Disturbed fibrinolysis in patients with inflammatory bowel disease. A study in blood plasma, colon mucosa, and faeces

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SUMMARY Using specific assays, we studied fibrinolytic activity in plasma and colonic mucosa biopsies of 28 patients with inflammatory bowel disease (IBD) (12 with Crohn's disease, 16 with ulcerative colitis) and 28 control patients without inflammatory bowel disease or colon malignancy. Blood coagulation was studied using standard techniques. In plasma of IBD patients significantly decreased tissue type plasminogen activator activity (t-PA) ($p < 0.02$), increased plasminogen activator inhibition (PAI) ($p < 0.01$) and fibrinogen ($p < 0.001$), and prolonged thrombin time ($p < 0.001$) and prothrombin time ($p < 0.001$) were found. In colon mucosa the percentage of t-PA to urokinase type plasminogen activator (u-PA) was 80:20% in the control group and 71:29% in the IBD group in non-inflamed mucosa. In inflamed mucosa the plasminogen activator percentage was 55:45%, significantly different ($p < 0.01$) from the control group. There was also a significant absolute increase in u-PA activity and decrease of t-PA activity in the inflamed mucosa compared to the control group ($p < 0.001$ and $p < 0.01$, respectively). Patients with inflammatory bowel disease therefore have significant changes in components of the fibrinolytic and coagulation system both systemically and locally in colon mucosa. These changes might contribute to an increased risk for thromboembolic complications and possibly to the pathogenesis of the colitis and to the local complications such as bleeding.

Kwaan *et al*^{1,2} showed for the first time an increased fibrinolytic activity in rectal mucosa in patients with an active colitis. Kondo *et al*³ found the same results, and suggested that this increased local fibrinolytic activity could influence anal blood loss in these patients. They treated ulcerative colitis patients with the antifibrinolytic agent tranexamic acid, administered as an enema. The patients in whom an increased local fibrinolytic activity was found improved clinically on this therapy with a significant reduction in blood loss, which was also reported in patients with ulcerative colitis treated with tranexamic acid orally.⁴ These two studies, however,

were uncontrolled and limited to 17 patients in whom only local fibrinolysis was investigated with the few tests on fibrinolysis available at that time. Kwaan *et al*^{1,2} used a histochemical fibrin slide method, which is a non-specific and non-quantitative method for measuring activity of components of the fibrinolytic system. Kondo *et al*³ used plasminogen rich and plasminogen poor fibrin plates, showing that the increased fibrinolytic activity was indeed caused by plasminogen activator(s). This method, however, does not discriminate between the two types of plasminogen activators that are distinguished to date: urokinase type plasminogen activator (u-PA) and tissue type plasminogen activator (t-PA). Both plasminogen activators are glycoproteins, capable of converting plasminogen into plasmin, and both glycoproteins are present in blood plasma as well as

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in many body tissues. Disturbances in one or both proteins have been detected in various disorders such as thrombotic disease,⁵ unexplained bleeding,⁶ and cancer.^{7,8} From the literature we know that thromboembolism is an accepted complication of inflammatory bowel disease, but the involvement of coagulation proteins is still a matter of debate.⁹⁻¹³ Recent research has indicated that disturbed fibrinolysis can also account for thromboembolic events.¹⁴ Until now, however, no specific data on fibrinolysis proteins in the systemic circulation of patients with inflammatory bowel disease are available. Therefore, in order to clarify specifically the nature and magnitude of the disturbances in fibrinolysis and their relation with anal blood loss and disease activity in patients with inflammatory bowel disease, we investigated fibrinolysis, systemically as well as locally, in two groups of gastroenterology patients, with and without inflammatory bowel disease, using recently developed specific chromogenic substrate assays.^{15,16}

Methods

PATIENTS

Plasma samples, colon mucosal biopsies and 24 hour faeces samples were collected from 56 patients who visited the outpatient department for gastroenterology. The patients were subdivided into a colitis and a control group. The colitis group included 28 patients with histologically proven inflammatory bowel disease. Twelve patients had Crohn's disease, 16 patients had ulcerative colitis. Disease activity indexes were calculated according to van Hees¹⁷ or Truelove¹⁸ (Table 1). Nine patients suffered from pancolitis, and six patients were in remission at the time of the investigation. The control group, comprised 28 patients having non-inflammatory bowel disease (Table 1), who visited the outpatient department of gastroenterology at the same day and hour as their matching case of the colitis group. The exclusion criteria for both groups were malignant tumours, objectively diagnosed thromboembolic disorders, anticoagulant therapy, blood transfusion within the last two weeks or other concomitant diseases at the time of the investigation. In the colitis group, biopsies were taken from normal colon mucosa (n=19) as well from inflamed mucosa (n=22). In the control group, colon mucosal biopsies were taken only from macroscopically normal mucosa. The study was approved by the Medical Ethic committee of the University Hospital and informed consent was obtained from all patients.

COAGULATION AND FIBRINOLYSIS IN PLASMA

Venous blood was collected, between 9 am and 5 pm

Table 1 Characteristics of the patient groups

	Colitis patients	Control patients
N	28	28
Age (mean in years)	41.2	46.1
Men	14	14
Women	14	14
Crohn's disease	12	—
Ulcerative colitis	16	—
Activity index:*		
Mild	15	—
Moderate	8	—
Severe	5	—
Irritable bowel syndrome	—	4
Polyps	—	8
Diverticulosis	—	2
No abnormalities	—	14

*According to van Hees¹⁷ and Truelove¹⁸.

after a rest period of at least 10 minutes. The first few millilitres of blood were discarded. Nine millilitres of blood was collected with minimal stasis in a polycarbonate tube containing 1 ml ice cold disodium citrate dihydrate (0.11 mol/l) or potassium-EDTA (1.5 mg/ml blood). Plasma was separated immediately by centrifugation for 30 minutes at 2000 g at 4°C, snap frozen and stored at -70°C in small aliquots. Activated partial thromboplastin time (APTT) was determined with ActinTM (Merz Dade, Dijdingen, Switzerland) (N: 25-35s). Thrombin time (ThT) was measured by adding 200 µl thrombin (±3 IU/ml) to 200 µl prewarmed plasma²⁰ (thrombin obtained from Merz Dade, Dijdingen, Switzerland) (N: 9-13s). Prothrombin time (PT) was assayed according to Quick,²¹ with Simplastin^r (General Diagnostics, Morris Plane, New Jersey, USA) (N: 15-19s). Factor II was measured using human brain thromboplastin and Factor II deficient plasma (Diamed, Salm and Kipp, Amsterdam, The Netherlands) and expressed in IU/ml (N: 0.80-1.20). Anti-thrombin III (AT-III) activity was measured using an amidolytic method²² with the synthetic peptide substrate S-2238 (Coatest^r antithrombin KabiVitrum BV, Amsterdam, The Netherlands) and expressed in IU/ml (N: 0.85-1.20 U/ml). Alpha-2-plasmin inhibitor (Alpha-2-AP) activity was assayed according to Friberger *et al*²³ using Coatest^r antiplasmin (KabiVitrum BV, Amsterdam, The Netherlands) and expressed in IU/ml (N: 0.85-1.20 U/ml). Plasminogen was measured according to Friberger²³ using streptokinase (KabiVitrum BV, Amsterdam, The Netherlands). Fibrinogen was measured according to Clauss.²⁴ Tissue type plasminogen activator (t-PA) activity was measured by the spectrophotometric assays according to Verheijen *et al*.¹⁶ Before measuring t-PA, euglobulin fractions were prepared

from rapidly thawed plasma by diluting 1 volume of plasma in 9 volumes ice cold distilled water and 0.75 volumes of 0.25% (v/v) acetic acid. The mixture was incubated in melting ice for 30 minutes, and subsequently centrifuged at 3000 g at 4°C for 10 minutes. After removal of the supernatant the precipitate was dissolved in 1 volume 0.10 mol/l Tris. HCl, 0.1% (v/v) Tween-80, pH 7.7. T-PA activity was measured by mixing 25 µl euglobulin fraction to a final volume of 250 µl with 0.10 mol/l Tris. HCl pH 7.5, 0.1% (v/v) Tween-80, 0.30 mmol/l H-D-Val-Leu-Lys-pNA (S-2251, KabiVitrum, Stockholm, Sweden). Each euglobulin sample was assayed in the presence and in the absence of rabbit IgG against human t-PA. The absorbances at 405 nm were recorded at regular intervals during two to six hours on a Titertek Multiscan spectrophotometer (Flow Laboratories). The difference between the absorbance with and without antibodies was used to calculate the t-PA activity. The intra-assay reproducibility is 5.5% (standard deviation) and the inter-assay reproducibility measured on different days 12.5% (standard deviation) both for a single measurement of a sample with 25–50 mIU/ml t-PA in the assay. T-PA activity was expressed in International Units (IU) per ml of plasma.²⁵ Inhibition of t-PA in plasma was measured according to Verheijen *et al.*²⁶ Briefly, varying amount of purified t-PA were added to a fixed amount of plasma (10–25 µl). The remaining t-PA activity was measured by the spectrophotometric assay. The inhibition level was determined by extrapolation and expressed in IU t-PA²⁵ inhibited per ml of plasma.

FIBRINOLYSIS IN COLON MUCOSAL BIOPSIES

Colon mucosal biopsies were obtained during routine endoscopic procedures. The samples were wrapped in parafilm and put into small Eppendorf cups, snap frozen and stored at –70°C. To prepare the homogenates, all biopsies were washed in 0.9% NaCl to remove visible traces of blood. After determining the individual weights of each biopsy, homogenates were made using a Elvehjem homogeniser and 500 µl Tris/Tween buffer (0.1 M Tris HCl, 0.1% Tween-80, pH 7.5). After centrifugation for 10 minutes at 10 000 g at 4°C, the supernatant was stored at –70°C in small aliquots. Using the supernatant of the centrifuged homogenates, urokinase-type (u-PA) and tissue type plasminogen activator (t-PA) activity were measured by a modification of the method according to Verheijen *et al.*¹⁶ Briefly, 10 µl of each supernatant was measured without antibodies, in the presence of rabbit anti-t-PA IgG or goat anti-urokinase monospecific antibodies and in the presence of both antibodies. The differences in absorbances at 405 nm of samples with and without antibodies were used to

calculate the t-PA, the u-PA activity and the residual amidolytic activity (RAA). In this method the conversion of plasminogen to plasmin by plasminogen activator activity (PA) is inhibited by anti-t-PA, anti-u-PA or both antibodies respectively. The absorption increase caused by t-PA and u-PA was proportional to the concentration of t-PA and u-PA according to the square of time (plasminogen is converted by PA to active plasmin, resulting in an increase of plasmin concentration, proportionally with time). The formed plasmin simultaneously degrades the chromogenic substrate S-2251, measured at 405 nm, resulting in an increase of absorption with squared time). When both antibodies were added, only a linear increase of absorption in time was observed (complete quenching of PA), resulting from residual amidolytic activity. Cross reactivity was excluded by summation of the quenching rates of both antibodies and residual amidolytic activity, which resulted in absorption equal to the unquenched sample. T-PA was expressed in international units (IU)²⁵ per mg biopsy weight. The amount of u-PA in the various samples was calculated from calibration curves obtained with standard u-PA preparations obtained from the National Institute for Biological Standards and Control, London, UK and expressed per mg biopsy weight. The amount of residual amidolytic activity

Table 2 Coagulation and fibrinolytic parameters in plasma

Test	(normal values)	Control patients n=28	Colitis patients n=28
		median (range)	median (range)
APTT	(25–35) (sec)	29 (20–33)	30 (23–38)
ThT 10	(9–13) (sec)	11 (9–13)	13‡ (10–15)
PT	(15–19) (sec)	17 (13–20)	18‡ (16–23)
Faktor II	(0.8–1.2) (IU/ml)	0.89 (0.72–1.00)	0.92 (0.70–1.00)
AT-III	(0.85–1.2) (IU/ml)	1.17 (0.93–1.48)	1.13 (0.69–2.10)
Fibrinogen	(1.6–4.0) (g/l)	2.10 (1.20–3.20)	2.95‡ (1.50–4.60)
Alpha-2-AP	(0.85–1.2) (IU/ml)	1.09 (0.56–1.38)	1.18 (0.77–1.54)
Plasminogen	(0.85–1.2) (IU/ml)	1.01 (0.69–1.53)	1.09 (0.68–1.54)
T-PA	(0–200) (mIU/ml)	123 (0–1138)	1* (0–905)
PAI	(0–15) (IU/ml)	3.8 (0–12.5)	5.5‡ (0.6–25.0)

Activated partial thromboplastin time (APTT), Thrombin time (ThT 10), Prothrombin time (PT), Antithrombin-III (AT-III), Alpha-2-plasmin inhibitor (Alpha-2-AP), Tissue-type plasminogen activator (t-PA) and Plasminogen activator inhibition (PAI), see also Figs 1 and 2. *= $p<0.02$; †= $p<0.01$; ‡= $p<0.001$, Wilcoxon's.

Fig. 1 *T-PA in plasma in the patient groups, expressed in mIU/ml. Colitis group median 1, range 0-905; control group median 123, range 0-1138 ($p<0.02$, Wilcoxon's).*

was expressed in Δ -A405 nm/hour incubation at 25°C per mg biopsy-weight.

DETERMINATION OF FAECAL BLOOD LOSS

Twenty four hour faeces samples were collected, from 23 patients in the colitis group and 24 patients in the control group, in a polyethylene container seven days after the sigmoid or colonoscopic examination and stored at 4°C. The blood content of the samples was determined by means of a modified faecal haem assay^{27 28} and expressed as μ l per gram dry weight of faeces.

STATISTICAL ANALYSIS

The Wilcoxon's test for independent samples was used for statistical analysis. Correlation of data was established by means of the Kendall rank correlation test. The probability that the dependent and the independent data were not correlated was calculated non-parametrically. Any probability less than 0.05 was considered to represent a significant difference between the samples studied.

Results

COAGULATION AND FIBRINOLYSIS IN PLASMA

Thrombin time and prothrombin time were prolonged ($p<0.001$), and fibrinogen concentrations were increased ($p<0.001$) in patients with colitis, compared with the control group (Table 2). Tissue type plasminogen activator activity in plasma was reduced ($p<0.02$) (Fig. 1) and plasminogen activator inhibition in plasma (PAI) was increased ($p<0.01$)

Fig. 2 *PAI in plasma in the two patient groups, expressed in IU/ml. Colitis group median 5.5, range 0-25.0; control group median 3.8, range 0-12.5 ($p<0.01$, Wilcoxon's).*

(Fig. 2) in the colitis group compared with the control group. No difference was observed in activated partial thromboplastin time, Faktor II, antithrombin III, alpha-2-plasmin inhibitor and plasminogen between both patient groups.

Table 3 *Fibrinolytic activity in colon mucosa*

	Controls	Colitis patients	
		Non-involved mucosa	Involved mucosa
<i>T-PA (mIU/mg biopsy)</i>			
n=28		n=19	n=22
Median	50.88	45.30	29.58
Range	32.76-134.22	11.46-107.16	6.00-81.12
Controls v colitis involved mucosa $p<0.001$ (Wilcoxon's)			
Controls v colitis non-involved mucosa $p<0.02$ (Wilcoxon's)			
<i>U-PA (mIU/mg biopsy)</i>			
n=28		n=19	n=22
Median	2.30	2.69	3.45
Range	0-9.42	0.24-9.13	1.57-13.39
Controls v colitis involved mucosa $p<0.01$ (Wilcoxon's)			
Controls v colitis non-involved mucosa ns (Wilcoxon's)			

Results are given as a median and range and are expressed as milli international units (mIU).

Fig. 3 Percentages of fibrinolytic activity in colon mucosal biopsies. T-PA, u-PA and residual amidolytic activity (RAA) after one hour incubation at 25°C, are presented as a percentage of the total absorbance – 405 nm.

FIBRINOLYTIC ACTIVITY IN COLON MUCOSAL BIOPSIES

Fibrinolytic activities in colon mucosal biopsies are given in Table 3 and Figure 3. In the control patients the percentage of u-PA activity in the mucosa was 20% of the plasminogen activator content. In the colitis group the percentage of u-PA activity in the non-involved mucosa was 29% of the plasminogen activator content, comparable with the control group. The percentage of u-PA in the involved mucosa of colitis patients (45% of the plasminogen activator content) was different, however, compared with the control patients ($p < 0.01$). Differences were found for t-PA in non-involved colitis and involved colitis mucosa *versus* control mucosa ($p < 0.02$ and $p < 0.001$ respectively), but for u-PA only for involved colitis mucosa *versus* control mucosa ($p < 0.01$). No differences between the residual amidolytic activities (PA quenched by two antibodies) of the three biopsy groups were observed. The total amidolytic activity (unquenched sample expressed in Δ -A405 nm per hour of incubation at 20°C) was also not different in the three biopsy groups. No correlations were observed for t-PA and u-PA in involved or non-involved mucosa *versus* disease activity indexes.

BLOOD LOSS IN FAECES

The results of the determination of blood loss in 24-hours faeces samples are given in Figure 4. No significant difference was observed between the colitis and the control group. No significant correlations were observed for t-PA and u-PA in colon mucosa or disease activity indexes *versus* faecal blood loss.

Discussion

The results of our study can be divided into the results from plasma, the biopsies supernatants and faeces. In plasma we found a small but significant rise

Fig. 4 Faecal blood loss in the two patient groups, expressed in µg dry weight. Colitis group median 53, range 23–363; control group median 43, range 12–585 (ns).

of plasminogen activator inhibition (PAI) and fibrinogen in the colitis group, which is compatible with the acute phase reaction described for these proteins.^{29,30} The decrease of plasma t-PA might be the result of complex formation with the increased inhibitor(s) (PAI). It is now known that patients with high PAI concentrations are at risk for developing deep venous thrombosis¹⁴ and our observation of increased PAI concentrations in colitis patients could help explain the known risk of thromboembolic disorders in colitis patients.^{12,31} The significant difference between thrombin time and prothrombin time in the colitis group could be caused by increased fibrinogen concentrations and decreased vitamin K absorption, which is known to occur in these patients.³²

The results of the fibrinolysis parameters in colon mucosa showed a shift towards the urokinase-type plasminogen activator in the inflamed mucosa of the colitis patients. This was also published in an abstract by Elliot *et al*³³ presenting qualitative data. Our quantitative t-PA concentrations were significantly decreased in non-involved, and even more in involved mucosa, an observation that correlates well with the finding of Kwaan *et al*³ who found prolonged lysis times, indicating decreased plasminogen activation concentrations, in relation to submucosal blood vessels in mucosal biopsies obtained from patients with active colitis. Whether locally increased plasminogen activator inhibitor, as a possible consequence of the observed increase of PAI in plasma of

the patients with colitis, could be responsible for the decreased levels of t-PA measured in the biopsies remains to be investigated. Quantitatively, urokinase-type plasminogen activator concentrations were significantly increased in involved colitis mucosa compared with non-involved and control mucosa, which is comparable with results recently published in an abstract.³⁴ Others have also found an increase of u-PA levels of tumour mucosa in malignant disorders of the colon.^{35,36} They concluded that this might be a marker of malignancy. It is, however, also possible that the described increase could be caused by an inflammatory process surrounding the tumour cells. In our study, no difference in residual amidolytic activity could be observed. A decrease in this activity was observed in human colorectal carcinomas by De Bruin *et al.*³⁵ As neither the nature of the substances responsible for this activity nor the importance of the differences in PA-activity between inflammation and malignancy are clear at present, this difference merits further study. The results of blood loss in faeces showed no difference between the two patient groups. This indicates that the disturbances in fibrinolysis in colon mucosa we found in this study do not imply a significant increase in faecal blood loss in these patients. In conclusion we can say that complex disturbances in fibrinolysis are present in patients with inflammatory bowel disease, locally as well as systemically. In plasma these disturbances may contribute to the known risk for thromboembolic complications, but in colon mucosa it is not clear whether these disturbances might contribute to increased or sustained mucosal bleeding.

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