Calibrated automated thrombin generation in paediatric patients with inflammatory bowel disease

H. Bernhard; A. Deutschmann; B. Leschnik; M. Novak; A. Hauer; H. Haidl; A. Rosenkranz; W. Muntean
Department of Paediatrics, Medical University of Graz, Austria

Keywords
Calibrated automated thrombography, endogenous thrombin potential, inflammatory bowel disease

Summary
In adults, inflammatory bowel disease (IBD) is associated with an increased risk of thromboembolic complications. The pathogenesis of IBD is not really clear and a high thrombin activity might contribute to disease progression. We wanted to see whether children with IBD have a higher thrombin generation (TG). Patients, material, methods: Plasma samples were collected of 20 patients with IBD and of 60 healthy controls (age range from 10 to 19). TG was measured by means of Calibrated automated thrombography (CAT). The disease activity was estimated, using the Pediatric Crohn’s Disease Activity Index (PCDAI) for Crohn’s disease and the Pediatric Ulcerative Colitis Disease Activity Index (PUCAI) for Ulcerative Colitis. In addition, we investigated F1+F2, TAT, TFPI and fibrinogen. Results: There was a significant increase of endogenous thrombin potential (ETP), lag time and time to peak in patients with IBD, while peak showed no difference to healthy controls. ETP and F1+F2 in children with IBD also showed a significant correlation with PCDAI (PUCAI) and fibrinogen. Conclusion: IBD in children is associated with high TG, but this seems to be caused mainly by the inflammatory process and not by any individual disposition.

Schlüsselwörter
Kalibrierte automatisierte Thrombographie, endogenes Thrombinpotenzial, chronisch-entzündliche Darmerkrankung

Zusammenfassung

Hämostaseologie 2009; 29 (Suppl 1): S90–S93

Correspondence to:
Wolfgang Muntean, MD
Professor of Paediatrics
Medical University of Graz
Department of Paediatrics
Auenbruggerplatz 30, 8036 Graz, Austria
Tel. +43/316/38 51 26 79, Fax +43/316/38 51 32 64
E-mail: wolfgang.muntean@meduni graz.at

Thromboembolism is a disease-specific extraintestinal manifestation of inflammatory bowel disease (IBD) (1, 2). In adults, IBD is associated with an increased risk of vascular complications. In clinical studies, thromboembolic events are demonstrated to occur in 1–8% and disease activity is also probably a risk factor for thromboembolic events (3). Bench research and clinical experience demonstrate, that in both forms of IBD, a hypercoagulable state and a prothrombotic condition exist. This might be caused by increased levels of haemostatic parameters like factors V, VII, VIII and fibrinogen as well as reduced levels of anticoagulation factors like antithrombin, protein C, protein S, and TFPI and fibrinolytic activity caused by the chronic inflammatory process (4).

On the other hand, the pathogenesis of IBD is not really clear and a high thrombin activity might contribute to disease progression. There is a relationship between multiple infarctions of the intestinal mucosa and the pathogenesis of Crohn’s disease (5, 6). Sensitive markers of activation of coagulation such as prothrombin fragment 1+2 (F1+2), the thrombin-antithrombin complex (TAT), fibrinopeptid A and B (FPA, FPB), indicating subclinical activation of coagulation are high in IBD (4).

This study was conducted to investigate the role of hypercoagulation during course (active and quiescent state) of IBD in paediatric patients.
tric patients. Therefore, we measured thrombin generation by means of CAT (Calibrated Automated Thrombography) developed by Hemker et al. to detect this hypercoagulable condition in children with IBD. We wanted to see whether such children have a higher thrombin generation and if there is a connection between hypercoagulability and activity of the inflammation process.

Patients, methods, material

This study was approved by the local ethics committee. In this study we compared 20 paediatric patients with IBD (16 children with Crohn’s disease and 4 patients with ulcerative colitis) with 60 healthy children. The age was between 10 and 19 years. The disease activity was estimated using

- PCDAI (Pediatric Crohn’s Disease Activity Index) for Crohn’s disease and
- PUCAI (Pediatric Ulcerative Colitis Activity Index) for ulcerative colitis.

After their consent to participate in this study, blood was taken from peripheral veins. Nine parts of blood were collected into one part of 0.1 mol/l citrate using S-Monovette (Sarstedt, Germany). Immediately afterwards platelet-poor plasma (PPP) was prepared by centrifugation of whole blood at 2800g for 10 min at room temperature and stored at –70°C.

For the fluorogenic assay via CAT imidazol buffer solution was obtained from Dade Behring (Marburg, Germany). Fluobuffer contained 20 mmol/l HEPES and 60 mg/ml bovine serum albumin (BSA) were both from Sigma (St. Louis, MO, USA). The fluorogenic substrate Z-Gly-Gly-Arg-amino-methylcoumarin (AMC) was purchased from Bachem (Bubendorf, Switzerland) and was solubilised in pure dimethylsulfoxide (DSMO), purchased from Sigma. Calcium chloride was obtained from Merck (Darmstadt, Germany). Thrombin calibrator and the PPP reagent with the content of 5 pmol/l tissue factor and 4 μmol/l phospholipids were purchased from Thrombinoscope BV (Maasricht, The Netherlands).

Assays were performed by means of Fluoroskan Ascent plate reader (Thermo Labsystems, Helsinki, Finland) for 60 min and Thrombinoscope software (Thrombinoscope BV, Maasricht, The Netherlands) as described by the manufacturer. We used the method which was developed and described by Hemker et al. (8, 9). Fluo-Buff was mixed with CaCl₂ in a glass tube: to 2625 μl of buffer (Hepes 20 mmol/l, pH 7.35) containing 60 g/l BSA, 300 μl of 1 mol/l CaCl₂ was added, and incubated for at least 5 min at 37°C. 75 μl of a 100 mmol/l DSMO solution of the fluorogenic substrate was added and immediately well mixed. The resulting clear solution was referred to as FluCa.

Thrombin calibrator was used to compare the simultaneously measured thrombin activity in the sample to that from a known and stable concentration in the calibrator well. Before starting the experiment all reagents were warmed up to 37°C. To each well of the 96-well round-bottom microtiter plate of 80 μl of platelet-poor plasma and 20 μl of the solubilized PPP-reagent were added. Plasma was activated with low amounts of tissue factor (TF) and calcium. Plasma measurement process was performed in triplicate. The plate was placed in the fluorometer. The instrument dispensed 20 μl of FluCa to all of the wells to be measured at the start of experiment.

This continuous measurement is based on the conversion of a thrombin-specific substrate Z-Gly-Gly-Arg-AMC. Thrombin activity was calculated as a function of time by comparing the fluorescent signal with that from a known and stable sample. During the measurement the program calculates and displays the thrombin concentration in time.

Commercially available ELISA assay systems (Enzygnost F1+F2 and Enzygnost TAT, Dade Behring, Marburg, Germany) were used for measurement of prothrombin fragment 1+2 (F1+F2) and thrombin-antithrombin (TAT) complex. Actichrome TFPI activity assay was obtained from American Diagnostica Inc. (Greenwich, CT, USA).

Statistical analyses were performed with help of SPSS software (SPSS inc., Chicago, IL, USA) by using Mann-Whitney-U-Test and Pearson’s correlation and p-values less than 0.05 were considered to be significant.

Results

Thrombin generation was measured in platelet-poor plasma by means of calibrated
Automated thrombography. The thrombogram describes the concentration of thrombin in clotting plasma. It starts with the lag time in which no thrombin is formed. After a steep increase the thrombin generation curve arrives at its peak, the maximum concentration of thrombin. The endogenous thrombin potential (ETP) or the area under the curve represents the amount of thrombin built. The time for reaching the peak is the time to peak (TTP) and the start tail marks the endpoint of thrombin generation (Fig. 1).

The ETP was significantly higher in paediatric patients when compared to controls. The time until the thrombin peak was reached (TTP) and the lag time was significantly prolonged in paediatric patients with IBD in active state, while no significant difference was found for the peak height in the phases of active disease. This data show that children with IBD in active state have the potential to generate significantly higher amounts of thrombin. The lag time and TTP are significantly elongated in comparison to healthy children.

Prothrombin fragment F1+2 concentration is a specific indicator for the amount of thrombin generated and consequently a sensitive marker for coagulation activation. Increased F1+F2 and fibrinogen concentration were higher in the active state representing in-vivo an increased activity of the coagulation system, which is in accordance with previous studies on coagulation in young patients with IBD (13). Tissue factor pathway inhibitor (TFPI) is a specific inflammatory inhibitor of the TF-FVIIa complex regulating both its procoagulant and pro-inflammatory properties. TFPI was significantly lower in our patients with IBD.

**Discussion**

In our study, we investigated all parameters of thrombin generation in paediatric patients with Crohn’s disease and Colitis ulcerosa. Importance of TG measurement lies in the key enzyme role of thrombin, so this method may become a new tool better reflecting overall haemostasis. CAT reflects the influence of all plasmatic pro-and anticoagulation factors and is appropriate to detect hypercoagulability. There is some evidence that high ETP might reflect risk of thrombosis (10) and Besser et al. (11) demonstrate an association between high ETP and recurrent venous thrombosis. In recent studies we have shown an age dependency of TG measured by CAT (12).

The coagulation system is known to be activated in inflammatory bowel disease (3). This is confirmed in our study, showing that endogenous thrombin potential and pro-thrombin activation markers do increase with higher activity index (PCDAI, PUCAI). Results of CAT show a high inter-individual variability, but measures rather constant over time in one person.

Significant higher levels of ETP and prolonged TTP and lag time were observed, while no significant difference was found for peak height in the phases of active disease. This data show that children with IBD in active state have the potential to generate significantly higher amounts of thrombin. The lag time and TTP are significantly elongated in comparison to healthy children.

Prothrombin fragment F1+2 concentration is a specific indicator for the amount of thrombin generated and consequently a sensitive marker for coagulation activation. Increased F1+F2 and fibrinogen concentration were higher in the active state representing in-vivo an increased activity of the coagulation system, which is in accordance with previous studies on coagulation in young patients with IBD (13). Tissue factor pathway inhibitor (TFPI) is a specific inflammatory inhibitor of the TF-FVIIa complex regulating both its procoagulant and pro-inflammatory properties. TFPI was significantly lower in our patients with IBD.

**Conclusion**

Our study shows that inflammatory bowel disease in children is associated with high thrombin generation, but this seems to be caused mainly by the inflammatory process and not by an individual disposition.

**Conflict of interest**

All authors declare that there is no conflict of interest.
References