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REGULAR ARTICLE

Interaction of Fibrinolysis and Prothrombotic Risk Factors in Neonates, Infants and Children With and Without Thromboembolism and Underlying Cardiac Disease-A Prospective Study

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Abstract

To evaluate the role of plasminogen activator inhibitor-1 (PAI-1) and tissue-type plasminogen activator (t-PA) in children with an estimated risk of vascular occlusion reported to range from 7% to 16%, we conducted a prospective study in infants and children with underlying cardiac disease. One hundred and twenty-five children (neonate — 16 years) were investigated. In 9 infants out of the 125 children vascular occlusion occurred, closely related to cardiac catheterisation and arterial or venous lines during major cardiac surgery. Six of the nine neonates and infants with ($n = 6$) and without ($n = 3$) prothrombotic risk factors showed evidence of a basically impaired fibrinolytic system. Five of the nine infants showed increased PAI-1 clearly correlated to the 4G/4G genotype of the plasminogen activator-1 promoter polymorphism along with elevated t-PA concentration before the first diagnostic cardiac catheterisation was performed. One infant presented with increased t-PA concentration only. Five of the six children with reduced fibrinolytic capacity had further prothrombotic risk factors. **Conclusion:** Data of this study indicate that neonates and infants with underlying cardiac disease and basically increased PAI-1 due to the 4G/4G variant of the PAI-1 promoter polymorphism along with elevated t-PA levels in combination with further prothrombotic risk factors are at high risk of developing early thromboembolism during cardiac catheterisation.

Key Words: PAI-1; t-PA; Factor V G1691A; Lipoprotein (a); Cardiac disease

Within the general population, the incidence of thromboembolism in children is estimated at 0.07/10,000 compared with 2.5–5% for the adult population [1]. Newborns and infants <1 year of age show the greatest risk of vascular occlusions, and the incidence decreases significantly following the first year of life. However, the majority of affected infants and children have several associated risk factors prior to thrombosis, e.g., peripartal asphyxia, foetal diabetes, dehydration,

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underlying cardiac diseases, septicaemia, central lines, cancer, cancer-associated polychemotherapy, trauma, or surgery [1]. In addition, as in adults [2–4] genetic risk factors of familial thrombophilia, i.e., deficiencies of the protein C pathway or antithrombin in the majority of cases, were increasingly recognised as being involved in childhood thromboembolism [5–8]. Apart from familial thrombophilia causing hyper coagulability, little is known concerning the role of fibrinolysis in this field.

Increased tissue-type plasminogen activator (t-PA) antigen levels and/or elevated plasminogen activator inhibitor (PAI-1) concentrations reflecting reduced fibrinolysis [9,10] were reported to be associated with a risk of recurrent myocardial infarction and with a high risk of future stroke [11–13]. However, besides the recently described interaction of a thrombin-activable fibrinolysis inhibitor (TAFI) leading to increased levels of PAI-1 [14], elevated PAI-1 levels are associated with the 4G-allele of a 4G/5G insertion/deletion polymorphism located in the promoter region 675 bp upstream from the transcription start sequence of the PAI-1 gene [15].

Percutaneous insertion of catheters into vessels was first described in 1953 [16]. Since then catheterisation has become important in diagnosis and treatment of congenital heart disease in children. The rate of thromboembolism following cardiac catheterisation in paediatric patients has been estimated to be as high as 4–16% [17,18]. To evaluate the role of fibrinolytic proteins and the possible interaction with prothrombotic risk factors, we conducted this prospective study in children at high risk for vascular accidents.

1. Methods

1.1. Ethics

The present prospective study is part of a multicentre case-control study and was performed in accordance with the ethical standards laid down in a relevant version of the 1964 Declaration of Helsinki and approved by the medical ethics committee at the Westfälische Wilhelms-University, Münster, Germany.

1.2. Patients

With parental consent 125 children suffering from congenital heart disease (neonate — 16 years) consecutively admitted to perform diagnostic cardiac catheterisation and subsequently to operative therapeutic interventions, were investigated during a 36-month-period.

1.3. Anticoagulation Administered

During venous cardiac catheterisation paediatric patients received low-dose flush heparin as recently described [20]. In addition, in patients with arterial cardiac catheterisation standard heparin (300–400 IU/kg b.w.) were administered for further 24 h. Infants and children with central lines received standard heparin 100–300 IU/kg b.w. per day until the catheter was removed. Paediatric patients suffering symptomatic thrombosis received vitamin K-antagonists adjusted to an INR between 2.5 to 3.5 for at least 6 months, switched over to heparin therapy when operative interventions were performed.

1.4. Study Endpoint and Imaging Methods

The diagnosis of clinically suspected venous or arterial thrombosis, used as the endpoint of this study, was made if echogenic material was found within the lumen of the vasculature on gray scale, and if partial or complete absence of flow was demonstrated by pulse-wave and Colour Doppler ultrasonography. Thromboembolic stroke was confirmed by MR-imaging methods.

1.5. Blood Samples

Blood samples for coagulation studies were obtained by separate venous puncture immediately before, at the end of, and 24 h after catheterisation [19,20]. In addition, in neonates and infants with a history of thrombosis, blood samples were obtained at least 3–6 months after the thrombotic episode. With parental consent blood samples were drawn from a peripheral vein into premarked 3-ml plastic tubes (citrate 3.8% /blood 1:10; Saarstedt), immediately placed on iced water and centrifuged at 4°C at 3000 g for 20 min. Platelet poor

plasma was snap-frozen and stored in plastic tubes at −79°C. For genetic analysis we obtained venous blood in EDTA-treated sample tubes (Sarstedt, Nümbrecht, Germany), from which cells were separated by centrifugation at 3000 g for 15 min. The buffy coat layer was then removed and stored at −70°C pen-ding DNA extraction by standard techniques.

1.6. Assays of Haemostatic Factors

PAI-1 activity was measured with chromogenic substrate (Chromogenix, Mølndal, Sweden), PAI-1 and t-PA concentrations were analysed with ELISA technique (Chromogenix). In addition, the response to activated protein C (Chromogenix), factor V, prothrombin (factor V and factor II deficient plasma: Instrumentation Laboratory, Munich, Germany), protein C (chromogenic substrate S 2765: Chromogenix), free protein S antigen (ELISA: Asserachrom, Diagnostica Stago, Asnières-sur-Seine, France), antithrombin (chromogenic substrate S 2765: Chromogenix), antiphospholipid antibodies (IgM/IgG: Stago) and lipoprotein (a) (Lp(a): Chromogenix) were measured as described earlier [6,7,21,22]. Total protein S, protein C antigen (Asserachrom, Stago) and heparin cofactor II antigen (HCII-EIA: Diagnostik International, Karlsdorf, Germany) were measured with ELISA technique.

1.7. Assays for Genotyping

The PAI-1 4G/5G polymorphism, the FV G1691A gene mutation and the prothrombin G20210A variant were measured as recently reported [4,23,24].

1.8. Classification of Risk Cut-Off

As recently described, a heterozygous type I deficiency state (protein C, antithrombin) was diagnosed when functional plasma activity and immunological antigen concentration of a protein were below 50% of normal of the age-related limit [25, 26]. A homozygous state was defined if activity levels and antigen concentrations were less than 10% of normal. A type II deficiency was diagnosed with repeatedly low functional activity levels along with normal antigen concentrations. The diagnosis of protein S deficiency was based on reduced free protein S antigen levels combined with decreased or normal total protein S antigen concentrations, respectively. Criteria for the hereditary nature of a haemostatic defect were its presence in at least one further first or second degree family member and/or the identification of a causative gene mutation.

1.9. Statistics

Calculations of medians, ranges, median absolute deviation (MAD) and nonparametric statistics (Wilcoxon signed rank; Wilcoxon–Mann–Whitney) were performed with the Stat. View 4.02 programme.

2. Results

2.1. Patient Population

Since 1996, 125 consecutively recruited Caucasian patients were enrolled in the study. The underlying noncyanotic CHD ($n=92$) were atrial septal defect ($n=22$), ventricular septal defect ($n=20$), coarctation of aorta ($n=11$), atrioventricular septal defect ($n=9$), hypoplastic left heart syndrome ($n=6$), total anomalous pulmonary venous connection ($n=6$), patent ductus arteriosus ($n=7$), aortic valve stenosis ($n=5$), subaortic stenosis ($n=3$), and pulmonary valve stenosis ($n=3$). Cyanotic CHD were found in 33 out of 125 childhood patients presented here: d-transposition of great arteries ($n=8$), tetralogy of Fallot ($n=9$), double inlet ventricle ($n=6$), pulmonary atresia ($n=5$), tricuspid atresia ($n=3$), and Ebstein's anomaly ($n=2$), respectively.

2.2. Prevalence of Prothrombotic Polymorphisms

The prevalence rate of prothrombotic polymorphisms in the population studied was within the range reported for healthy paediatric Caucasian individuals [6,7]: six patients carried the heterozygous FV G1691A mutation, three the PT G202010A variant, six increased Lp(a), and two showed confirmed protein C type I deficiency. The PAI-1 4G/4G genotype not combined with

the abovementioned risk factors was presented in 26 children (21%). In addition, the PAI-1 4G/4G genotype was found in six children carrying the abovementioned risk factors [FV G1691A ($n=2$); increased Lp(a) ($n=2$); protein C type I deficiency ($n=2$)]. No antithrombin-, protein S-, or heparin cofactor II deficiency was diagnosed in the patients investigated.

2.3. Thromboembolism in Children with Underlying Cardiac Disease

Within the 36-month study period 9 neonates and infants out of the 125 children investigated developed vascular occlusion, closely related to diagnostic cardiac catheterisation and arterial or venous lines during the course of major cardiac surgery. Vascular occlusion was located near the catheter implantation site in all infants, and additional arterial thromboembolism had occurred in one infant, respectively.

PAI-1 and t-PA single values, measured before the first cardiac catheterisation, genetic risk factors of familial thrombophilia and the number of thrombotic events are shown in Table 1. Interestingly, four of the nine infants with vascular occlusion developed recurrent thromboembolism during the study period, in six cases associated with established prothrombotic risk factors of childhood thrombophilia.

No thromboembolism was observed in the 11 childhood carriers of established prothrombotic risk factors [FV G1691A ($n=4$); PT G20210A ($n=3$); increased Lp(a) ($n=4$)] undergoing cardiac catheterisation beyond infancy. In addition, besides the five symptomatic infants mentioned above, none of the remaining subjects with homozygous PAI-1 4G/4G polymorphism not com-

Table 1. Patients' data

Patients diseases	Thrombotic onset after catheterisation	PAI-1 <75 ng/ml, PAI-1 <15 AU/ml	t-PA <8 ng/ml	Genetic risk factor	No. of thrombotic events and risk factor
F, 1 week TGA	24 h	35, 7	1	FV G1691A	1, arterial line
F, 6 weeks ASD	48 h	145, 22	20	FV G1691A, PAI-1 4G/4G	1, venous diagnostic catheter
F, 3 months TGA	24 h, 7 days, 21 days	185, 37	14	Protein C, PAI-1 4G/4G	3, diagnostic venous catheter and central line
M, 5 months TOF	13 days, 10 days	120, 18	18	Protein C, PAI-1 4G/4G	2, central venous lines
M, 4 months TGA	48 h, 10 days	75, 8	11	Lipoprotein (a)	2, diagnostic venous catheter and venous central line
F, 5.5 months VSD	36 h	35, 10	1.4	–	1, diagnostic venous catheter
F, 6 weeks VSD	24 h	21, 8	2	–	1, diagnostic venous catheter
F, 3 weeks VSD	12 h	125, 25	15	PAI-1 4G/4G	1, diagnostic venous catheter
M, 2 weeks TGA	24 h, 3 days	110, 28	12	Lipoprotein (a), PAI-1 4G/4G	2 and stroke, spontaneous and diagnostic arterial catheter

Underlying cardiac diseases, PAI-1 and t-PA values before the first diagnostic catheterisation, genetic risk factor of familial thrombophilia and number of thrombotic events within the first 12 months of life. Additionally, cut-off values for PAI-1 and t-PA are given.
Abbreviations used: ASD: atrial septal defect; F: female; M: male; TGA: transposition of the great arteries; TOF: tetralogy of Fallot; VSD: ventricular septal defect.

bined with established prothrombotic risk factors ($n = 17$) suffered catheter-related thrombosis.

2.4. Course of PAI-1 in Children not Suffering From Thrombosis or Prothrombotic Risk Factors

Fig. 1a and b shows the course of PAI-1 antigen (a) and t-PA antigen (b) before, at the end of and 24 h after cardiac catheterisation in 82 infants and children not suffering from thrombosis and not carrying established prothrombotic risk factors: Compared with starting values, concentrations of PAI-1 followed a similar pattern, showing a clearly significant increase at the end of cardiac catheterisation and returning to pretreatment values 24 h later. In contrast, t-PA antigen concentrations remain still significantly elevated 24 h after cardiac catheterisation.

As recently shown, the course of PAI-1 in children not suffering from prothrombotic risk factors or thrombosis was similar to the course of soluble thrombomodulin and prothrombinfragment F1 + 2 in childhood patients undergoing venous cardiac catheterisation (data not shown: [20]).

2.5. Course of PAI-1 and t-PA in Children Suffering from Thrombosis

Fig. 2a and b shows the course of PAI-1 (a) and t-PA (b) in the infants before cardiac catheterisation, at the end of catheterisation, 24 h after catheterisation (in seven of nine patients identical with the symptomatic thrombotic onset), and 3–6 months later. Both PAI-1 and t-PA were markedly increased after catheterisation and during the onset period. Baseline values were found 3–6 months after thromboembolism had occurred. In five of the nine infants with thrombosis, baseline PAI-1 and t-PA plasma values were above the normal age-related reference range, and one infant presented with increased t-PA concentrations alone. However, when comparing single values of the nine patients with vascular accidents with values obtained from asymptomatic patients the result did not reach statistical significance (Wilcoxon–Man–Whitney: P=.75), mainly due to the small numbers of symptomatic children. In four out of five infants with increased PAI-1 the 4G/4G promoter polymorphism was found. Interestingly five of six children with reduced fibrinolytic

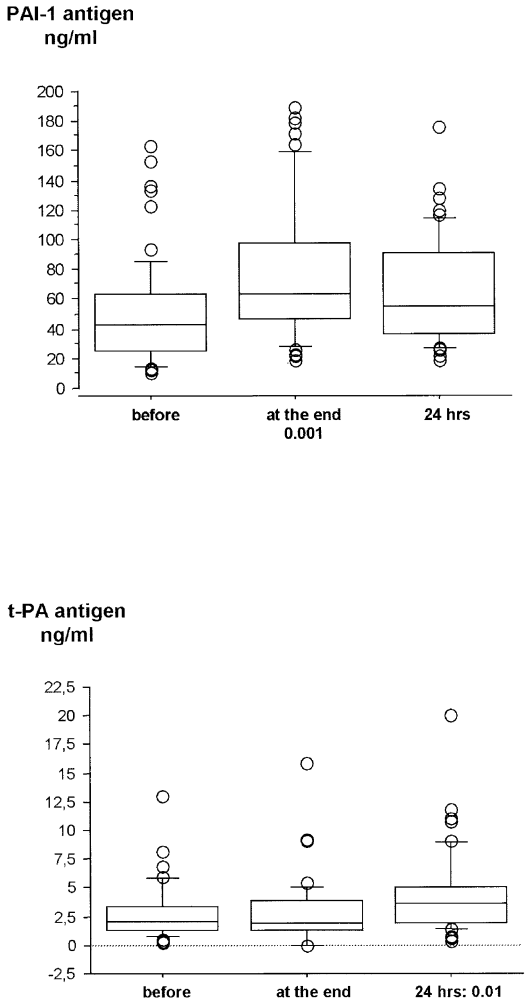

Fig. 1. (a) Plasma values (median and median absolute deviation: MAD) of PAI-1 and (b) antigen in children with underlying cardiac disease without familial thrombophilia. Compared with pretreatment values, PAI-1 concentrations were significantly increased at the end of catheterisation and returned to pretreatment values 24 h later (shaded area: paediatric reference range).

capacity before thrombotic onset had further prothrombotic risk factors, and in two neonates and infants suffering symptomatic thrombosis no genetic risk factor could be identified so far.

3. Discussion

With reference to literature data [17,18], 6 to 12 children in this study were expected to develop vascular occlusion during the study period: In 9 infants out of 125 children investigated vascular occlusion occurred, closely related to cardiac catheterisation and arterial or venous lines dur-

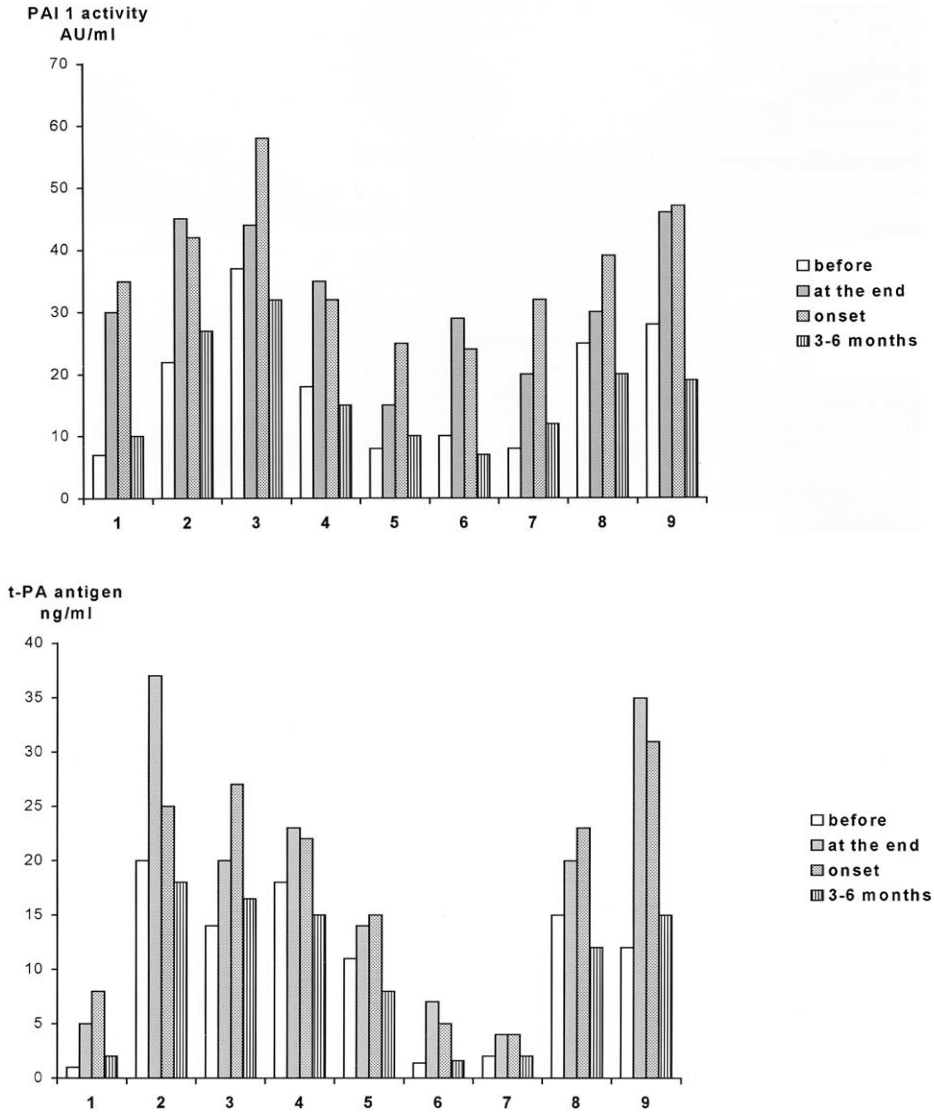

Fig. 2. (a) PAI-1 activity (single values) and (b) t-PA antigen (single values) in neonates and infants with first vascular occlusion following diagnostic cardiac catheterisation (white column: before catheterisation), at the end of the catheter (dotted dark column), onset of vascular occlusion (dotted light column), and 3–6 months later (striated column).

ing major cardiac surgery. Since neither age, weight, duration of catheterisation, nor the use of balloon catheters was found to be a risk factor for venous thrombosis following paediatric cardiac catheterisation [17,18], further risk factors possibly involved have to be discussed: six of the nine neonates and infants with and without familial thrombophilia showed evidence of a basically impaired fibrinolytic system, not caused by the thrombotic onset alone. Compared with healthy sex- and age-matched controls [data not shown: 25 26 27], concentrations of PAI-1 and// or t-PA were clearly increased before the first thrombotic episode, comparable to values reported by Corrigan and Jeter [28] in stressed human newborns. In addition, the symptomatic infants showed higher initial PAI-1 and t-PA values compared with the remaining nonsymptomatic childhood patients (Figs. 1a,b and 2a,b). This result, however, did not reach statistical significance due to the small number of symptomatic children.

In the majority of children affected, increased PAI-1 levels were found along with the 4G/4G variant of the recently described polymorphism in the PAI-1 promoter region [15]. This is in

accordance with recent reports that the release of t-PA or PAI-1 antigen occurs many years before vascular occlusion and that either of these factors may increase the risk of future vascular accidents [12,13,19,29]. In addition, t-PA and PAI-1 concentrations are increased not only in stressed human newborns [28] and in children with the factor V G1691A mutation [21] but also in children with further genetic risks of familial thrombophilia [30], i.e., protein C deficiency and elevated lipoprotein (a).

However, as previously discussed [14,22], to understand the paradoxical association of thromboembolic events with plasma levels of t-PA, a factor that promotes fibrinolysis [10], it should be borne in mind that t-PA covalently binds to a series of inhibitors including PAI-1. In contrast, the enzymatically active fraction of t-PA is not bound to PAI-1 or to any other inhibitor [28]. In addition, elevated t-PA antigen levels have been reported in subjects with a high plasma PAI-1 [31,32]. Considering the negative correlation between t-PA antigen and t-PA activity in plasma samples [33], an increase of t-PA antigen is thought to reflect an inhibitory effect of PAI-1 antigen rise with the increase in PAI-1 inhibition. Thus, high levels of either factor reflect impaired fibrinolysis.

Bajzar et al. [14,34] demonstrated that plasma of subjects with factor V G1691A mutation produces a clot that is more resistant to the profibrinolytic effect of APC. The authors proposed that impaired regulation of thrombin generation, which results in an increase of thrombin concentration in plasma, induces activation of the recently published TAFI [34]. TAFI modulates t-PA-induced activation of Glu-plasminogen [34], resulting in low t-PA and high PAI-1 activities being measured in children with factor V G1691A before and after venous occlusion [21]. Thus, since TAFI is produced by increased thrombin generation in patients with the common mutation in the factor V gene [14], we hypothesise that TAFI might also be produced in individuals with genetic thrombophilia such as protein C deficiency or familiarly increased Lp (a) values.

Data of this study indicate that neonates and infants with underlying cardiac disease and basically increased PAI-1 due to the 4G/4G variant of the PAI-1 promoter polymorphism along with elevated t-PA levels in combination with further prothrombotic risk factors are at high risk of developing early thromboembolism during endothelial damage. However, whether elevated values of PAI-1 or t-PA in neonates and infants with or without genetic risk factors of familial thrombophilia may predict future vascular occlusion in subjects at risk [20], requires confirmation in a large multicentre study.

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