Thromboembolism in inflammatory bowel disease: role of platelets

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Abstract

Patients with inflammatory bowel disease are susceptible to thromboembolism and recently small vessel thrombosis has been implicated as an aetiological factor in Crohn's disease. This study therefore investigated platelet function in 104 patients with inflammatory bowel disease of whom eight had previous thromboembolism. Thirty five patients had reproducible spontaneous platelet aggregation of more than 30% (0 in controls) ($p < 0.0001$). A further 20 patients showed hypersensitivity of platelets to low concentrations of aggregating agents ($p < 0.001$). Plasma thromboxane B_2 and β thromboglobulin levels were significantly higher than controls ($p < 0.001$ and $p < 0.001$), but platelet lifespan studies were normal. There was no correlation with disease activity. Patients with inflammatory bowel disease have abnormal platelet activity, which may contribute to the inflammatory process.

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An increased risk of major thromboembolism has been described in patients with inflammatory bowel disease¹⁻⁶ with incidences as high as 39% in some postmortem studies.⁴ Young patients with active ulcerative colitis appear to be particularly susceptible with significant mortality.¹⁻² A climate of blood hypercoagulability has been described in patients with inflammatory bowel disease (IBD) and more recently, thrombosis, vasculitis, and tissue infarction have been proposed as contributing factors in Crohn's disease.⁷ Examination of the clotting cascade has revealed significant abnormalities⁸⁻¹² and abnormalities of fibrinolysis have also been described¹³⁻¹⁵ but little attention has been focused on the role of platelets which are known to contribute to all aspects of normal haemostasis. We have therefore examined the risk of thromboembolic disease and undertaken a study of platelet function, in patients with inflammatory bowel disease attending a gastroenterology follow up clinic.

Patients and methods

One hundred and four consecutive patients were studied. All had a proved diagnosis of Crohn's disease or ulcerative colitis confirmed by histological examination and barium x ray studies. Eighty four healthy controls were also studied and age and sex matched with the patient group. A detailed clinical history was taken from each patient and notes scrutinised for previous evidence of thromboembolism. Disease activity was assessed using previously described methods.^{16,17} Biochemical and haematological markers of disease activity were also estimated (C

reactive protein, albumin, α_1 acid glycoprotein, haemoglobin, and erythrocyte sedimentation rate). These were used to confirm disease activity status, but if a clear discrepancy arose then more emphasis was given to these indicators and disease activity estimated by an independent observer. A 60 ml venous blood sample was obtained with minimal stasis after a fat free breakfast, of which 20 ml aliquot was allowed to stand for measurement of C reactive protein, α_1 acid glycoprotein, and anticardiolipin antibodies. The remainder was immediately anticoagulated using freshly prepared 3.8% disodium citrate solution (at a ratio of nine parts blood to one part anticoagulant).

A separate 11.5 ml sample of blood was also taken, of which 9 ml was placed directly in a pre-cooled tube containing 0.95 ml of EDTA and a 0.04 M solution of indomethacin (for measurement of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$) and the remaining 2.5 ml of blood was placed in another pre-cooled tube containing a solution of EDTA and theophyllin (Amersham International plc, Bucks) (for measurement of β thromboglobulin). Both tubes were rapidly cooled, centrifuged at 1200 rpm at 4°C, and the supernatant removed for assay.

The following studies were then undertaken: (a) Platelet count, platelet distribution width (PDW), mean platelet volume (MPV), and platelet crit (using a Technicon H1 blood analyser); (b) erythrocyte sedimentation rate (Westergren); (c) platelet 'adhesiveness' (Salzmann); (d) platelet aggregation¹⁸; (e) plasma β thromboglobulin (BTG), thromboxane B_2 (TBxB₂) and 6-keto-prostaglandin $F_{1\alpha}$ (Amersham International plc); (f) platelet lifespan.

PLATELET ADHESIVENESS (29 patients)

This was assessed using Salzmann's method and was facilitated by the use of a commercially available 'Adeplat T' kit (Simmelweis, Milan, Italy).

PLATELET AGGREGATION STUDIES (104 patients)

Throughout the platelet aggregation studies platelets were only exposed to plastic or siliconised surfaces. All tests were performed in duplicate and in parallel with tests on age and sex matched controls and were performed at 37°C using an LEA 4 channel platelet aggregometer (Bio/data Corporation). Platelet rich plasma (PRP) was generated by centrifugation of whole blood at 600 g for 12 minutes and the platelet count adjusted to $300 \times 10^9/l$ using platelet poor plasma (PPP). A 0.45 ml aliquot of PRP from each patient and control was added to each channel of the aggregometer, a stir bar added,

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the velocity of stir bar rotation reduced to 800 rpm, and the samples examined for spontaneous platelet aggregation (SPAG). Subsequently, serial dilutions of adenosine diphosphate (ADP), collagen, ristocetin, and arachidonic acid (at starting concentrations of 10 $\mu\text{mol/l}$, 5 $\mu\text{g/ml}$, 1.25 $\mu\text{g/ml}$, and 25 $\mu\text{mol/l}$ respectively) were freshly prepared and used as aggregating agents, with 44.7 μl of aggregating agent being added to each successive 0.45 ml aliquot of patient and control PRP. The maximum aggregation velocity for test *v* control was recorded as well as the endpoint dilution of aggregating agent at which platelet aggregation would still occur.

In those patients in whom spontaneous platelet aggregation was found, further studies were performed. Gel filtration¹⁹ was used to separate the platelets from plasma in PRP samples of patients who had shown SPAG (platelet viability was assessed by aggregation studies performed on platelets resuspended in autologous plasma). Patients' platelets were then resuspended in ABO compatible control plasma and tested again for spontaneous aggregation. Similarly, plasma from patients in whom SPAG was seen was added at a ratio of 1:1 by volume to the PRP of controls and tested for SPAG. The 104 patients were followed up over an 18 month period. Platelet aggregation studies were repeated for those patients in whom there was a change of disease activity status.

PLASMA BTG, TBXB₂, AND 6-KETO-PROSTAGLANDIN F₁ α (62 patients)

Plasma β thromboglobulin, thromboxane B₂, and 6-keto-prostaglandin F₁ α (the stable metabolites of thromboxane A₂ and prostacyclin)

were measured by radioimmunoassay (Amersham International plc).

PLATELET LIFE SPAN (10 patients)

Platelet life span was assessed in 10 patients with active IBD using indium-111 oxine labelled platelets. Platelets were separated from plasma and labelled according to methods previously described.²⁰ Viability following the labelling procedure was assessed by aggregation studies and by comparison of hepatic and blood pool curves (where increasing hepatic sequestration without a plateau being reached implied significant platelet damage).²¹ Statistical significance of differences between groups was determined by Mann-Whitney U test. Correlations were sought with Spearman's rank correlation test.

Results

In the study group of 104 patients, 40 had Crohn's disease and 64 ulcerative colitis (of whom 14 had undergone previous panproctocolectomy). Table I gives details of disease distribution. Twenty two patients were assessed as having active disease at the time of study. Seven patients had extra intestinal manifestations of disease, two had established primary sclerosing cholangitis (PSC), three had probable early PSC, and two had ankylosing spondylitis. No patient in the ulcerative colitis panproctocolectomy group had evidence of extra intestinal disease.

Sixty two patients were taking 5-amino-salicylic acid products and 18 of these were also receiving steroid medication. Cardiolipin antibodies were absent in all patients apart from one 49 year old Asian man who was later found to have type IIb hyperlipidaemia. The importance of this is unexplained.

TABLE I Patient and control details

Study group	No	Age	Men	Women	Active disease	Medical RX (steroids)	Previous surgery
Crohn's disease	40	49	14	26	12	17 (7)	18
Ulcerative colitis	64	51	35	29	10	45 (11)	12
Control	89	45	48	41			
Site of disease							
	Total		Diffuse disease		Segmental small bowel		Colitis
Crohn's disease	40		10		18		12
			Distal disease		Left sided disease		Total colitis
Ulcerative colitis	64		4		32		28

TABLE II Comparison of SPAG and history of thrombosis

Patient no	Diagnosis	Site of thrombosis	Age	SPAG
1	Ulcerative colitis	Deep vein thrombosis/pulmonary embolus	50	+
2	Crohn's disease	Deep vein thrombosis/pulmonary embolus	55	+
3	Crohn's disease	Deep vein thrombosis/pulmonary embolus, fatal myocardial infarction	55	+
4	Ulcerative colitis	Deep vein thrombosis/pulmonary embolus, transient ischaemic attacks	32	+
5	Crohn's disease	Pulmonary emboli fatal cortical vein thrombosis/pulmonary embolus	36	-
6	Ulcerative colitis	Deep vein thrombosis/pulmonary embolus	62	+
7	Crohn's disease	Deep vein thromboses severe ischaemic heart disease	62	+
8	Crohn's disease	Pulmonary embolus	31	+

Spontaneous platelet aggregation (SPAG) in patients with a history of thromboembolic complications. ('Age' denotes age at which first thromboembolic event occurred.)

THROMBOEMBOLISM (Table II)

Eight patients were found to have a previous history of thromboembolism and in seven there were recurrent complications.

Two patients died from causes directly attributable to thromboembolic complications over the 18 month follow up period.

PLATELET MORPHOLOGY AND NUMBER (Table III)

Eight of 40 patients with Crohn's disease and seven of 64 patients with ulcerative colitis had significantly raised platelet counts ($<420 \times 10^9/l$). The counts were higher in the patients with Crohn's disease than in those with ulcerative colitis but did not reach statistical significance. Platelet counts were generally higher in patients with active disease but not significantly so. The platelet distribution width was normal in all patients, but the mean platelet volume was generally lower in patients than in controls. No statistical significance was achieved.

PLATELET ADHESION

This test was discontinued after studying 29 patients and matched controls. Results obtained

TABLE III Quantitative and morphological aspects of platelets

	(n)	Thrombocytosis (n)	Mean platelet ($\times 109/l$) (SD)	MPV (fl) (SD)	PDW (%)	Platelet aggregates (n/field)
Crohn's disease	40	8	329 (118)	7.78 (0.94)	49	4
Ulcerative colitis	50	7	286 (91)	8.13 (0.93)	48	4
Panproctocolectomy	14	0	337 (78)	8.11 (0.93)	48	1
Controls	84	0	271 (65)	8.20 (0.94)	48	1

Platelet count, mean platelet volume (MPV), platelet distribution width (PDW), and platelet aggregates (per high power field) in controls and patients with Crohn's disease, ulcerative colitis, and patients with ulcerative colitis who have undergone panproctocolectomy.

TABLE IV Platelet aggregation studies

	(n)	SPAG (n)	SPAG (%) (SD)	SPAG (%/l) (SD)	Platelet hypersensitivity (n)
Crohn's disease	40	18*	48 (35)	70 (35)	29*
Ulcerative colitis	50	11*	54 (32)	65 (30)	19*
Panproctocolectomy	14	6*	54 (36)	65 (34)	7*
Controls	84	0	0	0	0

Spontaneous platelet aggregation and platelet hypersensitive in controls, patients with Crohn's disease, ulcerative colitis, and patients with ulcerative colitis who have undergone panproctocolectomy.

* $p < 0.001$.

were non-reproducible and therefore impossible to interpret. In those patients tested there was no significant abnormality of adhesion detected and no difference between patients and controls.

PLATELET AGGREGATION STUDIES (Table IV)

(a) Spontaneous platelet aggregation. Reproducible SPAG was observed in 18 of 40 patients with Crohn's disease and 17 of 64 patients with ulcerative colitis (including six of 14 patients with ulcerative colitis who had undergone previous panproctocolectomy), and in nine of the 36 patients with only distal disease (Fig 1). This phenomenon was not observed in the control group and was a highly significant finding ($p < 0.0001$ and $p < 0.0001$) for both Crohn's disease and ulcerative colitis groups. There was no difference between the Crohn's disease and ulcerative colitis groups and no correlation with disease activity, site of disease, and current treatment.

Eight of nine patients whose disease activity status changed during the course of the study retained this abnormality. Two patients who were taking non-steroidal anti-inflammatory drugs (which exert an anti-aggregatory effect) at

Figure 1: Reproducible spontaneous platelet aggregation in a patient with Crohn's disease (upper curve). The control platelets eventually aggregate when adenosine diphosphate is added (lower curve).

the time of study, also showed SPAG. Seven of eight patients with previous thromboembolism showed SPAG.

(b) Aggregation studies with aggregating agents. A further 11 patients in the Crohn's disease group and nine in the ulcerative colitis group (including one in the panproctocolectomy group) had marked sensitivity of platelets to low concentrations of aggregating agents when compared with controls (this arbitrarily being defined as more than a threefold difference in endpoint dilution of aggregating agent). (Fig 2). There was a highly significant difference between patients and controls for each of the three aggregating agents, adenosine diphosphate, collagen, and arachidonic acid ($p < 0.001$, $p < 0.001$, and $p < 0.001$). There was no difference for ristocetin, or between patients with Crohn's disease and ulcerative colitis and no correlation with disease activity.

(c) Gel filtered platelets. Gel filtration of PRP produced on average a platelet yield of approximately 60%. The platelets functioned adequately in 12 of 20 (60%) patients with SPAG when resuspended in autologous plasma. The platelets from six patients (50%) showed SPAG when suspended in ABO compatible control plasma, even with PRP platelet counts of less than $100 \times 10^9/l$. Conversely, the plasma from the same patients was not seen to induce SPAG in control PRP.

BTG, TBXB₂, AND 6-KETO-PROSTAGLANDIN F₁α (Table V)

Plasma thromboxane B₂ and β -thromboglobulin were significantly higher in patients than in controls ($p < 0.001$, $p < 0.001$). There was no difference between patients with Crohn's disease and those with ulcerative colitis and no correlation with disease activity. There was no significant difference between the patients and control groups in the 6-keto-prostaglandin F₁α levels

Figure 2: Box and whisker plots showing distribution and median values for endpoint concentration of aggregating agents, at which platelets would still aggregate for patients (□) and controls (▨).

TABLE V Platelet and endothelial cell release proteins

		Thromboxane B_2 (pg/tube)	6-keto-prostaglandin $F_{1\alpha}$ (pg/tube)	β Thromboglobulin 6-keto-prostaglandin $F_{1\alpha}$ (pg/tube)	β Thromboglobulin (ng/l)
Patients	62	53.11*	10.58	5.62	84.6†
(SD)		(17.33)	(3.18)	(2.86)	(54.0)
Controls	62	36.0	9.10	6.38	36.0
(SD)		(14.87)	(3.46)	(8.47)	(18.0)

Plasma thromboxane B_2 , 6-keto-prostaglandin $F_{1\alpha}$, β thromboglobulin, and the ratio of thromboxane B_2 to 6-keto-prostaglandin $F_{1\alpha}$ in patients and controls.

* $p < 0.001$, † $p < 0.001$.

Figure 3: Box and whisker plots showing distribution and median values for plasma levels of thromboxane B_2 , 6-keto-prostaglandin $F_{1\alpha}$ and β thromboglobulin for patients (□) and controls (▨).

and in the $TBxB_2$:6-keto-prostaglandin $F_{1\alpha}$ ratio (Fig 3).

PLATELET LIFESPAN

The *in vitro* aggregation response of patients' PRP to $10 \mu M$ ADP after separation and labelling with ^{111}In oxide compared favourably with that of controls. Hepatic sequestration after reinjection reached a plateau and matched the curve for the blood pool in all patients. These results suggest that minimal damage was incurred by the platelets as a result of the separation and labelling procedures.²⁰ The platelet life span in all 10 patients was normal (seven to ten days), although two patients had borderline low results (Fig 4).

Figure 4: Platelet survival in patients with Crohn's disease, ulcerative colitis, and idiopathic thrombocytopenic purpura (ITP).

Screening of the abdomen for abnormal uptake showed no evidence of sequestration of platelets in the bowel wall at sites of active disease. There was no difference in platelet survival between the Crohn's disease and ulcerative colitis groups and no correlation with platelet count or spontaneous platelet aggregation.

Discussion

The association between thromboembolism and inflammatory bowel disease was first described by Bargen and Barker in 1936. The work, based mainly on clinical observation, described severe and life threatening thromboembolism, particularly in young patients with active ulcerative colitis, and the authors concluded that a coagulopathy was to blame and that it probably related to 'platelet function or the ease in which thromboplastin is generated.'¹ Studies of blood coagulation have shown several abnormalities⁷⁻¹² with raised levels of fibrinogen, fibrinopeptide A, factors II, V, and VIII having been described. In all cases fluctuating levels of these factors have matched that of the underlying bowel disease implying an acute phase response.

Fibrinolysis has also been shown to be abnormal in patients with inflammatory bowel disease,¹³⁻¹⁵ with raised levels of plasminogen activator inhibitor and decreased tissue plasminogen activator having been described, although no correlation with disease activity was found in this instance. Platelets have received less attention, which is surprising considering the essential role they play in normal haemostasis and the contribution they make to normal coagulation and fibrinolysis. Although abnormalities have been described,²²⁻²⁴ studies have generally been small and inconclusive.

We have shown a significant risk of recurrent thromboembolism in patients with inflammatory bowel disease (8/104) and in most cases this complication occurred during disease quiescence (and in some cases preceded the diagnosis of the underlying bowel disorder). Platelet aggregation and in particular spontaneous aggregation was found to be markedly active in a high proportion of patients and spontaneous platelet aggregation was observed in seven of the eight patients with previous thromboembolism. Spontaneous platelet aggregation has been described in other disorders associated with thromboembolic disease.²⁵⁻²⁷ In addition, circulating plasma levels of the platelet release proteins β thromboglobulin and thromboxane B_2 were also found to be raised, providing *in vivo* evidence for increased platelet activation. No correlation between abnormal platelet function and disease activity was observed and the features of abnormal platelet function were retained in patients who experienced a change in disease activity status during the study period. In addition, patients with ulcerative colitis who had undergone previous panproctocolectomy also showed these abnormalities. The mean platelet volume of patients especially of those with SPAG, was generally lower than that of controls. This would tend to rule out the possibility of an abnormal population of younger, more active (and larger) platelets circulating in patients with inflam-

matory bowel disease because of high platelet turnover.²⁸ Platelet life span was normal in all 10 patients studied (although two patients had borderline low values), which suggests that the increased platelet consumption taking place is of a low grade nature and more likely to occur at the microvascular level. These results would support the hypothesis of a small vessel vasculitic/thrombotic cause in patients with Crohn's disease,⁷ although in our study patients with ulcerative colitis were found to be equally at risk.

Our studies with gel filtered platelets suggest that the abnormalities observed are due to an inherent platelet defect and not due to circulating platelet activators, although potential candidates for circulating activators would have to include bacterial lipopolysaccharides (which have been shown to include surface tissue factor expression in monocytes, which is a potent activator of platelets)^{12,29} and bacterial toxins such as verotoxins (which have been shown to induce SPAG in children with the haemolytic uraemic syndrome).³⁰ It is interesting that two patients currently taking cyclooxygenase inhibiting drugs (non-steroidal anti-inflammatory drugs) also had SPAG, implying that prostaglandin synthesis is not a primary mechanism in the development of this phenomenon.

In conclusion, we have shown a high risk of thrombosis in patients with inflammatory bowel disease in addition to markedly abnormal platelet function. Both abnormalities are unrelated to the underlying bowel disease activity and imply that platelet hyperaggregation may be important in developing thrombosis in such patients. In view of our findings, we suggest that tests for spontaneous platelet aggregation should be included routinely particularly in patients who are at increased risk of thromboembolism. Prophylactic antithrombotic drugs could then be given should the test prove positive. Abnormal platelet function might also contribute to the underlying pathological process and supports the hypothesis of a microvascular thrombotic cause in the pathogenesis of IBD.

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