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α_2-Macroglobulin Remains as Important as Antithrombin III for Thrombin Regulation in Cord Plasma in the Presence of Endothelial Cell Surfaces

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ABSTRACT

Infants and children rarely develop thrombotic complications compared with adults, suggesting that there are protective mechanisms in place for the young. Because endothelial cell surfaces regulate thrombin formation and inhibition, we compared thrombin regulation by human umbilical vein endothelial cell surfaces exposed to defibrinated cord and adult plasmas. After activation by either 10% activated partial thromboplastin reagent (strong activator) or coagulant phospholipids (weak activator) the following were measured: free thrombin, thrombin bound to antithrombin III (ATIII), heparin cofactor II, α_2-macroglobulin (α_2M), and prothrombin concentration. Free thrombin activity was expressed as remaining activity, after subtraction of thrombin-α_2M activity. After 10% activated partial thromboplastin reagent, 100% of prothrombin was consumed and significant amounts of thrombin generated by 2 min. Cord plasma generated significantly less thrombin than adult plasma, reflecting the lower initial plasma concentration of prothrombin. Correspondingly, concentrations of thrombin inhibitor complexes were significantly greater in adult plasma than in cord plasma. After coagulant phospholipids, 50% of prothrombin was consumed and negligible thrombin activity measured for both adult and cord plasma. Similar amounts of thrombin inhibitor complexes were formed. ATIII was the predominant inhibitor of thrombin in adult plasma, whereas α_2M was as important as ATIII in cord plasma for both activators. When cord plasma concentrations of ATIII were increased to adult values, the proportion complexed to α_2M decreased. We conclude that on human umbilical vein endothelial cells, the capacity to generate thrombin is decreased in adult and cord plasmas. Furthermore, α_2M is at least as important an inhibitor as ATIII in cord plasma, even in the presence of endothelium. ***(Pediatr Res* 37: 373–378, 1995)**

Abbreviations

α_2M, α-2-macroglobulin
ATIII, antithrombin III
HCII, heparin cofactor II
APTT, activated partial thromboplastin time reagent
IIa, free thrombin
HUVEC, human umbilical vein endothelial cells
RDS, respiratory distress syndrome
FV, factor five
FVIII, factor eight

Thromboembolic complications rarely occur in fetuses and newborns, suggesting that protective mechanisms are in place in the young. Biologically, the ability to regulate thrombin formation and activity are of central importance to the prevention and formation of large-vessel thrombi. Regulation of thrombin under physiologic circumstances involves both plasma coagulation and inhibitors, as well as interactions with endothelial cell surfaces (1). Under physiologic conditions, endothelial cells present an anticoagulant surface that maintains vessel wall thromboresistance (2).

There are age-dependent differences in the regulation of thrombin that may, in part, explain the low rates of thrombotic complications in the young. Decreased amounts of thrombin are generated in cord plasmas compared with adult plasmas, reflecting low plasma concentrations of prothrombin in cord plasmas (3). In addition, α_2M is as important an inhibitor of thrombin as ATIII in fetal plasma (4). Consequently, the antithrombin effect of α_2M may remain significant throughout childhood, inasmuch as it is elevated above adult levels until after adolescence (5). However, these previous studies of

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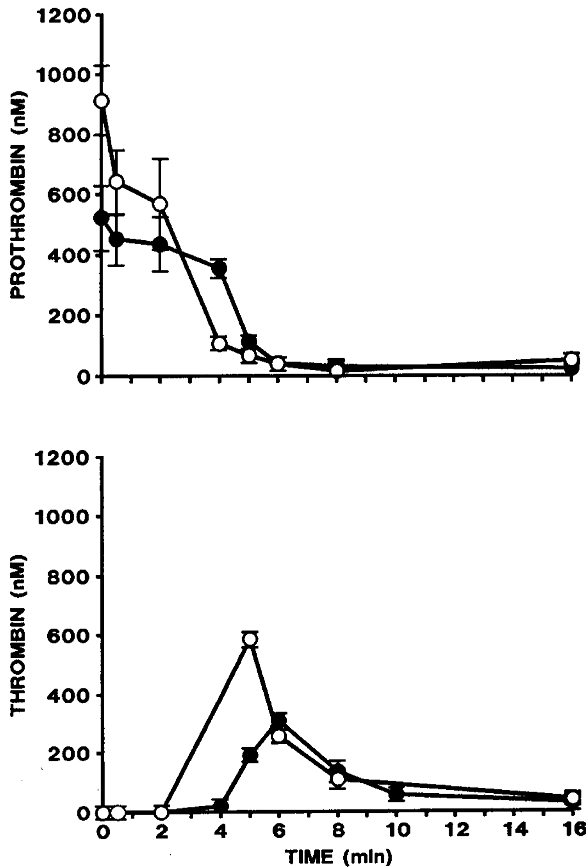

Figure 1. Comparison of prothrombin consumption and free thrombin activities in adult plasma (○) and cord plasma (●) in the presence of a plastic surface after activation with coagulant phospholipids. The data are presented in nM as means ± 2 SEM of at least five experiments.

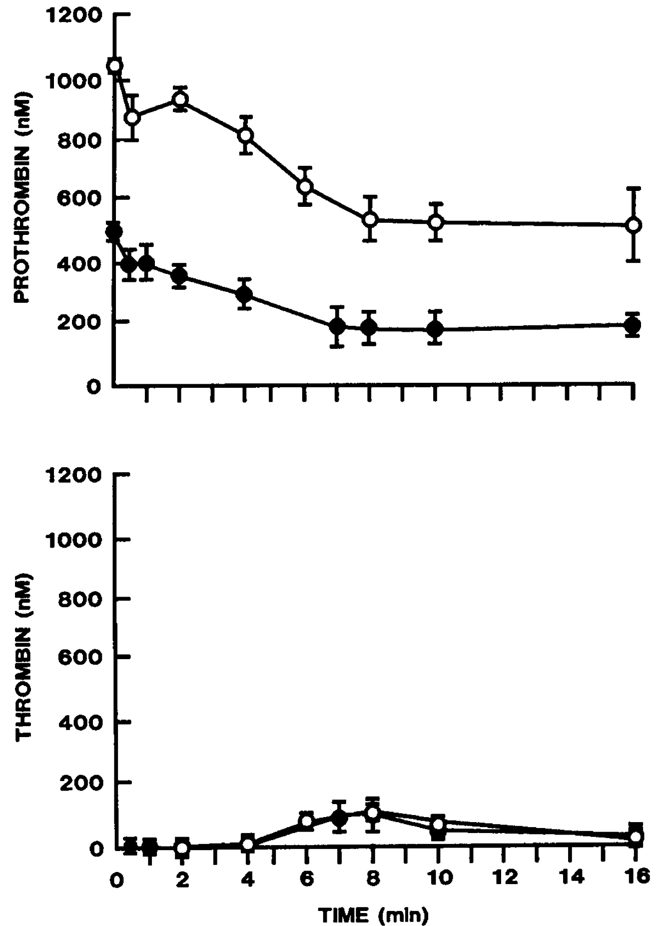

Figure 2. Comparison of prothrombin consumption and free IIa activities in adult plasma (○) and cord plasma (●) in the presence of a human endothelial cell surface after activation with coagulant phospholipids. The data are presented in nM as means ± 2 SEM of at least five experiments.

thrombin regulation in cord plasmas were conducted in the presence of plastic surfaces (3, 4). No previous studies have evaluated endothelial cell-surface regulation of thrombin formation or inhibition in a fetal system, even though this is known to be very important in the mature adult system. Therefore, the overall objective of this study was to explore ontogenic influences on thrombin regulation in the presence of endothelial cell surfaces.

METHODS

Materials. Fetal bovine serum, M-199 media, penicillin-streptomycin, L-glutamine and trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA) were purchased from GIBCO Laboratories (Grand Island, NY); tissue culture flasks (25 × 25 cm) were from Becton Dickinson (Lincoln Park, NJ; 24-well plates were purchased from Nunc (Roskilde, Denmark); collagenase and p-nitrophenyl phosphate were from Sigma Chemical Co. (St. Louis, MO). APTT reagent, standard heparin, and platelin were purchased from Organon Teknika Corp. (Durham, NC); the chromogenic substrate H-D-Phe-Pip-Arg-pNA (S-2238) was from Kabi Vitrum (Stockholm, Sweden); and ATIII was from Cutter Laboratories (Berkeley, CA). Antihuman prothrombin antibody, antihuman ATIII antibody, and purified human thrombin were obtained from Affinity Biologicals (Yarker, Ontario, Canada). Affinity-purified sheep IgG antihuman (pro)thrombin antibody was produced as described previously (6), and rabbit antihuman prothrombin antibody was from Behring Corporation (Montreal, Quebec, Canada). Goat antirabbit IgG-alkaline phosphatase–conjugated antibody was purchased from Dimension Laboratories (Toronto, Ontario, Canada). Arvin (ancrod) was obtained from Connaught Laboratories Ltd. (Toronto, Ontario, Canada), and goat antihuman

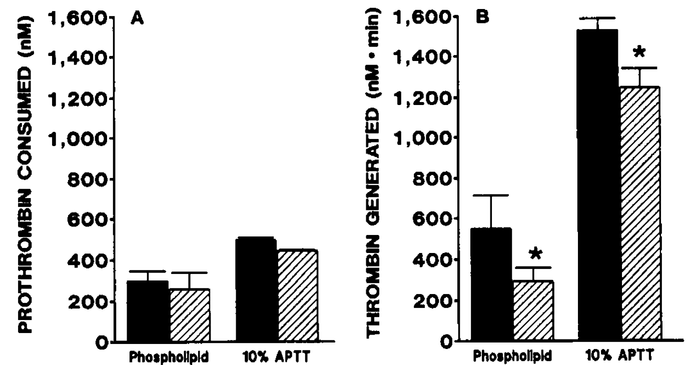

Figure 3. Comparison of prothrombin consumption (*A*) and free IIa (*B*) generation in cord plasma ■ and cord plasma supplemented with antithrombin III □ after activation with either coagulant phospholipids or 10% activated partial thromboplastin time reagent. The data are presented in nM as means ± 2 SEM of at least five experiments.

heparin cofactor II antibody was purchased from Behring Hoechst (Montreal, Quebec, Canada). Human HCII was purified from plasma using the method of Griffith *et al.* (7). Dermatan sulfate was obtained from Mediolanum (Milan, Italy).

Collection and preparation of plasma samples. Cord blood was obtained immediately after deliveries by withdrawing blood from double-clamped umbilical cords. All deliveries were uneventful full-term deliveries (37–41 wk gestational age) with appropriate birth weight and Apgar scores. Adult blood samples were obtained from 20 healthy donors and were similarly anticoagulated. Cord plasmas were pooled (five cords per pool), and plasmas from 20 adult donors were pooled. Analysis of adult and cord plasma pools for IIa-inhibitor complexes (IIa-ATIII, IIa-α_2M, and IIa-HCII analyzed as described below) showed that levels were less than 2 nM in each case. Therefore, activation of coagulation, as measured by thrombin-inhibitor complex formation, was minimal during plasma collection and preparation in relation to that during thrombin generation.

Human umbilical endothelial cell culture. Endothelial cells were isolated from HUVEC using 0.06% collagenase by a technique described by Jaffe *et al.* (8). HUVEC were maintained in M-199 media supplemented with 20% (vol/vol) fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 nM L-glutamine in flasks (25 × 25 cm) at 37°C in a 5% CO_2 atmosphere. Subculturing into 24-well plates was performed by treating confluent monolayers with trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA) after growing for 5 d in the flasks. Cells were plated into 24-well plates at a density of 8×10^5 to 1×10^6 cells per well and cultured using the same media conditions as with flasks. Confluent monolayers of cells passed only once into 24-well plates were used for experiments within 48–72 h after plating. Endothelial cells were identified by their morphology and by indirect immunofluorescence for Von Willebrand factor (9).

Thrombin generation and prothrombin consumption. Total amidolytic thrombin activity on cell surfaces or plastic surfaces was quantitated by previously described procedures (10, 11). Cell monolayers were washed twice with 1 mL of acetate-barbital–buffered saline, pH 7.4 (0.036 M sodium acetate, 0.036 M sodium diethylbarbitarate, and 0.145 M sodium Chloride). After this, 200 μL of defibrinated plasma was incubated for 3 min with endothelial monolayers and 100 μL of either platelin (coagulant phospholipids) or 10% APTT reagent. Calcium chloride (100 μL of 0.04 M $CaCl_2$ in buffered saline) was then added. At timed intervals (1–16 min) after the addition of calcium chloride, 25-μL aliquots were removed from the wells. Each aliquot was mixed with 475 μL of 0.005 M EDTA at 4°C. A 25-μL aliquot of each time sample-EDTA mixture was immediately added to 75 μL of 0.16 mM S-2238 in buffered saline and maintained at 37°C for 10 min. Amidolysis of S-2238 was then stopped by addition of 200 μL of 50% acetic acid, and absorbance at 405 nm was determined. A standard curve was generated using increasing concentrations of purified human thrombin. Based on known concentrations of purified thrombin in acetate-barbital buffered saline, an absorbance of 1.0 represented 1796 nM of thrombin in defibrinated plasma.

Thrombin bound to α_2M retains amidolytic activity against S-2238 (12), thereby contributing to the total amidolytic activity measured in thrombin generation experiments. Therefore, free IIa activity was determined as described previously (11). Briefly, the amidolytic activity of thrombin-α_2M complex was determined [by measuring residual activity with S-2238 after neutralization of free IIa with a large excess of ATIII (12U/mL) and heparin (71U/mL), followed by addition to EDTA (0.005 M)] and subtracted from the total amidolytic thrombin activity measured as described above. Prothrombin concentrations were measured by ELISA in the EDTA aliquot used for quantitating thrombin activity (6). Thrombin-ATIII and thrombin-HCII were also measured by ELISA (Affinity Biologicals, Yarker, Ontario, Canada).

Statistical analysis. All results are expressed as mean values of at least five experiments ± 2 SEM. An unpaired *t* test was used to compare differences in thrombin generation between adult and cord plasmas after activation with either coagulant phospholipids or 10% APTT reagent. $p < 0.05$ were considered significant. To further assess the difference between adult *versus* cord plasma, a one-way analysis of variance was used.

RESULTS

Prothrombin consumption and thrombin generation on plastic or HUVEC surfaces after exposure to 10% APTT reagent. Prothrombin consumption and IIa activity were measured in adult and cord plasmas in the presence of plastic or HUVEC surfaces after exposure to 10% APTT reagent (a relatively strong stimulus). On either plastic or HUVEC, in adult plasma or in cord plasma, approximately 90% of the starting prothrombin concentrations were consumed by 2 min. Coincidentally, on either plastic or HUVEC, a peak of thrombin activity appeared in either adult plasma (632 ± 59 nM at 2.5 min on plastic and 643 ± 13 nM at 2.5 min on HUVEC) or in cord plasma (408 ± 30 nM at 2 min on plastic and 389 ± 23 nM at 2 min on HUVEC). Therefore, with a strong stimulus, prothrombin was nearly completely consumed and maximal thrombin generated within a short period of time, regardless of surface or plasma. Thus, the fact that more thrombin was generated in adult plasma compared with cord plasma, on either surface, likely reflected the higher prothrombin concentrations in adult plasma than in cord plasma (1006 ± 114 nM and 527 ± 42 nM for adult and cord, respectively).

Prothrombin consumption and thrombin generation on plastic or endothelial cell surfaces after exposure to coagulant phospholipids. Prothrombin consumption and IIa activity were compared in adult and cord plasmas in the presence of plastic or HUVEC surfaces after exposure to coagulant phospholipids (a relatively weak stimulus that may simulate *in vivo* conditions). On plastic, greater than 90% of starting prothrombin concentrations were consumed in both adult and cord plasmas by 6 min (Fig. 1, *upper panel*) whereas detectable IIa activities were significant, maximally at 5–6 min (Fig. 1, *lower panel*). However, on HUVEC, in both adult and cord plasmas, only 50% of starting prothrombin concentrations were con-

sumed by 16 min (Fig. 2, *upper panel*). Detectable IIa activity was minimal relative to plastic, appearing at more than 4 min in both plasmas on HUVEC (Fig. 2, *lower panel*).

Thrombin inhibitor complex formation. To explore potential mechanisms responsible for differences in regulation of thrombin generation and inhibition in adult and cord plasmas, because of HUVEC surface, thrombin-inhibitor complexes were measured. After exposure to 10% APTT reagent, total plasma concentrations of thrombin-inhibitor complexes in adult plasma were significantly greater than in cord plasma on plastic or HUVEC (Tables 1 and 2). ATIII remained the major inhibitor of thrombin in adult plasma, whereas α_2M remained as important as ATIII in cord plasma (Tables 1 and 2).

After exposure to coagulant phospholipids, total plasma concentrations of thrombin-inhibitor complexes in adult plasma were significantly greater than in cord plasma on a plastic surface (Table 1). In contrast, interaction of plasma with coagulant phospholipids on an HUVEC surface resulted in similar total concentrations of thrombin-inhibitor complexes in adult and cord plasmas (Table 2). On both surfaces, ATIII was the major inhibitor of thrombin in adult plasma, whereas α_2M was at least as important as ATIII in cord plasma (Tables 1 and 2).

These data suggested that low plasma concentrations of ATIII in cord plasma may limit the ability of any HUVEC-surface heparan sulfate to potentiate the inhibition of thrombin by ATIII, especially in the case of a strong stimulus. To test this hypothesis, cord plasma was supplemented with purified ATIII and thrombin-generation experiments repeated in the presence of HUVEC surfaces.

Effect of ATIII supplementation of cord plasma on prothrombin consumption and thrombin generation on HUVEC surfaces. It has been shown that plasma ATIII levels in the fetus at full term are lower than in adults [60% of adult values (13)]. Purified human ATIII (0.55 U/mL) was added to cord-pooled plasma to increase the final concentration to a measured value of 1.1 U/mL and experiments repeated in response to coagulant phospholipids and 10% APTT reagent. After either stimuli, prothrombin consumption was similar in cord plasma plus ATIII compared with cord plasma (Fig. 3). However, the amount of thrombin activity measured was significantly decreased in cord plasma plus ATIII compared with cord plasma (Fig. 3).

Table 1. *Thrombin-inhibitor complex formation on plastic surface in adult and cord plasma*

		Thrombin inhibitor complexes		
Activator	Complexes total (nM)	IIa-ATIII (%)	IIa-α_2M (%)	IIa-HCII (%)
Phospholipid				
Adult	605 ± 28	70 ± 3	29 ± 3	1
Cord	263 ± 22*	50 ± 2*	49 ± 4*	1
10% APTT				
Adult	640 ± 26	72 ± 2	27 ± 2	1
Cord	238 ± 18*	49 ± 4*	49 ± 4*	2

The total amount of complexes are expressed as mean values of at least five experiments. Individual complexes are presented as mean percentages ± 2 SEM of the total complexes formed.

* Significant difference between adult and cord values ($p < 0.05$).

Table 2. *Thrombin-inhibitor complex formation on HUVEC surface in adult plasma, cord plasma, and cord plasma plus ATIII*

		Thrombin inhibitor complexes		
Activator	Complexes total (nM)	IIa-ATIII (%)	IIa-α_2M (%)	IIa-HCII (%)
Phospholipid				
Adult	153 ± 12	75 ± 8	22 ± 8	2
Cord	115 ± 16*	43 ± 5*	55 ± 5*	3
Cord + ATIII	83 ± 8†	35 ± 4	61 ± 4	3
10% APTT				
Adult	635 ± 49	70 ± 3	29 ± 3	1
Cord	279 ± 22*	44 ± 3*	54 ± 3*	2
Cord + ATIII	302 ± 16	73 ± 2†	26 ± 1	1

The total amount of complexes are expressed as mean values of at least five experiments. Individual complexes are presented as mean percentages ± 2 SEM of the total complexes formed.

* Significant difference between adult and cord values ($p < 0.05$).

† Significant difference between cord plasma and cord plasma plus ATIII ($p < 0.05$).

Effect of ATIII supplementation of cord plasma on thrombin-inhibitor complex formation. Thrombin-inhibitor complexes were measured in these same experiments. After exposure to coagulant phospholipids, total plasma concentrations of thrombin-inhibitor complexes were similar in cord plasma plus ATIII and cord plasma. α_2M remained as important as ATIII for the inhibition of thrombin in the presence of plasma supplementation with ATIII (Table 2). After exposure to 10% APTT reagent, total plasma concentrations of thrombin-inhibitor complexes were similar in cord plasma plus ATIII and cord plasma. However, ATIII was now significantly more important than α_2M for the inhibition of thrombin (Table 2).

DISCUSSION

Although endothelial cell-surface anticoagulant properties have been characterized in the presence of the adult coagulation system (1, 11, 14), there are no previous studies assessing a developmentally immature hemostatic system. Our study addressed this question and showed that endothelial cell surfaces regulated prothrombin consumption and thrombin generation in a fetal system, with a pattern similar to that in the mature adult system. After exposure to a strong stimulus (10% APTT), all prothrombin was consumed, regardless of plasma, resulting in more IIa and a higher proportion of thrombin-ATIII than thrombin-α_2M in adult plasma than in cord plasma. This result may have been predicted, given the higher starting prothrombin concentration (stated above) and the higher ATIII concentration (1 and 0.6 for adult and cord, respectively) in adult plasma. However, with a mild stimulus (coagulant phospholipids), total prothrombin consumption on an HUVEC surface was limited to 50% of starting concentrations in either adult or cord plasmas compared with more than 90% consumption in the absence of cells. Also, with the mild stimulus, less thrombin was generated in the fetal system, and α_2M, not ATIII, was the major inhibitor of thrombin. The latter observations reflected the decreased plasma concentration of prothrombin (3, 4), increased concentration of α_2M, and decreased levels of ATIII in the fetal system [48, 139, and 63% of adult levels, respectively (13)].

Under physiologic circumstances, endothelial cell surfaces regulate thrombin activity through several mechanisms. First, endothelial cells synthesize and secrete glycosaminoglycans, including heparan sulfate (15–17). Both *in vitro* studies and *in vivo* studies in rats have clearly shown that endothelial cell-surface heparan sulfate catalyzes thrombin inhibition by ATIII (16, 17). Second, a specific endothelial cell-surface thrombin receptor, thrombomodulin, complexes thrombin, thereby altering its substrate specificity (18). Thrombin complexed to thrombomodulin no longer functions as a coagulant by cleaving fibrinogen and activating FV and FVIII (19); it functions as an inhibitor of coagulation by activating the protein C/protein S inhibitory system (20, 21). Activated protein C down-regulates thrombin generation by limited proteolysis of FVa and FVIIIa (22). Third, endothelial cells decrease thrombin generation by receptor-mediated endocytosis and degradation of factor Xa (23, 24).

There are several reasons for suspecting that endothelial cell-surface regulation of thrombin may differ in fetal plasma than in adult plasma. First, fetal plasma concentrations of the thrombin inhibitors ATIII and HCII are approximately 50% of adult values (3, 25, 26). Therefore, catalysis of ATIII and HCII inhibition of thrombin activity by endothelial cell-surface glycosaminoglycans may not be as efficient in fetal than in adult plasma. Second, plasma concentrations of prothrombin are only 50% of adult values, causing less thrombin to be generated in the presence of a plastic surface (Fig. 1, *lower panel*). Third, an anticoagulant with characteristics of dermatan sulfate is present in maternal and fetal plasma (27). Dermatan sulfate catalyzes the inhibition of thrombin by HCII and acts synergistically with heparin to inhibit thrombin (28). Fourth, in contrast to ATIII and HCII, newborn plasma concentrations of α_2M are increased compared with adult values (13). Our results show that α_2M inhibits as much thrombin as ATIII in fetal plasma in the presence of plastic or HUVEC, which agrees with the fact that thrombin-α_2M complexes are present in plasma from sick newborns (29). Fifth, activated protein C activity in fetal plasma is decreased, reflecting protein C levels less than 50% of adult values, and potentially a fetal form of protein C (30).

In this study, the effect of endothelial cell-surface regulation of thrombin was compared in adult and fetal systems after exposure to either a weak (coagulant phospholipids) or a strong activator (10% APTT reagent). The weak activator likely simulates *in vivo* conditions more closely than the strong activator. The pattern of response was similar in cord and adult plasma for both activators. On an HUVEC surface, after the weak activator, decreased amounts of prothrombin were consumed compared with plastic surface and less IIa generated compared with the stronger activator. However, the magnitude of the response differed significantly in adult and fetal plasmas. Initial prothrombin concentrations were decreased in fetal plasma compared with adult plasma, resulting in decreased absolute amounts of prothrombin consumed and thrombin generated, as has been shown previously on plastic surfaces (3). ATIII was the major inhibitor of thrombin in adult plasma. However, α_2M was as important as ATIII in fetal plasma.

Together, these observations suggested that endothelial cell-surface heparan sulfate catalysis of ATIII inhibition of thrombin was impaired in fetal plasma because of low concentrations of ATIII. This hypothesis was tested by supplementing fetal plasma with purified ATIII and thrombin generation experiments repeated in the presence of endothelial cell surfaces. After either the weak or strong activator, similar amounts of prothrombin were consumed but less thrombin generated in ATIII-supplemented fetal plasma than in unsupplemented plasma. Although the amounts of thrombin inhibitor complexes generated were similar, ATIII, not α_2M, was the predominant inhibitor of thrombin in ATIII-supplemented plasma after the strong activator.

Our findings provide insight into potential mechanisms that protect healthy newborns from thrombotic complications, despite their low plasma concentration of ATIII. The decreased capacity to generate thrombin may slow the formation of fibrin after a pathologic stimulus. This was illustrated in experiments on HUVEC using 10% APTT and cord plasma, supplemented with prothrombin to adult levels, in which IIa generated and prothrombin consumed approached that of adult plasma (n = 3; data not shown). Also, increased plasma concentrations of α_2M compensate for low levels of ATIII and contribute significantly to the inhibition of thrombin, even in the presence of endothelial cell surfaces. Although not widely appreciated, α_2M is present on the luminal surface of endothelial cells in normal human arteries, veins, and lymphatics (31). Whether endothelial cells interact with α_2M, thereby influencing thrombin regulation by this inhibitor, remains to be determined (31, 32).

The results of this study are also relevant to the treatment of RDS in newborns. RDS is characterized by fibrin deposition in the lung, intravascularly, extravascularly, and intraalveolarly (33, 34). Plasma concentrations of ATIII are decreased even further in infants with RDS compared with their healthy counterparts (35–38). This observation has provided the rationale for previous and ongoing intervention studies in neonatal RDS with ATIII concentrates.

In summary, endothelial cell-surface regulation of thrombin differs in fetal and adult plasma. Decreased concentrations of prothrombin and ATIII and increased concentrations of α_2M in fetal plasma provide at least part of the explanation for our findings. Future studies are needed to investigate the physiologic role of thrombomodulin, protein C, and protein S on thrombin regulation in fetal and adult systems.

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