Circulating tissue factor, tissue factor pathway inhibitor and D-dimer in umbilical cord blood of normal term neonates and adult plasma

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The investigation of many hemostatic defects in newborns is restricted by the lack of normal reference values. The coagulation system of the neonate differs in many ways from that of the adult. The present study was designed to compare the concentrations of tissue factor (TF), tissue factor pathway inhibitor (TFPI) and D-dimer (DD) in the umbilical cord blood of healthy newborns and in adult plasma. TF antigen was quantified using an in-house enzyme-linked immunosorbent assay, whereas TFPI and DD levels were measured with commercial kits. The mean TF level in cord blood (mean ± standard deviation, 183.94 ± 103.63 pg/ml) was significantly higher ($P = 0.008$) than that in adults (136.64 ± 65.09 pg/ml). Cord blood exhibited enhanced fibrinolysis, as was reflected by a significantly higher level of DD (924.57 ± 733.87 ng/ml, $P < 0.001$) than that in adults (45.57 ± 17.21 ng/ml). Conversely, cord blood (30.88 ± 10.16 ng/ml) demonstrated significantly lower ($P < 0.001$) TFPI levels than that in adults (55.77 ± 21.16 ng/ml). However, no significant differences of these three hemostatic markers were noted between both gender groups in newborns and adults. Our findings indicate that an active and dynamic state of hemostasis exists in cord blood, as the fluidity of cord blood remains preserved in the presence of birth injury. ***Blood Coagul Fibrinolysis*** **14:125–129** **© 2003 Lippincott Williams & Wilkins.**

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Introduction

Cellular initiation of the extrinsic coagulation serine protease cascade is mediated by tissue factor (TF), which is a ubiquitous membrane-anchored low molecular weight (~47 kDa) glycoprotein. TF is a high-affinity cellular receptor and co-factor for factor VII and its activated form, FVIIa [1–4]. TF expression *in vivo* is highly cell-type specific [5]. It is not constitutively expressed by endothelium or blood cellular components. Instead, it is strongly expressed in a cell-specific manner in tissues surrounding vascular structures and in cells delimiting organ boundaries. The normal cellular distribution of TF represents a hemostatic envelope ready to activate coagulation when vascular integrity is disrupted [5,6]. TF triggers blood coagulation by forming a stoichiometric bimolecular complex with factor VII in plasma, thereby initiating a cascade of proteolytic reactions resulting in thrombin production — the penultimate step in hemostasis [7]. The activity of the TF–FVIIa complex is neutralized by tissue factor pathway inhibitor (TFPI). TFPI forms a stable, inactive quaternary complex composing TFPI, TF, FVIIa and activated factor X, thereby blocking the generation of thrombin [8].

Intricately interwoven with the clotting mechanism is the process of fibrinolysis, which is achieved by a potent plasma proteolytic enzyme, plasmin. The digestion of fibrin by plasmin produces fibrin degradation products, and one of these is fibrin D-dimer (DD), a cross-linked fragment that is not produced by the digestion of fibrinogen [9]. The fibrinolytic system is essential for removing fibrin deposits as part of vessel healing to re-establish blood flow and to preserve vascular patency.

There are limited data available on plasma concentrations of many components of the hemostatic system during childhood. Recent studies of adults, pre-term and term infants demonstrate that levels of blood procoagulants and anticoagulants may not be similar in children and adults [10,11]. Although the concentrations of most blood procoagulants and anticoagulants achieve near-adult values by 6 months of life, there remain significant discrepancies from adults for both mean values and ranges of normal [12]. Furthermore, some studies have reported that the plasma levels of certain coagulation factors and inhibitors might not reach the adult values until early or late childhood [13,14].

The objectives of the present study were: (a) to

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determine the plasma concentrations of TF, TFPI and DD in umbilical cord blood of normal term neonates and in healthy adults, and (b) to compare the plasma levels of these three hemostatic markers between these two groups of subjects. Quantification of plasma TF antigen was achieved by utilizing an in-house enzyme-linked immunosorbent assay (ELISA), whereas commercial kits were employed for the measurement of plasma TFPI and DD.

Materials and methods

Subjects

Umbilical cord blood

Umbilical cord blood samples were obtained from term neonates of normal pregnancy, delivered vaginally in the labor ward of the Hospital Universiti Kebangsaan Malaysia, from July to November 2000. The syringe technique was applied to draw 1 ml cord blood from the umbilical vein of the freshly delivered placenta, after obtaining informed consent from the mothers of the neonates under study. Samples from infants who were found to be abnormal physically by the attending pediatricians or doctors after birth were excluded from the study. A total of 50 healthy term neonates, comprising 25 male (50%) and 25 female (50%) infants, were included in the study.

Adults

A total of 50 healthy volunteer adults from the biomedical student population and blood donors were recruited into the adult group. The subjects were in good health without any diagnosed acute or chronic illness, and receiving no medications at the time of blood sampling. They were 25 males (50%) and 25 females (50%), with a mean age of 28.35 years.

Plasma preparation

Citrated platelet-poor plasma was obtained by drawing nine volumes of blood into one volume of 3.8% (129 mmol/l) trisodium citrate, and by centrifugation at $1660 \times g$ for 30 min at 4°C. The plasma was then aliquoted into 1.5 ml polypropylene microtubes, snap-frozen and stored at −80°C for further testing. Blood separation was performed within 2 h of collection to minimize any effects of storage. Just before being tested, frozen plasma was allowed to thaw for 15 min in a waterbath at 37°C. Clotted and grossly hemolyzed samples were excluded from the study.

Materials

Mouse anti-human TF monoclonal antibody (mAb) and horseradish peroxidase-labeled sheep IgG anti-human TF (IgG-TF-HRP) were obtained from Enzyme Research Laboratories Inc. (South Bend, Indiana, USA) and Affinity Biologicals Inc. (Hamilton, Ontario, Canada) respectively. Recombinant TF (rTF) was supplied by American Diagnostica Inc. (Greenwich, Connecticut, USA). All were stored and reconstituted according to the manufacturers' instructions. Immulon® 2HB Removawell® microplates were provided by Dynex Technologies (Chantilly, Virginia, USA). Unless stated otherwise chemicals were purchased from Sigma Co. (St Louis, Missouri, USA). Asserachrom® Total TFPI and TintElize® D-dimer commercial enzyme immunoassay kits were purchased from Diagnostica Stago (Asnières-Sur-Seine, France) and Biopool® International (Ventura, California, USA) respectively.

In-house TF ELISA

TF antigen was measured by an in-house direct sandwich ELISA that employed two antibodies against human TF. Briefly, the microtiter plate was coated at 4°C overnight with 100 μl/well mAb-TF (1250 ng/ml in 50 mmol/l carbonate–bicarbonate buffer, pH 9.6) and washed five times with phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% (v/v) Tween 20 (USB). It was blocked for 2 h at 27°C by floating each well with PBS containing 5% (w/v) skimmed milk and 0.5% Tween 20. The plate was then washed five times before it was reacted for a period of 1 at 27°C with 100 μl/well of samples or standards (rTF), which were diluted 1 : 2 in dilution buffer (PBS with 1.5% skimmed milk and 0.5% Tween 20). After five washes, the samples were incubated with 100 μl/well IgG-TF-HRP (2 μg/ml in dilution buffer) at 27°C for 1 h. Finally, after another five washes, 100 μl/well freshly prepared substrate buffer (50 mmol/l phosphate-citrate buffer, pH 5.0, containing 0.03% sodium perborate and 0.1 mg/ml 3,3′ ,5,5′ -tetramethylbenzidine) was added, and the plate was left in the dark for 10 min at 27°C. The reaction was terminated by acidification with 100 μl/well of 1 mol/l sulfuric acid (BDH, London, UK), and the absorbance was measured spectrophotometrically in the Multiskan MS microplate reader (Labsystem, Vantaa, Finland) equipped with 450 and 630 nm optical filters. Except for the initial coating step, which was done without agitation overnight in the fridge, all other incubations were carried out with agitation (30 rpm) in a VorTemp 56 microplate mixer (Labnet, New Jersey, USA). Plasma from patients with disseminated intravascular coagulation was used as positive controls for the assay. Samples were assayed in triplicate and the respective TF levels were interpolated from the linear portion (10–4000 pg/ml) of the standard curve constructed from rTF. The assay demonstrated low intra-assay [2.50–9.23% coefficient of variation (CV)] and inter-assay (5.65–13.57% CV) variability, as well as satisfactory analytical recovery (91.55–103.95%) and good parallelism.

Enzyme immunoassay of total TFPI (Asserachrom® Total TFPI)

The assay was based on a one-step ELISA method, measuring the total biologically available level of TFPI

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(free and lipoprotein-bound TFPI). It employed a capture mAb reactive against an epitope on the second Kunitz domain, and a detecting mAb coupled with peroxidase reactive against an epitope on the first Kunitz domain. Both the mAbs reacted with native, full-length, complexed and truncated forms of TFPI. TFPI was used as external calibrator, whereas samples were diluted 1:20 for expected TFPI < 200 ng/ml, and were diluted 1:40 for expected levels up to 400 ng/ml. Samples and calibrators were assayed in duplicate. The TFPI level was derived from the calibration curve. The assay range was 0.5–10 ng/ml with a detection limit of 0.15 ng/ml and recovery rates of 95–109%. Intra-assay and inter-assay CVs were < 6.5 and < 8%, respectively [15].

Enzyme immunoassay of DD (TintElize® D-Dimer)

The TintElize® D-Dimer enzyme immunoassay utilized the double antibody principle. Plasma samples or standard containing DD was added to a microtest well pre-coated with a mAb against DD. After an incubation period of 0.5 h, which was sufficient to allow > 85% of the DD to bind to the capture mAb, HRP-labeled Fab fragments of anti-DD IgG were added to react with the adsorbed DD. The presence of DD was then visualized by addition of peroxidase substrate. Human plasma depleted of DD and enriched with DD was used as 0 and 1000 ng/ml standards, respectively. Samples and standards were assayed in duplicate. The absorbance values of the samples were interpolated from the standard curve to derive their respective DD levels. The assay range was 0–1000 ng/ml with a maximal sensitivity of 40 ng/ml. The intra-assay and inter-assay CVs were ~4% at 200 ng/ml and ~3% at 360 ng/ml, respectively.

Statistical analysis

Microsoft® Excel 97 was employed to generate titration plots and standard curves. Statistical analysis was carried out using SPSS Version 9.0 (SPSS Inc., Chicago, Illinois, USA). Tests of normality were performed to verify the distribution of continuous variables. The unpaired Student's *t* test (or the Mann–Whitney U test for not-normally distributed variables) was used to compare variables between two groups. All statistical tests were two-tailed and were considered to be statistically significant at $P \leqslant 0.01$.

Results

Table 1 compares the plasma TF, TFPI and DD levels between umbilical cord blood of healthy neonates and blood of normal adults. Umbilical cord blood had significantly higher TF (183.94 ± 103.63 pg/ml, $P = 0.008$) and DD (924.57 ± 733.87 ng/ml, $P < 0.001$), but lower TFPI (30.88 ± 10.16 ng/ml, $P < 0.001$) levels than adults. There were no significant differences in the levels of plasma TF and DD between the male and female infants. On the contrary, male infants demonstrated statistically lower levels of TFPI (27.84 ± 9.03 ng/ml) than the female infants (33.93 ± 10.48 ng/ml, $P = 0.032$). There was no significant difference in levels of TF (136.64 ± 65.09 pg/ml), TFPI (55.77 ± 21.16 ng/ml) and DD (45.57 ± 17.21 ng/ml) between adult males and females.

Discussion

No reference range of TF concentration in cord blood has been reported in the literature to date. In this study, TF levels in umbilical cord blood showed a mean value of 183.94 ± 103.63 pg/ml [95% confidence interval (CI), 154.49–213.39], which was significantly higher than those of adults ($P = 0.008$). The mean level of plasma TFPI in cord blood (30.9 ng/ml; 95% CI, 28.0–33.8) was close to the range established by Reverdiau-Moalic *et al.* (mean, 38.1 ng/ml; 95% CI, 22.7–55.8; $n = 60$) [16]. Our results were consistent with those reported by Warr *et al.* [17], where TFPI was shown to be significantly lower than in adults

Table 1. The mean plasma levels of tissue factor, tissue factor pathway inhibitor and D-dimer in umbilical cord blood of healthy neonates and blood of normal adults

	All subjects ($n = 50$)	Male ($n = 25$)	Female ($n = 25$)	*P* values between males and females
Tissue factor (pg/ml)				
Cord blood	183.94 ± 103.63* (154.49–213.39)	186.60 ± 121.21 (136.57–236.63)	181.28 ± 84.98 (146.20–216.36)	0.9
Adults	136.64 ± 65.09 (118.14–155.14)	137.36 ± 61.01 (112.18–162.54)	135.92 ± 70.19 (106.95–164.89)	0.9
Tissue factor pathway inhibitor (ng/ml)				
Cord blood	30.88 ± 10.16* (28.00–33.77)	27.84 ± 9.03 (24.11–31.56)	33.93 ± 10.48 (29.61–38.26)	0.03
Adults	55.77 ± 21.16 (49.75–61.78)	52.13 ± 23.13 (42.58–61.67)	59.41 ± 18.75 (51.66–67.15)	0.2
D-dimer (ng/ml)				
Cord blood	924.57 ± 733.87* (716.01–1133.14) 1122.04 (103.18–1802.73)[a]	893.94 ± 742.00 (587.66–1200.23) 408.18 (103.18–1780.45)[a]	955.20 ± 739.63 (649.90–1260.50) 1226.36 (103.18–1802.73)[a]	0.9
Adults	45.57 ± 17.21 (40.68–50.46)	44.82 ± 18.43 (37.21–52.42)	46.33 ± 16.24 (39.62–53.03)	0.8

Values are presented as mean ± standard deviation (95% confidence interval). [a]Values are presented as median (range). * $P < 0.01$ compared with adult values.

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($P < 0.001$). As for the DD levels in cord blood (924.57 ± 733.87 ng/ml), our results confirmed the findings of Schneider *et al.* [18], who reported significantly higher DD levels in cord blood than adults. Intriguingly, two peaks of plasma DD levels were observed in cord blood and natural logarithm transformation failed to normalize the distribution. This was unlikely due to the process of sampling or the quality of samples, as the other two parameters (TF, TFPI) were normally distributed. We surmise that this unique distribution may stem from minor trauma that occurs at birth, for instance cord blood around the neck or excessive handling of the umbilical cord during delivery.

The present study was able to demonstrate the presence of TF in the plasma of normal healthy adults, with a mean level of 136.64 ± 65.09 pg/ml, similar to the findings of the other investigators [19–29]. In agreement with most previous studies [19,20], with the exception of one [25], the present study showed that plasma TF levels were not age dependent. As reported by other workers [19,20,25], gender did not appear to have an effect on the levels of TF. These data suggested that physiologic variables had no influence on plasma TF levels. The mean plasma TFPI level (55.77 ± 21.16 ng/ml) in normal adults measured in the present study was quite close to that reported elsewhere [16,21,23,26,29–35] and fell within the range provided in the kit (81.2 ± 30.4 ng/ml). No significant difference was found between men and women, which coincided with the findings of Warr *et al.* [17] However in contrast to Warr *et al.* [17], no correlation with age was noted. The mean level of plasma DD (45.57 ± 17.21 ng/ml; 95% CI, 40.68–50.46) fell within the range provided in the kit (39–130 ng/ml), and was similar to that reported by Kamikura *et al.* (49.5 ± 11.7 ng/ml; $n = 30$) [33]. Besides, this value was quite similar to that established by van der Bom *et al.* [36] from a group of 300 normal subjects (mean, 40 ng/ml; 95% CI, 35–44). However, the levels of DD in female adults measured in the present study (46.33 ± 16.24 ng/ml) were lower than those reported by Koh *et al.* (162 ± 90 ng/ml; $n = 20$) [37].

In healthy individuals, plasma levels of TF are expected to be rather low, since normally TF-expressing cells are sequestered from the blood circulation. Although the origin of plasma TF remains unclear, it has been theorized, at least in part, to result from its detachment from cellular membranes [20], or from its constant generation by activated monocytes and macrophages [38]. On the contrary, the higher level of TF in cord blood might be related to the traumatic effect of delivery and cutting of the umbilical cord, which caused the release of this protein from the injured tissues and the endothelium. The increase of TF level would directly induce the inhibitory effect of TFPI, in order to maintain the delicate balance between these systems. This resulted in large consumption of TFPI and reduction of TFPI in the cord blood. Meanwhile, the fibrinolytic system was also initiated by the increased procoagulant activity in cord blood, leading to the formation of a great amount of its end products, including DD.

Fibrin degradation products and DD had been reported in 65% of normal-term infants, presumably in response to the trauma of labor and delivery [39]. DDs are large, so placental passage is unlikely; therefore, the umbilical cord DD level may reflect activation of fetal fibrinolysis following labor [40]. Other possible explanations were that DD might represent low-grade activation of coagulation cascade, as a result of the circulatory adjustments of closure of the ductus venosus and ductus arteriosus after birth. Or perhaps there was a delay in the renal clearance of DD in newborns [39]. However, the significantly elevated fibrinolytic marker denotes that newborns are able to remove fibrin efficiently.

In summary, the significantly higher levels of TF and DD with a lower TFPI level in cord blood when compared with those in adult plasma denote the existence of an active and dynamic state of coagulation as well as fibrinolysis process in the newborns. The fluidity of cord blood remains preserved in the presence of birth injury that occurs during the childbirth process.

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