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Full Length Article

Differences in the mechanism of blood clot formation and nanostructure in infants and children compared with adults

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ABSTRACT

Introduction: Infants and children have a lower incidence of thrombosis compared with adults. Yet, the mechanism of blood clot formation and structure in infants and children, as the end product of coagulation, has not been studied. This study aimed to establish differences in the mechanism of thrombin generation, fibrin clot formation and response to thrombolysis in infants and children compared with adults.
Materials and methods: We studied thrombin generation, fibrin clot formation, structure and fibrinolysis in healthy infants, children and adults.
Results: Younger populations had a decreased potential to generate thrombin, at a slower velocity compared with adults, correlating positively with age. Clot formation at venous shear rate was decreased in infants and children compared with adults, with increased time for fibrin formation, decreased fibrin formation velocity, resulting in decreased tendency for fibrin formation in younger populations. These differences were less pronounced at arterial shear rate. Studies of the fibrin clot structure in paediatric age groups showed a significantly larger pore size compared with adults, suggestive of a clot that is less resistant to fibrinolysis. The presence of tissue plasminogen activator (tPA) resulted in a significant decrease in the pore size of infants and children, but not in adults.
Conclusions: This is the first study to suggest that the mechanism of blood clot formation and nanostructure, as well as response to thrombolytic therapy is different in infants and children compared with adults.

1. Introduction

The extremely low incidence of thrombosis in infants and children suggests that they are protected from clinical events. Apart from the enormous differences in rates of spontaneous thrombosis, thrombosis secondary to vascular access occurs at lower rates in children compared to adults, although such secondary thrombosis are the major proportion of thrombosis observed in children. The lower prevalence in thrombosis, for the same iatrogenic insult, relates to physiological differences in the hemostatic system of children and adults. There is compelling evidence from multiple studies, that the hemostatic system is profoundly affected by age [1–6]. Vitamin K dependent coagulation factors are reduced by 50% in newborns compared with adults and remain about 10–20% lower during childhood. In neonates antithrombin levels are reduced by 20%, but throughout the rest of childhood are increased by 10% compared with adults. Protein C and protein S levels are also reduced in neonates, with protein C remaining reduced throughout childhood [1].

The age-related differences in the quantitative and functional measurements of the majority of hemostatic proteins [1–5], are described conceptually as Developmental Haemostasis. Recently, the platelet phenotype has also been shown to change with age [6]. However, the majority of studies of Developmental Haemostasis to date have focused on individual components of the hemostatic system. Very few studies have focused on the functionality of the hemostatic system as a whole.

Thrombin generation tests are known to correlate with the overall function of the hemostatic system and are related to a bleeding and/or thrombotic risk [7]. Such tests are increasingly being recognized as a versatile diagnostic tool in thrombosis and haemostasis. Thrombin generation has previously been found to be decreased in infants and children compared with adults [5,8,9] and has been suggested to be

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the result of an increased inhibition of thrombin by the naturally occurring coagulation inhibitor alpha-2-macroglobulin (α2M) [10]. However, the exact mechanism behind the age-specific differences in thrombin generation is not well understood.

Formation of a clot via the thrombin-mediated conversion of fibrinogen into fibrin is the final step in the process of coagulation. The fibrinolytic and viscoelastic properties of the clot are directly dependent on the structure of the fibrin clot [11]. The thrombin concentration present during the conversion influences fibrin clot nanostructure. In the presence of low amounts of thrombin, fibrin fibres are thick and highly susceptible to lysis. On the other hand, in the presence of high thrombin levels, fibres are thin but tightly packed and impermeable [12]. The fibrin network architecture regulates the distribution of lytic enzymes [13], thus fibrin clots composed of compact (small pores), highly branched networks and with thin fibres are more resistant to fibrinolysis. Plasma from adults with a personal or family history of thrombosis produces such thin, highly-branched, impermeable fibrin networks that are resistant to fibrinolysis [14]. Adults with myocardial infarction and stroke have clots that are less permeable and hence more resistant to fibrinolysis [15,16], whilst adults with venous thromboembolism (VTE) have decreased clot permeability, lower compaction and prolonged clot lysis time compared with healthy controls [17].

The exact mechanism of the protection of children for thrombosis is unknown and improved understanding of this mechanism may provide new insights in the prevention and treatment of hemostatic disturbances in children and adults. This study investigates the clot nanostructure of children compared with adults and the effect of tissue plasminogen activator (tPA) on the properties of the fibrin clot.

2. Materials and methods

2.1. Samples

Blood samples used in this study were collected and processed according to established protocols [1,5,6,18]. Specifically, samples were obtained from healthy children attending the hospital for minor day-surgery without a family history of coagulation disorders (e.g. thrombosis and haemorrhage). Children were assigned to 4 different groups according to age: 1 month to 1 year, 1 to 5 years, 6 to 10 years and 11 to 16 years, based on previous studies of developmental haemostasis [1,5,6]. The paediatric cohort excluded adolescents taking oral contraceptive and smokers. Adult samples were obtained from healthy volunteers aged between 20 and 45 years of age who were not taking any medication (including oral contraceptive), were not smokers and did not have a family history of coagulation disorders. The incidence of thrombosis is not significantly different in adults within this age range and hence these were analysed as one age group. Family history was assessed via a brief interview with the parents of the children and the adult volunteers themselves.

This study was approved by the Royal Children's Hospital Ethics in Human Research Committee, reference number 20,031. Written informed consent was obtained from parents of children and from adult participants themselves.

Blood samples were collected in S-Monovette® tubes (Sarstedt, Australia), containing 1 volume of citrate per 9 volumes of blood. Samples were then centrifuged at 3000 g for 10 min at 10 °C (Megafuge 1.0R, Heraeus), and platelet poor plasma was stored at −80 °C until testing. Informed consent was obtained from the parents of children and from the adult participants themselves. All participants were healthy individuals, without previous thromboembolic events, family history of bleeding or thrombosis, and not subjected to any form of anticoagulant therapy. All laboratory analyses of plasma samples were performed in a blinded fashion.

2.2. Thrombin generation

Thrombin generation (TG) was measured using the Calibrated Automated Thrombography (CAT) as described previously [19–22]. In detail, coagulation was triggered with 1 and 5 pM tissue factor (TF) in the presence of 4 μM phospholipids (60% dioleoyl phosphatidyl choline (PC), 20% dioleoyl phosphatidyl serine (PS) and 20% dioleoyl Phosphatidyl ethanolamine (PE); prepared as described elsewhere [23]. Parameters measured from the TG curve included: lag time, maximal thrombin concentration (peak), time to peak, as well as the area under the curve (endogenous thrombin potential, ETP) and velocity index (VI = Peak height / (time to Peak-lag time)). The raw data from TG was also used to calculate the overall velocity constant of the total thrombin decay, the thrombin decay constant ($k_{dec.Thrombin}$). The thrombin decay constant was split mathematically into specific decay by α_2M ($k_{dec.\alpha2M}$) and by AT ($k_{dec.Serpin}$), as previously described [24]. The latter includes the inhibitory action of a group of miscellaneous serpins that play a minor role in the inhibition of thrombin during thrombin generation.

2.3. Assessment of fibrin formation in the setting of continuous flow using rheometry

Fibrin clot formation was measured in the presence of laminar flow using rheometer MCR301 (Anton Paar, Oosterhout, the Netherlands). All measurements were performed with a CP40 measuring system (Anton Paar, angle 1°), at the zero gap position suggested by the manufacturer. A quartz glass plate (Anton Paar) was used as sample holder. 220 μl plasma was pre-incubated with 55 μl TF/phospholipids (PL) (yielding the indicated final concentration) for 5 min at 37 °C. The measurement was started upon addition of $CaCl_2$ (final concentration 16.67 mM). All experiments were performed at 37 °C. Resulting viscosity plots are characterized by the parameters base level (viscosity level before coagulation), lag time (time it takes before an increase of the viscosity is observed), plateau level (maximum viscosity level), plateau-base level (increase in viscosity due to the clotting of the sample), time-to-plateau (TTP, time to reach the plateau level), and velocity (slope of the ascending curve between the lag time and the TTP).

2.4. Fibrin clot formation and fixation for SEM

Fibrin clots were the final result of a thin layer thrombin generation method on paper discs, as described previously [25]. The decision to use thin layer thrombin generation versus the standard CAT approach for preparation of fibrin clots was the final reaction volume of 5 μl versus 120 μl, respectively. The volume of 5 μl is easily fixed and results in significantly improved SEM images compared to larger sample volumes. In short, a 96 well microplate was used in which paper discs (Whatman 589/1, Whatman GmbH) were placed at the bottom of the wells. The final reaction mixture included 50% plasma with the following final concentrations: 4 μM phospholipids (60% dioleoyl PC, 20% dioleoyl PS and 20% dioleoyl PE, prepared as described elsewhere [23]), 5×10^{-9} mmol/L tissue factor and 16.7 mmol/L $CaCl_2$; with and without additional 50 ng/ml tPA. The concentration of tPA was guided by previous studies [26,27].

The resulting fibrin clots were prepared using a modification of a previously published method [27]. This protocol was initiated at exactly 10 min post thin layer thrombin generation for all samples across all age groups, standardizing the time from initiation of clotting to fibrin clot preparation, which is especially important for samples prepared in the presence of tPA. In detail, the fibrin clots were fixed with 2.5% glutaraldehyde; washed with PBS and fixed with osmium tetroxide OsO_4 1% in sodium cacodylate 200 mM, pH 7.4. The clots were dehydrated in ethanol and hexamethyldisilazane and were placed on carbon adhesive