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REGULAR ARTICLE

Plasminogen activator inhibitor 1 expression in platelets is not influenced by the 4G/5G promoter polymorphism

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Abstract

In the present study we investigated the influence of the 4G/5G promoter polymorphism of the PAI-1 gene on the levels of PAI-1 mRNA and protein in platelets. After a screening of healthy male subjects, thirty-eight subjects homozygote for either the 4G or 5G allele were investigated. mRNA levels were quantified by real-time PCR and PAI-1 antigen in platelets and plasma was analysed by ELISA. The platelet PAI-1 mRNA levels correlated significantly with the PAI-1 antigen content, but there was no association between the polymorphism and mRNA levels, or protein levels in platelets. Also, plasma levels of PAI-1 antigen were not associated with homozygosity of the 4G/5G polymorphism, but as expected BMI and triglycerides emerged as significant predictors of plasma PAI-1 levels. The importance of the 4G/5G polymorphism on PAI-1 levels is controversial and the present study shows that although levels of platelet mRNA are related to its content of PAI-1 protein, there is no association between the 4G/5G promoter polymorphism and platelet PAI-1 mRNA or protein expression.

Introduction

Intravascular fibrinolysis is initiated by tissue plasminogen activator (tPA) that converts plasminogen to plasmin. The main physiological regulator of the activity of tPA is plasminogen activator inhibitor type 1 (PAI-1), which resides in the circulation both in plasma and in platelet alpha-granules. The majority of circulating PAI-1 is found in platelets while plasma PAI-1 constitutes only approximately 10% [1]. Platelet PAI-1 is released locally upon activation and it is generally assumed that it thereby contributes to stabilisation of thrombi by

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inhibiting endogenous fibrinolysis [2]. This view has, however, been somewhat difficult to reconcile with the fact that the majority of platelet PAI-1 is considered to be inactive [1,3].

Platelets are anucleate and cannot synthesize mRNA. However, it was shown already in 1967 by Booyse et al. that platelets retain megakaryocyte-derived mRNAs [4,5], and it was subsequently confirmed by microarray analysis that platelets contain mRNA transcripts for a large number of genes [6,7]. In 2004 we reported that platelets contain mRNA for PAI-1 and that they have a constitutive de novo synthesis of active PAI-1 [8]. This observation may provide a mechanistic explanation for how platelet PAI-1 can make clots resistant to fibrinolytic degradation.

The PAI-1 promoter contains a common polymorphism which appears to be of importance for its transcriptional activity and possibly also PAI-1 levels in plasma. This polymorphism consists of a single base-pair insertion/deletion (4G or 5G) located -675 bp upstream of the transcription start site. It has been shown in expression experiments in HepG2 cells by Dawson et al. that, when stimulated with IL-1, the 4G allele produces 6 times more PAI-1 mRNA [9]. Indeed, an association between the 4G/5G polymorphism and cardiovascular disease has been observed in some studies [10,11]. However, although previous studies have suggested 42 to 60% heritability rates of PAI-1 levels [12,13], a large number of clinical studies that have evaluated the influence of the 4G/5G polymorphism for plasma PAI-1 concentration have shown divergent results [14–18].

Thus, it is possible that the association between the 4G/5G polymorphism and thrombotic events is mediated via an effect on platelet PAI-1 synthesis rather than on the plasma levels of the inhibitor. In this study we therefore investigated the potential association between the 4G/5G polymorphism and levels of PAI-1 mRNA and PAI-1 protein in platelets. Furthermore we studied factors known to influence the plasma PAI-1 levels and their correlation to platelet PAI-1 mRNA and antigen as well as their relation to the promoter polymorphism.

Materials and methods

Subjects

Samples for genotyping were collected from 86 apparently healthy male subjects (women were excluded due to the variations in PAI-1 levels during the menstrual cycle [19]). They were all non-smokers without any known disease, and aged between 21 and 50. The subjects were informed about the purpose of the study and gave their informed consent. The study has been approved by the Ethics committee of Göteborg University.

DNA extraction and genotyping of the 4G/5G polymorphism

Five ml EDTA-blood was collected and DNA was extracted using QIAamp® 96 DNA Blood Kit and QIAamp® DNA Blood mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Genotyping was performed using a 5′ nuclease Taqman genotype assay and the ABI PRISM® 7700 Sequence Detection System (Perkin-Elmer Applied Biosystem, Foster City, CA, USA).

Preparation of platelets and plasma

Blood samples for plasma and platelet preparations were drawn between 09:00 and 10:00 due to the diurnal variation of plasma PAI-1 [20]. Special care was taken not to activate the platelets and no stasis was used during the sampling. The test subjects had been informed not to take aspirin or non-steroid anti-inflammatory drugs 10 days prior to the blood sampling. They had been asked to refrain from extreme physical activity, alcohol and avoid high fat diet at least one day prior to the sampling.

Platelet isolation

Blood was drawn in acid-citrate-dextrose (ACD 1.5 ml/8.5 ml blood) containing 100 nM PGE_1 (Sigma, St Louis, MO, USA). Platelet-rich plasma (PRP) was prepared by centrifugation at 150 ×*g* for 20 min. The PRP was re-centrifuged at 150 ×*g* for 10 min and then pelleted at 800 ×*g* for 15 min. The plasma was discarded and the platelet pellet was resuspended and washed in Pipes/saline/glucose buffer (5 mM Pipes, 145 mM NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgCl_2$ and 5.5 mM glucose) containing 100 nM PGE_1. Finally the platelets were pelleted at 800 ×*g* 15 min.

Platelet and leukocyte count

Platelets were counted both manually, using a Bürker chamber, and automatically by flow cytometry (Celldyn 2000, Abbot Wiesbaden, Germany). The results presented are calculated on the basis of manually counted platelets (TPK). Analysis of leukocyte contamination was made in all platelet preparation and the number of leukocytes were less than 3–5 per 10^5 platelets in all samples which is considered to be below interfering leukocyte RNA levels [6].

Plasma preparation

3 ml of blood was collected in citrate (0.129 M) and immediately put on ice. Samples were centrifuged at 2000 ×*g*, at 4 °C, for 15 min, plasma was collected and stored at −70 °C until analysis.

Platelet mRNA quantification

Quantification of platelet mRNA has been described previously [8], in brief: Platelets were lysed in Trizol® (Invitrogen, Paisely, UK), RNA was extracted according to the manufacturers instructions and 0.25 μg platelet RNA was used for reversed transcription. GAPDH and cyclophilin were used as endogenous controls to correct for potential variation in RNA loading, or efficiencies of the reverse transcription or amplification reaction.

Analysis of PAI-1 antigen

Platelets were lysed (50 mM Tris–HCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, and 0.1% Triton X-100) for 30 min on ice. Enzyme linked immunosorbent assay (ELISA) (Innotest PAI-1; Hyphen BioMed, Andrésy, France) was used to determine the total PAI-1