Alpha-2-Macroglobulin Inhibits the Anticoagulant Action of Activated Protein C in Cord and Adult Plasma

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Key Words

Activated protein C ·
Alpha-2-macroglobulin · Thrombin
potential · Prothrombin activation

manner when a2-M levels were successively elevated, reflected in increased thrombin potential (TP), and enhanced at low a2-M levels, reflected in decreased TP.

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Abstract

Healthy newborns have a very low risk of thrombosis. It has been suggested that this is partly due to the anticoagulant effect of alpha-2-macroglobulin (a2-M). This broad-spectrum protease binding glycoprotein is physiologically elevated in newborns over adult values and has been shown to complex generated alpha-thrombin. In our present study, we point out that a2-M also acts as a procoagulant by inhibiting activated protein C (APC). In all experiments performed in cord and adult plasma the anticoagulant action of APC was diminished in a dose-dependent

Introduction

The risk for thromboembolic complications is considerably less for newborns and children than for adults for any given insult, due to lower plasma concentrations of vitamin K-dependent coagulation factors, i.e. prothrombin, and contact factors [1, 2]. The capacity of cord plasma to generate thrombin is decreased to 30–50% of adult values [3]. In addition, alpha-2-macroglobulin (a2-M) has been suggested as a major anticoagulant protein in infancy and has been demon-

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strated to complex up to 49% of generated alpha-thrombin in cord plasma [4, 5]. On the other hand, a2-M has been shown to bind activated protein C (APC) [6], one of the most important anticoagulant proteins. In addition, increased complex formation between a2-M and APC has been demonstrated to be associated with an unfavorable outcome in patients suffering from disseminated intravascular coagulation (DIC) [7], suggesting that a2-M might diminish the anticoagulant action of APC. Previously, we have demonstrated a dose-dependent anticoagulant effect of APC in cord and adult plasma using the determination of thrombin generation (thrombin potential, TP) [8]. The TP has been shown to be a reliable parameter to assess the combined effects of all the factors that may influence thrombin generation in a given sample [9–13].

We investigated the effect of different concentrations of a2-M on the anticoagulant action of APC in both cord and adult plasma since levels of a2-M and APC during delivery are elevated over adult values [14] by using the determination of the TP. In addition, since the TP does not exactly reflect the amount of prothrombin activated, we determined the generation of prothrombin fragments 1+2 (F1+2) in the presence of different concentrations of a2-M.

Materials and Methods

Reagents

Buffer A contains 0.05 M Tris-HCl at pH 7.4, 0.1 M NaCl and 0.5 M bovine serum albumin. Buffer B is analogous to buffer A but contains in addition 20 mM EDTA. a2-M concentrate from human plasma was purchased from Sigma, Vienna, Austria. A stock solution was prepared by dissolving 10 mg of the lyophilized glycoprotein in 500 µl of distilled water, dialysis against buffer A for 3 h at 4°C, aliquots of 100 µl were stored at –70°C. APC from human plasma was obtained from ICN Biomedicals GmbH, Esch-

wege, Germany. A stock solution was prepared by dissolving 10 µg of the lyophilized protein in 370 µl of distilled water, dialysis against buffer A for 3 h at 4°C, aliquots of 30 µl were stored at –70°C. Proteinase A, an enzyme from the venom of the Mexican west-coast rattlesnake (*Crotalus basiliscus*), was purchased from Pentapharm Ltd, Basel, Switzerland. To 0.175 mg of the lyophilized protein, 400 µl of distilled water was added and stored at 4°C. Unitest™ Assay for a2-M determination in human plasma was obtained from CoaChrom Diagnostics, Vienna, Austria. As a trigger for the extrinsic coagulation pathway, we used Thromborel S from Behring Diagnostics GmbH, Marburg, Germany, containing human placental thromboplastin and calcium chloride. The fibrin polymerization inhibitor H-Gly-Pro-Arg-Pro-OH (GPRP, Pefabloc FG) was purchased from Pentapharm Ltd. The chromogenic substrate used for thrombin determination was H-D-Phe-Pip-Arg-pNA.2HCl (S-2238) from CoaChrom Diagnostics. Test kit F1+2 Micro for determination of prothrombin F1+2 generation was obtained from Behring Diagnostics GmbH. Stopping solution for F1+2 determination consisted of Trasylol:EDTA:Na-citrate = 8:1:1 and 110 µM PPACK (D-Phe-Pro-Arg chloromethyl-ketone) from Sigma. Trasylol from Bayer, Vienna, Austria contained aprotinin, a protease inhibitor.

Collection and Preparation of Plasma

Cord blood was obtained immediately following the delivery of 12 full-term infants (38–42 weeks' gestational age). It was collected into 0.1 M citrate using a two-syringe technique, centrifuged at room temperature for 15 min at 2,800 g, pooled and stored at –70°C in propylene tubes until assayed. Clotting factors, AT, protein C, protein S, and a2-M in the pooled cord plasma were in the normal range for neonates. In the same way, plasma from 10 healthy adults was collected, prepared and checked.

Preparation of Plasma with Different a2-M Concentration

The a2-M activity of the pooled neonatal or adult plasma was decreased by supplementation of proteinase A, a metalloenzyme that forms a complex with a2-M at a molar ratio of 1:1 [15, 16]. To 1 ml of plasma, different amounts (0–20 µl) of proteinase A were added, resulting in a2-M activities from 1.8 to 3.6 µM for cord plasma and from 1.1 to 2.0 µM for adult plasma, respectively. To increase the a2-M concentration, to 1 ml of plasma, different amounts (0–80 µl) of purified a2-M concentrate were added, resulting in a2-M concentrations from 3.6 to 5.9 µM in

cord plasma and from 2.0 to 4.6 μM in adult plasma, respectively.

Determination of the a2-M Concentration

Determination of the a2-M concentration of pooled cord and adult plasma was performed using the standard test kit UnitestTM Assay for a2-M determination in human plasma from CoaChrom Diagnostics.

Determination of the PC Content

Determination of the PC content of pooled plasma was performed by means of a standard coagulation method using protein C-deficient plasma on a Behring Coagulation Timer from Behring Diagnostics GmbH.

Activation of Plasma

200 μl of plasma with different a2-M concentrations was incubated with 10 μl of buffer A (containing different amounts of APC) for 1 min at 37°C. After subsequent incubation with 20 μl buffer A containing 1.0 mg/ml H-Gly-Pro-Arg-Pro-OH (GPRP, Pefabloc FG) for 1 min at 37°C to inhibit fibrin polymerization, the plasma samples were activated by addition of 200 μl Thromborel S and thrombin generation was recorded. Clotting times were determined in the absence of Pefabloc FG on a Behring Fibrin Timer from Behring Diagnostics GmbH.

Determination of Thrombin Generation

We used a subsampling method derived from a recently described technique [10, 13]. Plasmas were prepared and activated as described above. At timed intervals, 10- μl aliquots were withdrawn from the activated plasma and subsampled into 490 μl buffer B containing 225 μM S-2238. The reagents were pre-warmed to 37°C. Amidolysis of S-2238 was stopped after 6 min by addition of 250 μl 50% acetic acid. The amount of thrombin generated was quantitated by measuring the absorbance by double wavelength (405–690 nm) in the Anthos microplate reader 2001, from Anthos Labtec Instruments GmbH, Salzburg, Austria. The total amidolytic activity measured is caused by the simultaneous activity of free thrombin and a2-M/thrombin complex [9]. The amount of free thrombin was determined by two different methods: (A) At timed intervals, aliquots of the extrinsically activated plasma were subsampled into buffer B containing heparin (20 U/ml) and AT (3.4 U/ml) to rapidly and completely inactivate free thrombin in the sample. The residual amidolytic activity was then determined as described above for the total amidolytic activity. The amidolytic activity of the a2-M/thrombin complex was subtracted from the total amidolytic activity [3].

(B) Free thrombin generation curves were calculated by mathematical treatment of total amidolytic activity curves using a method developed by Hemker et al. [10].

Determination of Prothrombin F1+2

Plasmas were prepared and activated as described above. At timed intervals, 5- μl aliquots were withdrawn from the plasma and subsampled into 495 μl stopping solution. After subsequent 1:10 dilution in stopping solution, the amount of F1+2 generated was quantitated by using a standard immunoenzymatic test kit.

Statistical Analysis

The results shown are expressed as means ($n = 3$). Data were statistically analyzed for significance using the Kruskal-Wallis nonparametric analysis of variance taking $p < 0.05$ to indicate a significant difference. Pearson's correlation was used to assess the relation between different a2-M levels and the respective thrombin potentials, end levels of the prothrombin generation curves, and clotting times.

Results

To determine free thrombin generation curves from total amidolytic activity curves, we used two different methods as described in Material and Methods. Both methods resulted in the same free amidolytic activity curves. Experiments were done in cord plasma over a concentration range of 1.8–5.9 μM a2-M and in adult plasma over a concentration range of 1.1–4.6 μM a2-M. Protein C concentration was 90% in pooled adult and 42% in pooled cord plasma. All experiments were done in triplicate; the average is shown in all figures. SEM were not shown for clarity of graph reading but represented less than 10% of the mean. The capacity of normal cord plasma to generate free thrombin was decreased to about 50% as compared to adult plasma.

Fig. 1. a Free thrombin generation in cord plasma containing 2.0, 3.6 or 4.3 μM $\alpha_2\text{-M}$ in the absence of APC (■, TP = 291 nM·min). Effects of supplementation of 21 nM APC to plasma with different $\alpha_2\text{-M}$ concentrations (▼, 2.0 μM , TP = 150 nM·min; ▲, 3.6 μM , TP = 236 nM·min; ●, 4.3 μM , TP = 291 nM·min) on free thrombin generation. **b** Reduction of the TP by addition of 21 nM (■) or 42 nM (●) APC to plasma containing 1.8–5.9 μM $\alpha_2\text{-M}$ related to corresponding plasma in the absence of APC.

Effect of APC Administration to Plasma with Different $\alpha_2\text{-M}$ Content on Thrombin Generation

In the absence of APC, the same amounts of free thrombin were generated in cord plasma containing different amounts of $\alpha_2\text{-M}$ (fig. 1). Reduction of the TP due to addition of 21 nM APC depended on the amount of $\alpha_2\text{-M}$ present in the cord plasma sample ($p < 0.05$; fig. 1). Essentially the same results were obtained in adult plasma (fig. 2).

Effect of APC Administration to Plasma with Different $\alpha_2\text{-M}$ Content on Prothrombin Activation

In the absence of APC, the same amounts of F1+2 were generated in cord plasma containing different amounts of $\alpha_2\text{-M}$ (fig. 3). Suppression of prothrombin activation due to addition of 21 nM APC depended on the $\alpha_2\text{-M}$ content of the cord plasma sample ($p < 0.05$; fig. 3). Essentially the same results were obtained in adult plasma (fig. 4).

Fig. 2. a Free thrombin generation in adult plasma containing 1.4, 2.0 or 3.2 μM a2-M in the absence of APC (■, TP = 627 nM·min). Effects of supplementation of 42 nM APC to plasma with different a2-M concentrations (▼, 1.4 μM , TP = 277 nM·min; ▲, 2.0 μM , TP = 428 nM·min; ●, 3.2 μM , TP = 627 nM·min) on free thrombin generation. **b** Reduction of the TP by addition of 21 nM (■) or 42 nM (●) APC to plasma containing 1.1–4.6 μM a2-M related to corresponding plasma in the absence of APC.

Effect of APC Administration to Plasma with Different a2-M Content on Clotting Time

Prolongation of the clotting time due to addition of 21 or 42 nM APC (final) depended on the amount of a2-M present in the given cord or adult plasma sample ($p < 0.01$; fig. 5).

Effect of a High-Dose Application of APC on TP in Plasma with Physiologic and Elevated a2-M Level

Addition of 56 nM APC (final) to cord plasma containing 3.6 μM a2-M (physiologic

concentration in cord plasma) resulted in a significant decrease of thrombin generation compared to plasma with physiologic concentration of a2-M in the absence of APC. The calculated area under the thrombin generation curve decreased from 291 to 157 nM·min ($p < 0.05$), shown in figure 6a. Administration of the same amount of APC to cord plasma containing 5.9 μM a2-M had no significant effect on thrombin generation compared to plasma with the same a2-M content in the absence of APC, shown in figure 6b. Comparable results were obtained in adult plasma. Addition of 81 nM APC (final) to

Fig. 3. a Prothrombin F1+2 activation in cord plasma containing 2.7 or 4.1 μM a2-M in the absence of APC (■, 0.6 μM F1+2 end level). Effects of supplementation of 21 nM APC to plasma with 2.7 μM (▲, 0.38 μM F1+2 end level) or 4.1 μM (●, 0.54 μM F1+2 end levels) a2-M on F1+2 generation. **b** Reduction of F1+2 generation by addition of 21 nM (■) or 42 nM (●) APC to plasma containing 1.8–5.9 μM a2-M related to corresponding plasma in the absence of APC.

plasma containing 2.0 μM a2-M (physiologic concentration in adult plasma) resulted in a significant decrease of thrombin generation compared to plasma with physiologic a2-M concentration in the absence of APC. The calculated area under the thrombin generation curve decreased from 627 to 241 nM·min ($p < 0.05$), shown in figure 7a. Addition of the same amount of APC to adult plasma at elevated a2-M level (3.8 μM) did not significantly decrease thrombin generation compared to plasma with the same a2-M content in the absence of APC, shown in figure 7b.

Discussion

APC exhibits its anticoagulant activity by proteolytic cleavage of activated factors V and VIII [17, 18] and thus prevents feedback activation of thrombin [19]. The effect of APC on thrombin generation in adult plasma of normal and APC-resistant individuals to detect a potential prothrombotic status has recently been reported [20]. Additionally, it has been shown by Hoogendoorn et al. [6] that a2-M binds and inhibits APC. They demonstrated a time-dependent inhibition of APC by a2-M using an activated partial thromboplastin

Fig. 4. **a** F1+2 activation in adult plasma containing 1.4 or 2.4 μM a2-M without APC administration (■, 1.2 μM F1+2 end level). Effects of supplementation of 42 nM APC to plasma with 1.4 μM (▲, 0.53 μM F1+2 end level) or 2.4 μM (●, 1.0 μM F1+2 end level) a2-M on F1+2 generation. **b** Reduction of F1+2 generation by addition of 21 nM (■) or 42 nM (●) APC plasma containing 1.1–4.6 μM a2-M related to corresponding plasma in the absence of APC.

time-based APC assay. Our data are in good agreement with these findings. However, it has been suggested that determination of clotting time does not necessarily reflect the coagulant potency of plasma since only a small amount of thrombin has been generated when the fibrin clot occurs [9]. Consequently, features of the thrombin-generation process that take place after clotting has occurred are not reflected in the clotting time. Therefore, determination of free thrombin generation has been suggested to be a more reliable parameter to assess the combined action of procoagulant and anticoagulant factors in plasma [11].

We have described previously a dose-dependent anticoagulant effect of APC using the determination of thrombin generation after intrinsic activation [8]. In cord and adult plasma, Protac [21]-induced APC reduced the amount and prolonged the lag times until the onset of free thrombin generation. These results were reproduced in the present study after addition of already activated protein C. Plasmas were activated by supplementation of thromboplastin (Thromborel S) and calcium ions. Complex formation between a2-M and APC effectively only occurs in the presence of divalent metal ions [22]. After addi-

Fig. 5. a Prolongation of clotting time by addition of 21 nM (■) or 42 nM (●) APC to cord plasma containing 1.8–5.9 μ M α 2-M related to corresponding plasma in the absence of APC. **b** Prolongation of clotting time by addition of 21 nM (■) or 42 nM (●) APC to adult plasma containing 1.1–4.6 μ M α 2-M related to corresponding plasma in the absence of APC.

tion of α 2-M, the anticoagulant effect of APC was diminished. In addition, the anticoagulant effect of APC was enhanced when α 2-M was inhibited by proteinase A [15, 16] in both cord and adult plasma. We found no difference in the inhibitory effect of α 2-M on APC anticoagulant function in cord and adult plasma. In accordance with the effect on free thrombin generation, α 2-M diminished the inhibiting effect of APC on prothrombinase activity assessed by determination of prothrombin F1+2 in both cord and adult plasma.

The observation that α 2-M diminishes the effect of APC on feedback activation of

thrombin is intriguing since it has been shown that α 2-M accounts for a large amount of thrombin inhibition, especially in cord plasma [4]. Studies dealing with thrombin inhibition by α 2-M determined thrombin/ α 2-M complex formation after a certain period of plasma incubation and did not assess the time course of thrombin generation and inhibition [23, 24]. The action between α 2-M and thrombin might not be fast enough [25] to prevent thrombin from feedback activation of factors V and VIII, thereby inducing prothrombinase formation. Thus, the same free thrombin generation curves result for plasma containing

Fig. 6. a Effect of supplementation of 0 nM (■) or 56 nM (●) APC to cord plasma containing 3.6 μ M a2-M on generation of free thrombin. **b** Effect of supplementation of 0 nM (■) or 56 nM (●) APC to cord plasma containing 5.9 μ M a2-M on free thrombin generation.

different amounts of a2-M at physiologic anti-thrombin levels. Proteinases are trapped within the 'bait region' of a2-M resulting in conformational change [26, 27]. Thus, although a2-M inactivates procoagulant proteins such as factor Xa and thrombin, inhibition of the anticoagulant factor APC counteracts its anticoagulant effect.

Beside a2-M, protein C inhibitor and alpha-1-antitrypsin (a1-A) contribute to APC inactivation [28, 29]. Scully et al. [7] investigated the activation of protein C and its distribution between its inhibitors in patients with DIC and suggested that a2-M and a1-A ap-

peared to assume greater roles in inhibition of APC in 2 fatal cases. a2-M increases during the initial phase of sepsis and subsequently decreases due to multiple complex formation with proteinases. Availability of APC seems to be predictive for the outcome of critically sick patients and application of APC results in favorable outcome [30, 31].

Although laboratory experiments obviously do not allow definite conclusions for various clinical situations, our data suggest that a2-M might modulate the anticoagulant property of APC in both cord and adult plasma.

Fig. 7. a Effect of supplementation of 0 nM (■) or 81 nM (●) APC to adult plasma containing 2.0 μ M a2-M on generation of free thrombin. **b** Effect of supplementation of 0 nM (■) or 81 nM (●) APC to adult plasma containing 3.8 μ M a2-M on free thrombin generation.

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