ANTICOAGULANT PROPERTIES OF THE ENDOTHELIUM STUDIED BY THE STANDARD VENOUS OCCLUSION TEST

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The endothelium plays a key role in the prevention of thrombosis. This role is effected through a system of thrombomodulin [4] and proteoheparan sulfates, secreted by endothelial cells [7]. There are two alternative pathways of thrombin inactivation: combining with antithrombin III (AT), which involves the participation of proteoheparan sulfates of the endothelium [10], and binding with thrombomodulin, the endothelial thrombin receptor. The complex of thrombin with thrombomodulin activates protein C (PC) [9], which interrupts the blood clotting reaction by cleavage of factors V and VIII. A special inhibitor binds activated PC in the course of 30 min. The anticoagulant properties of the thrombomodulin – PC system can be assessed experimentally with the aid of measured thrombin infusion. No method yet exists for assessing the potential ability of the endothelium to perform this particular function in man [5]. Clinically, the venous occlusion test (VOT), during which thrombin is generated, can be used [12].

The aim of this investigation was to correlate the degree of change of PC during VOT with other factors of hemostasis, and thus to analyze the anticoagulant potential of the endothelium during activation of blood clotting by venous occlusion.

EXPERIMENTAL METHOD

Tests were carried out on 98 men aged 46.1 ± 0.61 years (from 26 to 64 years). The patients' diagnosis was ischemic heart disease. Coronary atherosclerosis was not present in 14 subjects. The clinical characteristics are given in Table 1. Blood was taken into a siliconized vacuum container in the morning before breakfast. VOT was carried out by applying a sphygmomanometer cuff to the arm for 20 min and creating a pressure in it midway between systolic and diastolic, after which (before removal of the cuff) another blood sample was taken. Correction of the data due to the development of hemoconcentration during the VOT was achieved by determination of the total protein level. Citrated plasma was obtained by the standard method. To measure the level of tissue plasminogen activator (TPA), blood mixed with sodium citrate (3.8%, 9:1) was mixed with 1 M Na-acetate buffer, pH 3.9, in the ratio of 1:2, and centrifuged at 10,000*g* for 30 sec. The plasma was distributed among aliquots, frozen, and kept at −40°C. TPA was determined by a modified method [12], based on measurement of the amidolytic activity of plasmin formed in the reaction mixture, containing plasminogen (PG), the chromogenic substrate S-2251 ("Kabi"), fibrin-monomer, and plasma containing TPA. The acidified plasma was diluted in the ratio 1:100 with 0.1 M Trisacetate buffer,

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TABLE 1. Clinical Details of Patients

Number	98
Age	46.1±0.6
Smokers	77(78 %)
Hypertension	47(48 %)
Hypercholesterolemia (ChS > 250 mg/dl)	42(43 %)
Hypertriglyceridemia (TG > 140 mg/dl)	49 % (50 %)
Number of affected coronary arteries with stenosis >70%	
0	20(20 %)
1	38(39 %)
2	15(15 %)
3	10(10 %)
CAG not carried out	1(1 %)
Absence of coronary atherosclerosis	14(14 %)
Number of patients with active vasospastic angina	24
Number of patients with unstable angina	17

containing 0.01% Tween-80, pH 8.2 (buffer 1). To 50 μl of buffer 1, containing 2 mM S-2251 and 0.07 mg/ml of PG, 100 μl of diluted plasma was added. The reaction was started by 50 μl of fibrin-monomer (0.1 mg/ml). The mixture was incubated for 30-60 min at 37°C and absorption of light was measured at 405 and 500 nm. The second international standard of TPA [6], obtained from the Institute of Biological Standards in London, was used for calibration. The type 1 TPA inhibitor (TPAI) was measured by the method in [3, 8] with the following modifications. Plasma was diluted 1:10 with buffer 1. To 50 ml of diluted plasma or buffer (TPA activity control) 10 μl of TPA diluted with buffer 1 to a concentration of 25 I.U./ml was added, and the mixture was incubated at 30°C. After 10 min 50 μl of 1 M Na-acetate buffer, pH 3.9, was added and incubation was continued for a further 10 min at 30°C. The sample was then diluted 50 times with buffer 1, and to 100 μl was added 50 μl of a mixture containing Glu-PG (0.7 mg/ml) and S-2251 (2 nmoles/ml) in buffer 1. The reaction was started by 50 μl of fibrin monomer (0.1 mg/ml). The sample was incubated for 3 h at 37°C, the light absorption of the solution being measured periodically at 405 and 500 nm. One I.U. of TPAI was taken to be the quantity of inhibitor inhibiting 1 I.U. TPA. The TPAI antigen was measured by enzyme immunoassay, using the COALIZA PAI-1 kit ("Kabi"). PC was determined by a kinetic method [11]. To 10 μl of the sample was added 77 μl of a mixture (75 μl of 50 mM Tris-HCl buffer, pH 8.4, 0.1% BSA, and 2 μl of PC activator [2]), and incubation was carried out for 5 min at 37°C. The reaction was started by 115 μl of 50 mM Tris-HCl buffer, pH 7.4, with 100 mM CsCl, and absorption of light was recorded for 1 min (405 nm), AT, PG, and antiplasmin (AP) were determined with standard kits of reagents from "Boehringer Mannheim" (Germany), "Kabi" (Sweden), and "Behring" (Germany) respectively. Standard plasma ("Behring") containing AT 105%, PG 99%, AP 100%, and PC 95%, was used for calibration. Degradation products of fibrinogen/fibrin and fibrinopeptide A (FPA) in the blood serum were determined by enzyme immunoassay [1].

EXPERIMENTAL RESULTS

The mean PC level was 110.7 ± 1.65% (from 65.4 to 187.0%). The difference between the level after VOT and the initial level of PC (after correction for hemoconcentration) varied within wide limits – from −54.8 to 57.3%.

Depending on the degree of change in PC (DPC) the patients as a whole were divided into four groups. The boundary between groups 1 and 2 was conventionally taken to be the PC consumption equal to the median (14%) among patients with a significant (over 4%) decrease in PC during the VOT. Group 1 consisted of patients with a change of PC during occlusion compared with the initial level (DPC) of under −14%, group 2 with DPC from −14% to −4%; group 3 consisted of patients for whom PC after venous occlusion differed from its initial value by not more than 4%, i.e., by the level of error of the method; group 4 consisted of patients with a significant (more than 4%) increase in PC after VOT.

In patients with marked consumption of PC (group 1) its initial level was a little higher than in the other groups, and significantly so compared with group 4 (Table 2).

TABLE 2. Factors of Fibrinolytic and Anticoagulant Systems in Groups of Subjects with Different Degrees of Change in Protein C during Venous Occlusion

Parameter	Group 1 (n = 30)	Group 2 (n = 22)	Group 3 (n = 22)	Group 4 (n = 24)	p
PC_i, %	115.2±3,0	106.6±4.7	106,4±3,3	100,0±3,6	$p_{1-4}=0.002$
PC_v, %	91.9±2,6	98,4±4.8	105,9±3,4	116,4±4,3	$p_{1-3}=0,002$
					$p_{1-4}<0,001$
					$p_{2-4}=0,008$
DPC, %	−23,3±1,8	−8,2±0,6	−0,5±0,5	16,4±2,7	ND
AT_i, %	110,0±4,4	107,1±5,2	110,2±5,7	99,6±4,9	ND
AT_v, %	96,4±3,2	103,0±4,2	111,9±5,7	104,5±4,5	$p_{1-3}=0,025$
DAT, %	−13,6±4,0	−4,1±4,0	1,7±3,9	5,8±4,6	$p_{1-3}=0,009$
					$p_{1-4}=0,003$
AP_i, %	106,5±2,3	103,5±3,0	104,4±3,8	105,9±3,8	ND
AP_v, %	80,4±2,6	91,2±3,2	92,5±2,9	92,1±4,1	$p_{1-2}=0,008$
					$p_{1-3}=0,004$
					$p_{1-4}=0,021$
DAP, %	−25,8±2,7	−12,9±4,6	−11,7±2,8	−13,9±3,9	$p_{1-2}=0,013$
					$p_{1-3}=0,001$
					$p_{1-4}=0,016$
PG_i, %	109,0±2,9	111,4±7,7	103,6±4,7	104,7±4,2	ND
PG_v, %	95,3±3,8	94,1±4,1	104,1±5,1	104,0±4,4	ND
DPG, %	−13,7±2,9	−17,3±5,8	0,5±3,1	−1,0±2,4	$p_{1-3}=0,002$
					$p_{1-4}=0,001$
					$p_{2-3}=0,011$
					$p_{2-4}=0,014$
FDP_i, μg/ml	34,6±5,9	23,3±2,5	24,7±3,0	23,2±2,6	ND
FDP_v, μg/ml	39,1±7,2	48,5±21,4	28,3±4,5	37,1±5,9	ND
TPAI, IU/ml	12,3±1,4	14,6±1,4	15,1±1,7	15,0±2,0	ND
$TPAI_i$, Ag	20,6±5,3	21,6±2,5	24,3±2,8	20,5±2,4	ND
$TPAI_v$, Ag	19,8±4,3	28,7±4,1	30,5±5,7	30,1±5,9	ND
DTPAI	−8,4±3,7	8,3±3,9	8,8±3,4	5,6±4,9	$p_{1-2}=0,018$
					$p_{1-3}=0,002$
					$p_{1-4}=0,076$
TPA, IU/ml	70,5±12,0	66,9±11,1	76,9±15	69,7±16,8	ND

Legend. TPA) Tissue plasminogen activator, TPAI) Tissue plasminogen activator inhibitor, PC) Protein C, PG) plasminogen, AP) α_2-antiplasmin, AT) antithrombin III, FDP) fibrinogen/fibrin degradation products (i – initial, v – after venous occlusion test, D) change during occlusion compared with initial level. ND) Not determined.

The initial AT level in the groups did not differ. In group 1 marked consumption of AT (13.6 ± 4.0%) also was observed. In the remaining groups, AT consumption did not differ from one to another (Table 2).

FPA was found in 30 patients. The patients were divided into two groups depending on the change in PC. Group 1 contained patients for whom PC decreased by more than 4%, group 2 – of patients with no decrease in PC (Fig. 1). The initial levels of FPA and PC clearly did not differ. During VOT, FPA of patients with PC consumption remained unchanged, but in the absence of PC consumption FPA rose significantly.

The initial PG level was the same in all groups. In groups with PC consumption (1 and 2) a decrease in PG also was observed, evidence of significant activation of fibrinolysis in these groups during VOT. In the groups with no decrease in PC (3 and 4) PG did not change significantly. The initial AP level did not differ. In all groups AP fell significantly, but this decrease in group 1 was significantly greater than in the other groups. Release of TPA against the background of VOT did not differ in the groups. The initial level of TPAI (activity) did not differ significantly. A change in TPAI antigen demonstrated marked differences in the reaction of group 1 (with marked PC consumption), in which it fell significantly. In the remaining groups its level rose a little.

We thus demonstrated that the response of PC to venous occlusion may differ and that it is associated with changes in other factors of hemostasis.

The first question is: does activation of the clotting system always take place during VOT. The data in the literature do not give an unambiguous answer. The question can be answered by an analysis of the change in FPA during venous occlusion. According to our data, in patients with PC consumption during VOT, i.e., in our opinion, the anticoagulant system of the endothelium is functioning well, thrombin is neutralized by thrombomodulin

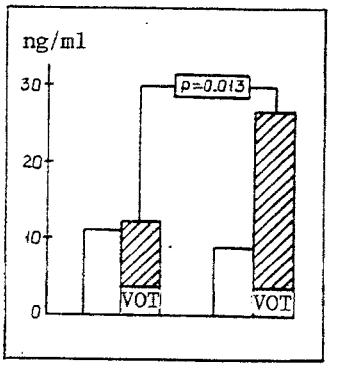

Fig. 1. Fibrinopeptide A in patients with and without protein C consumption during venous occlusion test. FPA) Fibrinopeptide A; VOT) venous occlusion test, p = 0.013) significance of differences between mean values of FPA in response to venous occlusion in groups of patients with and without consumption of protein C during VOT.

(which activates PC and thereby lowers its concentration) and by AT. In these patients thrombin did not react with its main substrates, fibrinogen, and the FPA level was unchanged. In patients without PC consumption, both anticoagulant failure of the endothelium and absence of thrombin secretion can be postulated. However, elevation of their FPA level strictly indicates that here also thrombin generation is taking place. In other words, the presence of FPA in the blood stream is evidence of the presence of thrombin which has not been inactivated by the anticoagulant system. In other words, data on the change in FPA concentration during VOT (together with data on the change in PC concentration) are evidence that, as a rule, thrombin generation does take place during VOT.

Information on agreement between changes in factors of the fibrinolytic system of the blood and changes in PC during venous occlusion is interesting. For instance, PG was significantly utilized only in those groups in which PC consumption took place. This fact is evidence that activation not only of the anticoagulant system, but also of the fibrinolysis system took place in this case. It is interesting to note that AP is consumed about equally in all groups (except group 1, where its consumption was almost twice as much as the change in the others). AP probably behaves as nonspecific inhibitor of proteases released during VOT. The initial level of TPAI activity was the same in all groups. During venous occlusion, however, in patients with marked PC consumption, the TPAI-antigen was not released into the lumen of the vessel, a fact which distinguishes this group from the others. Absence of release of TPAI may perhaps be only apparent. Since activated PC can interact with TPAI also, the possibility cannot be ruled out that TPAI, present in that complex, may change its antigenic properties and become inaccessible for determination by the usual method of enzyme immunoassay which we used. In other words, marked activation of PC leads not only to "arrest" of thrombin formation, but also to a fall in the TPAI concentration.

No definitive interpretation of the data was obtained so far as the causes of the increase in PC during VOT are concerned. The most likely explanation is the presence of tissue depots from which PC is utilized during VOT, though we are unaware of any evidence for their presence.

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