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Prothrombotic State and Signs of Endothelial Lesion in Plasma of Patients with Inflammatory Bowel Disease

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Recent investigations suggest that microthrombi formation in bowel capillaries could be a determinant factor in inflammatory bowel disease (IBD) pathogenesis. To evaluate the implication of the hemostatic system during these thrombotic events, we analyzed plasmatic values of prothrombotic state markers, physiologic inhibitors of coagulation, and endothelial lesion markers in 112 IBD patients. We found an increase in thrombin–antithrombin complexes and a decrease in antithrombin III, probably due to consumption, demonstrating an increase in thrombin generation. High levels of D-dimer reflect increased fibrin formation, but there is no correlation between thrombin generation markers and D-dimer, possibly suggesting the presence of inadequate fibrinolysis. Levels of tissue factor pathway inhibitor were higher in patients than in controls. Nine patients with Crohn's disease (35% of our sample) had levels of this marker under 70% (range 37–69%). Von Willebrand factor values were increased and those of thrombomodulin only in active patients. Most of the changes were detected in patients with inflammatory activity, and there were no differences between ulcerative colitis and Crohn's disease. In conclusion, these results support the hypothesis that there is an endothelial lesion with sustained coagulation activation in IBD patients.

KEY WORDS: D-dimer; inflammatory bowel disease; thrombin–antithrombin complexes; thrombomodulin; tissue factor pathway inhibitor; von Willebrand factor.

Ulcerative colitis (UC) and Crohn's disease (CD) are frequently complicated by thromboembolic events of variable location. They can appear both in arterial and venous systems (1). Previous clinical studies have found an incidence of thrombosis between 1.2 and 7.1%, although autopsy studies demonstrate an incidence up to 39% (2). Frequency of thromboembolic disease seems to increase with enhanced inflammatory activity (3).

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Since the late 1960s, several studies have analyzed hemostatic alterations in patients with inflammatory bowel disease (IBD) (2–6). An association between thrombotic episodes in IBD patients and decreased levels of some coagulation inhibitors such as antithrombin III, protein C, and protein S has occasionally been described (7).

In recent years and due to the development of new markers and techniques, some investigations have shown increased thrombogenesis (7–13) and abnormal platelet activity (9, 14) in these entities. In general, these studies have found a relationship between the degree of inflammatory activity and prothrombotic abnormalities.

All these laboratory pathologic findings are even more interesting in view of recent publications, which

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TABLE 1. CLINICAL DETAILS OF PATIENTS INCLUDED IN STUDY

	CD	UC	Total
Number	28	84	112
Male	10	42	52
Female	18	42	60
Active	11	22	33
Inactive	17	62	79
Treatment*			
Salazopyrine	8	46	54
5-ASA	10	21	31
Oral steroids	11	10	21
Topical steroids	1	8	9
Azathioprine	4	1	5
Metronidazol	1	0	1
None	6	10	16
Previous thrombotic disease	2	1	3

*Some patients were on more than one treatment.

suggest a strong relationship between multiple infarctions of the intestinal mucose and IBD pathogenesis (15). Animal models propose the implication of decreased blood flow as a previous step in the pathogenesis of inflammation and mucosal ulcerative changes (16). Necrosis, inflammation, and mucosal ulceration would then be secondary to changes in thrombogenic mechanisms, primarily located within the intestinal tissue. These changes could be responsible for the generation of capillary microthrombi and subsequent ischemia, as has been proposed in the past (17).

The purpose of this work was to analyze the prothrombotic tendency and the role played by the hemostatic system in the etiopathogenesis of IBD. In a wide sample of IBD patients, we analyzed plasma values of prothrombotic markers, physiologic coagulation inhibitors, and specific markers of endothelial damage. Correlations among these different markers were studied and their values with respect to both clinical diagnosis (UC and CD) and inflammatory state were correlated.

MATERIALS AND METHODS

Patients with IBD

A total of 112 IBD patients followed in the Department of Gastroenterology of our hospital were included in this study (Table 1). Median age was 43.5 years (range 16–80). Crohn's disease and ulcerative colitis were diagnosed by conventional clinical, radiological, endoscopic, and histological criteria. Disease activity in CD was determined by the Harvey-Bradshaw index. Active disease was considered to be present if the score was over 4 (18). Disease activity in patients with ulcerative colitis was assessed by a variation of Truelove-Witts criteria. Patients with a score above 5 were regarded as having active colitis (19). Patients with severe hepatic or renal dysfunction, proteinuria, malnutrition, or with a history of oral contraceptive use were not asked to participate. No participants were on anticoagulant therapy. Some patients had associated diseases: rheumatoid arthritis, 1; ischemic cardiomyopathy, 4; nephrolithiasis, 2; cholelithiasis, 2; chronic obstructive lung disease, 5; erythema nodosum, 3; psoriasis, 3; gastroduodenal ulcus, 3; diabetes mellitus, 1; sclerosing cholangitis, 2; and moderate alteration of hepatic enzymes, 3. Four patients with UC were totally colectomized. Two patients had a history of deep venous thrombosis in the lower extremities and one of thrombophlebitis in the forearm. Table 1 shows treatments at the moment of study.

Controls

Sixty healthy blood donors were used as normal controls.

Laboratory Studies

Blood Sampling. Blood samples were taken from an antecubital vein puncture with minimal venous stasis, in resting conditions. Blood was collected in two different types of plastic tubes: sodium EDTA (1.5 mg/ml blood) and 3.8% trisodium citrate (1 vol citrate plus 9 vol blood). Platelet-free plasma was obtained by centrifugation at 3000 rpm for 20 min, at 4°C, immediately after extraction. Plasma was then snap frozen in aliquots and stored at −40°C until assayed.

Coagulation Measurements. Platelets, activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), and fibrinogen were measured according to conventional methods. Thrombin–antithrombin complexes (TAT) and plasma levels of prothrombin fragment F_{1+2} (F_{1+2}) were assessed by ELISA techniques (Enzygnost, Bheringwerke, Marburg, FRG). D-Dimer was determined by ELISA according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Antithrombin III (AT III) activity was measured using the chromogenic substrate S-2238 (Coatest Antithrombin, Kabi Vitrum Diagnostica, Mölndal, Sweden). Protein C activity was quantified with the chromogenic substrate S-2366 (Coatest Protein C, Kabi Vitrum Diagnostica). Functional protein S levels were determined by means of a commercial assay (Staclot Protein S, Diagnostica Stago, Asnières, France). Tissue factor pathway inhibitor (TFPI) was determined using a functional assay. Von Willebrand (vW) factor was determined by ELISA using commercially available von Willebrand factor antisera (Dakopatts, Glostrup, Denmark). Soluble thrombo-

TABLE 2. PLASMATIC LEVELS (MEAN ± SD) of Prothrombotic State Markers and Endothelial Lesion Markers

Test	*Control*	*Patients*	*Active*	*Inactive*	*CD*	*UC*
TAT (ng/ml)	1.35 ± 0.31	7.86 ± 16.3*	11.5 ± 24.5*	6.3 ± 11*	4.43 ± 6.16*	9 ± 18.4*
F_{1+2} (nmol/liter)	0.79 ± 0.30	2.94 ± 12.8	3.77 ± 15.5	2.59 ± 11.6	0.91 ± 0.44	3.63 ± 14.8
D-dimer (ng/ml)	220 ± 133	410 ± 285*	576 ± 550*	341 ± 264*†	478 ± 423*	388 ± 371*
Willebrand (%)	106 ± 30	169 ± 82*	199 ± 79*	157 ± 80*†	174 ± 65*	167 ± 87*
TM (ng/ml)	32.3 ± 13.7	34.8 ± 18.5	41.7 ± 21.5*	31.9 ± 16.3†	31.1 ± 15.3	36 ± 19.3

*Significant difference with controls.
†Significant difference with active patients. See 95% confidence intervals of differences in text.

modulin (TM) in plasma was measured with an ELISA method (Asserachrom Thrombomodulin, Diagnostica Stago).

Statistical Analysis. Results are expressed as mean ± standard deviation. Statistical significance of differences in group mean values was determined by two-tailed Student's *t* test. Correlations were sought using Pearson's correlation coefficient. Chi-square test for paired data (McNemar test) was used to study discrepancy between prothrombotic state markers. Any probability less than 0.05 was considered to represent a significant difference between the samples studied. Confidence intervals of 95% (95% CI) were calculated for significant differences between means. Data were analyzed by means of the PC program Statgraphics.

RESULTS

Basal Hemostasis Data. There were no significant differences in platelet count, APTT, PT, or TT respect to the controls or between active and inactive patients. Fibrinogen was higher in active (4.43 ± 0.86 g/liter) than in inactive patients (3.97 ± 0.97 g/liter) and 95% CI was 0.08 to 0.85 g/liter, $P = 0.02$.

None of these parameters showed differences between CD and UC.

Markers of Prothrombotic State (Table 2). TAT values ($N = 111$) were significantly higher in all subgroups with regard to the control group with these 95% CI: total sample, 3.4–9.6 ng/ml, $P = 0.009$; active patients, 1.5–18.9 ng/ml, $P = 0.007$; inactive patients, 2.5–7.4 ng/ml, $P = 0.003$; CD, 0.7–5.5 ng/ml, $P = 0.001$; UC, 3.6–11.7 ng/ml, $P = 0.006$. Plasmatic levels in active patients were higher than in those with inactive disease but the difference was not significant ($P = 0.12$).

F_{1+2} values ($N = 111$) were higher in patients than in controls, although this difference was not significant in any subgroup. Levels were highest in active patients ($P = 0.09$). This marker showed a large dispersion, except in CD (see standard deviations, in Table 2) and statistical analysis is conditioned by this.

Plasma values of D-dimer ($N = 111$) were elevated in all analyzed subgroups of IBD patients with respect to the control group. The 95% CI of the differences were: total sample, 111–270 ng/ml, $P = 0.0002$; active patients, 158–554 ng/ml, $P < 0.0001$; inactive patients, 53–189 ng/ml, $P = 0.001$; CD, 91–425 ng/ml, $P = 0.0001$; UC, 81–256 ng/ml, $P = 0.003$. Patients with active disease had significantly higher levels than those with inactive disease (CI 32–438 ng/ml, $P = 0.003$).

There was a positive correlation between TAT and F_{1+2} values in inactive patients ($r = 0.80$, $P < 0.0001$). In active patients this correlation was still more intense ($r = 0.92$, $P < 0.0001$). Neither marker of thrombin generation, TAT and F_{1+2}, correlated with D-dimer. If we consider normal values of TAT those lower than 2 ng/ml and of D-dimer those lower than 500 ng/ml (corresponding to means + two standard deviations of our controls), 63.1% of patients with IBD had pathologic levels of TAT concomitant with normal levels of D-dimer. The statistical analysis of these proportions by means of the chi-square test demonstrated a significant discrepancy ($P < 0.0001$). The same was observed in the active subgroup, with a discrepancy in 51.5% of patients ($P = 0.0001$), and in the inactive subgroup, where 67.9% of patients showed no concordance ($P < 0.0001$).

Physiologic Inhibitors of Coagulation (Table 3). Mean values of AT III ($N = 112$) were lower in all the subgroups with respect to controls with the following 95% CI: complete IBD sample, 7.5–14.4%, $P < 0.0001$; active patients, 9.3–19.5%, $P < 0.0001$; inactive patients, 6.1–13%, $P < 0.0001$; CD, 8.4–16.6%, $P < 0.001$; UC, 6.8–14.1%, $P < 0.0001$. Patients with active disease presented with lower mean levels than inactive ones (1–8.8%, $P = 0.01$).

No differences in protein C levels ($N = 111$) were found in any of the subgroups with respect to control values.

TABLE 3. PLASMATIC LEVELS (MEAN ± SD) of Coagulation Inhibitors

	Control	*Patients*	*Active*	*Inactive*	*CD*	*UC*
AT III (%)	109 ± 10	97.6 ± 9.7*	94.2 ± 12*	99.1 ± 8.3*†	96.2 ± 7.6*	98.2 ± 10.3*
Protein C (%)	101 ± 13.8	103 ± 17.5	105 ± 16.2	102 ± 18.1	105.2 ± 18.3	102.4 ± 17.4
Protein S (%)	see text	99.9 ± 25.7	93.8 ± 24	102 ± 26	97.1 ± 26	100.8 ± 25.5
TFPI (%)	97 ± 22	110 ± 44*	126 ± 57*	104 ± 36*†	100 ± 48	114 ± 42*

*Significant difference with controls.
†Significant difference with active patients. See 95% confidence intervals of differences in text.

We have stratified normal values of functional protein S by sex and age: in women under 40 years, 88 ± 24%; in women over 40 years and in men, 110 ± 24%. In IBD patients (N = 109), women under 40 had a mean of 92 ± 23%, not different from their control counterparts. The mean value in the remaining women and the men was not different (103 ± 26%, P = 0.06). When comparing active with inactive patients, and CD with UC patients, without the distinction of age and sex, no significant differences were found.

Levels of TFPI (N = 106) in the whole sample were higher than in controls (95% CI 4.4 to 23.2%, P = 0.004). Active patients had a mean higher than inactive individuals (95% CI 0.6–44%, P = 0.02). Fifteen of the studied cases showed levels of TFPI lower than 70%. Nine were CD patients (range 37–69%), which represents 35% of our sample (9/26). One of these nine patients had experienced deep venous thrombosis.

AT III values correlated negatively with TAT complexes (r = −0.63, P = 0.0001) and with F_{1+2} (r = −0.75, $P < 0.0001$) in active IBD patients. Nevertheless, these correlations were not seen in patients with inactive disease.

Endothelial Lesion Markers (Table 2). Von Willebrand factor (N = 112) was higher in all subgroups with respect to the control group. The CIs were, in the whole group, 46–80%, $P < 0.0001$; in active patients, 64–122%, $P < 0.0001$; in inactive patients, 30–60%, $P < 0.0001$; in CD, 42–94%, $P < 0.0001$; in UC, 40–81%, $P < 0.0001$. Active patients had higher levels than inactive patients (95% CI 10–76%, P = 0.01).

Thrombomodulin values (N = 108) appeared elevated in active patients with respect to the control group (95% CI 1.2–17.6 ng/ml, P = 0.004) and to inactive patients (95% CI 1.2–18.3 ng/ml, P = 0.01).

A poor correlation (r = 0.31, P = 0.005), only present in patients without clinical inflammatory activity, was seen between von Willebrand factor and thrombomodulin.

DISCUSSION

The present study quantifies a significant number of hemostatic-related markers. To our knowledge, it includes a bigger sample than previous studies related to this subject.

Results obtained from markers of prothrombotic state show a maintained activation of coagulation in IBD, both in active- and inactive-phase patients. TAT complexes and F_{1+2} are specific markers of this activation because their increment implies increased thrombin generation (20). D-dimer is a final product of fibrinolysis, resulting from plasmin action on fibrin (21), and the observed increase corroborates two findings: coagulation activation with fibrin appearance and secondary activation of fibrinolysis. Nevertheless, and contrary to what we expected, neither TAT nor F_{1+2} correlated with D-dimer, and the majority of cases with altered values of TAT (higher than the mean + 2 SD) presented normal D-dimer values. This discordance suggests an insufficient fibrinolytic response in a hypercoagulable population. Other authors have described increases in plasmatic thrombin-generation markers (8, 9) and fibrinolytic system disturbances (22–24), and the work of Hudson et al (10) simultaneously reflects both alterations.

AT III is the main physiologic thrombin inhibitor. Active protein C and its cofactor protein S are also inhibitors by means of their action on factors Va and VIIIa. Congenital or acquired deficits of these inhibitors may be responsible for thrombosis (25). There are no data in our study that implicate protein C and S in the thrombogenic tendency displayed by these

patients. Decreased AT III levels have been interpreted as a sustained consumption, secondary to thrombin generation. The more decreased AT III levels in active patients indicate a higher consumption and more intense coagulation activation.

These results confirm former observations in smaller samples of patients (2, 4, 6) with a transitory decreased AT III during the inflammatory activation phase. The few reported cases that associate protein C or S deficits with thrombosis in IBD patients could be coincidental (7), but they probably explain the high thrombogenic tendency in this subgroup of patients, in whom two risk factors are present: protein C or S deficiency and IBD. Aadland et al recently found low free protein S plasma levels in 54 patients with CD (26). They have studied antigenic levels by means of an electroimmunoassay, which has been demonstrated to yield less accurate and more variable results than other immunological assay methods (27).

TFPI is a natural anticoagulant that directly binds and inactivates factor Xa and, in a factor Xa-dependent fashion, produces feedback inhibition of the factor VIIa–tissue factor catalytic complex (28). In our study, it appears elevated in IBD patients. Furthermore, active patients have levels higher than inactive ones, behaving as an acute-phase reactant, although previous work does not agree with this result (29). It is worth noting that 35% of our CD patients had low levels of TFPI. There is just one study related to this subject published so far that finds lower levels of TFPI in IBD patients regardless of whether they have CD or UC (13). There are no available data about the different incidence of thromboembolism between CD and UC. In our series, two of 28 (7%) CD patients have suffered from venous thrombosis, while one of 84 (1.2%) UC patients had this complication (Table 1).

Von Willebrand factor is a complex multimeric protein essential for the correct adhesion of platelets to endothelium and for platelet aggregation. The main synthesis and storage site is the endothelial cell. Clinical, *in vitro*, and animal studies support the concept that increased levels of vW factor reflect endothelial cell damage or injury (30).

The vW factor levels were statistically higher in our patients, both in active and inactive phases, than in controls. This increase was more intense in patients with clinical activity. Similar results have been obtained by Stevens et al (11) in 92 IBD patients and by Gris et al (24).

Thrombomodulin is a glycoprotein located on the endothelial cell surface, which serves as a receptor for thrombin. It is a cofactor for thrombin-catalyzed activation of the anticoagulant protease zymogen, protein C. Thrombomodulin is also present in other tissues and in plasma in a small soluble form. Plasma levels are increased in various diseases, presumably due to vascular endothelial cell damage (31).

In our study, active patients presented with increased levels, but patients in remission were not different from controls. To our knowledge, very few data on soluble TM in IBD patients have been published. A unique work including 26 cases (12) reports high levels in IBD patients, with no differences between active and inactive patients. TM is located on the endothelial cell membrane as a transmembrane protein (32), whereas vW factor is found inside the Weibel-Palade granules of the endothelial cell (33). It would be reasonable to think that TM could be released into plasma after minor injury, but cellular lysis would be necessary to release vW factor. If we accept that TM and vW factor are not only simple acute-phase reactants but also markers of endothelial lesions, our results support the hypothesis of the existence of endothelial intestinal injury in IBD patients.

Most of the alterations reported in this study are of moderate intensity, although statistically significant. If we consider that their origin is a relatively small and restricted vascular territory, such as the intestinal mucosa, plasmatic levels of these markers are the result of their dilution in the total volume. Therefore, subtle or moderate plasmatic alterations can reflect more intense local changes and a critical loss of the regional hemostatic balance.

Recently, Wakefield et al (34) have shown the existence of granulomas in the wall of the intestinal microvasculature of CD patients. This granulomatous vasculitis seems to be an initial pathologic event in the course of the disease. The same group has found a pathogenic sequence of events in the initial phases of the disease (15). The prothrombotic abnormalities described in our study and in others could be playing a central role in IBD pathogenesis through the amplification of the consequences of a primitive vascular lesion. Furthermore, there is an interesting argument in favor of thrombosis as a pathogenic factor in IBD. Gaffney et al reported clinical improvement and histological evidence of remission associated with use of heparin in three patients with UC refractory to conventional treatment (35).

In conclusion, IBD patients present persistent signs of endothelial damage concomitant with coagulation activation. Minor changes in physiologic inhibitors of coagulation do not explain the prothrombotic ten-

dency. Increased inflammatory activity is related to greater changes. From the point of view of the markers studied, physiopathological processes underlying UC and CD would be similar.

The intestinal mucosa would be the primary site of the observed alterations according to biopsy-based studies (22). In our opinion, more direct tissue studies are needed to investigate the coagulation pathways, endothelial cell damage, and fibrinolysis in IBD patients.

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