paid in choosing an appropiate cutoff value. Unfortunately, PS deficiency can not be detected adequately so that special tests for this defect should be applied first-line. In the future, these screening assays may possibly change to prognostic indicators.

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