Whole blood procoagulant activity in breast and colorectal cancer

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SUMMARY Whole blood procoagulant activity was determined by measuring the recalcification time of citrated blood, with and without the addition of bacterial endotoxin, in patients with breast cancer (n = 39), colorectal cancer (n = 20), benign breast disease (n = 15), benign colorectal disease (n = 11), normal volunteers (n = 15) and inpatients with non-malignant disease (n = 22). The median clotting times of those samples incubated with endotoxin were significantly shorter in the patients with breast and colorectal cancer compared with normal controls. Furthermore, significant differences between the median clotting times of stimulated and unstimulated samples within each subject group were observed only in the two cancer groups. There was no correlation between whole blood procoagulant activity and absolute monocyte counts, with histological staging or with plasma concentrations of plasma fibrinopeptide A.

The results suggest that blood from patients with cancer is more sensitive to endotoxin stimulation than that from normal or benign controls, but that in its present form the technique cannot be used to distinguish between malignant and non-malignant disease.

The increased incidence of clinical and laboratory abnormalities of haemostasis in malignant disease is well recognised,¹ and sensitive markers such as plasma fibrinopeptide A, often provide evidence of in vivo clotting activation in acute leukaemia^{2,3} and solid tumours.^{4,5} Although the mechanism of coagulation activation in cancer is probably multifactorial, procoagulants associated with tumours may have a role in this process. Various malignant tumours^{6,7} and leukaemic promyelocytes⁸ may release tissue factor, and specific factor X activating tissue procoagulants occur in a wide range of malignancies in man and animals.^{9,10}

Peripheral blood monocytes and tissue macrophages also express tissue factor, particularly after stimulation with endotoxin,^{11,12} and this activity may be increased in both animal¹³ and human¹⁴ malignancies. Furthermore, the correlation between unstimulated monocyte tissue factor activity and plasma fibrinopeptide A in patients with advanced lung cancer suggests that monocyte tissue factor expression is related to the increased rate of fibrin generation in vivo.¹⁴

Most studies have measured tissue factor activity in isolated monocytes, although manipulation of the cells during this procedure may artefactually increase tissue factor production.¹⁵ As monocytes are the only circulating blood cells capable of synthesising tissue factor, the shortening of the recalcification time of whole blood incubated with endotoxin may be attributed to an increase in monocyte tissue factor synthesis.¹⁶ Using this principle, Dasmahapatra *et al* showed that the recalcification time was significantly shorter in patients with cancer compared with controls,¹⁷ particularly in samples stimulated with endotoxin, and concluded that the test might distinguish such patients from both controls and subjects with benign tumours. Monocyte counts were not reported in this study, however, and the possibility that monocytosis in the cancer groups might have accounted for the increased procoagulant activity was not excluded. Plasma fibrinopeptide A concentrations were also not determined, and thus a possible correlation between monocyte tissue factor concentrations and ongoing intravascular coagulation was not assessed.

The whole blood recalcification time method of measuring monocyte tissue factor activity is a simple

and inexpensive test that can be rapidly performed, and which is potentially suitable for routine use. The aims of this study were to determine whether patients with breast or colorectal cancer generate more whole blood procoagulant activity than normal controls or patients with benign disease, and to correlate the extent of procoagulant activity with histological staging, absolute monocyte counts, and plasma fibrinopeptide A concentrations.

Patients and methods

A total of 122 subjects were studied. Patients with breast and colorectal cancer were selected from routine hospital admissions. The extent and nodal spread of the tumours were determined post-operatively, and staged according to the TNM classification for breast tumours, and Dukes' classification for colorectal cancer. Control groups comprised patients with histologically confirmed benign breast disease, patients found to have benign colorectal disease after colonoscopy (with subsequent histological confirmation), inpatients before surgery for hernia repairs and varicose veins, and normal healthy volunteers recruited from hospital staff. The numbers in each group, together with age ranges, median ages, and sex distribution are shown in table 1. The study had local ethical committee approval, and informed consent was always obtained before blood sampling.

Sterile glass bijou bottles were siliconised by immersion in 2% dimethyldichlorosilane (BDH Chemicals Ltd), thoroughly rinsed in distilled water, and finally dried at 100°C for 10 minutes. Venous blood (10 ml) was collected preoperatively using 21G butterfly needles; 3.6 ml was transferred to the siliconised bijou bottles containing 0.4 ml buffered sodium citrate (31.3 g/l tri-sodium citrate, 50 g/l HEPES); 4.5 ml was added to 0.5 ml of 9 g/l NaCl containing 1000 U/ml heparin and 1000 U/ml Trasylol (Bayer) and subsequently used for the measurement of fibrinopeptide A. The remainder was taken into disodium edetic and (1 g/l) for white cell count and differential.

The method used for measurement of whole blood procoagulant activity was based on that described by Dasmahapatra *et al.*¹⁷ As soon as possible after blood

sampling, a 2 ml aliquot of the citrated whole blood was transferred to a second siliconised glass bijou containing 40 µl of *Escherichia coli* endotoxin (0111:B4, 500 µg/ml, Sigma Chemicals, Poole). Samples with and without endotoxin were then incubated at 37°C for two hours, after which 200 µl aliquots from each tube were transferred to 12 × 75 mm sterile plastic tubes and allowed to warm at 37°C. Prewarmed calcium chloride solution (0.035 M, 100 µl) was then added and the clotting times recorded in quadruplicate. In practice the tubes were left undisturbed for the first 60 seconds, and then tilted every five to 10 seconds until the end point was observed. The mean clotting time (in seconds) for the stimulated and unstimulated samples was recorded and the percentage shortening in the presence of endotoxin was then calculated.

The reproducibility of the assay was assessed by performing replicate determinations (n = 10) on a normal blood sample, with and without endotoxin stimulation. The coefficients of variation for unstimulated and stimulated clotting times were 10.8% (range 246–347 seconds) and 15.4% (range 217–357 seconds), respectively.

Total white cell counts were determined electronically using a Coulter Counter (model Zn). Absolute monocyte counts were calculated from the total white cell counts and the percentage of monocytes observed in blood films stained by the Romanovsky method.

Plasma fibrinopeptide A concentrations were determined with a commercially available ELISA assay (Boehringer Diagnostics).

The siliconised glass tubes and the buffered citrate anticoagulant used in this study were tested with a commercially available chromogenic technique (Kabi Diagnostics) and found to be free of contaminating endotoxin.

Analysis of data was performed using the STATGRAPHICS statistical software system. The observations were not always normally distributed, and summary statistics were therefore expressed as medians and interquartile ranges. Differences between groups were assessed with the Mann-Whitney U test or the Kruskal-Wallis procedure for non-parametric data.

Results

WHOLE BLOOD PROCOAGULANT ACTIVITY Without endotoxin

The median values, interquartile ranges (IQR), and total ranges of the clotting times for unstimulated samples in each subject group are shown in table 2. Unstimulated clotting times in the patient groups did not differ significantly from those of the normal control group, and there were no differences between

Table 1 Details of patients studied

	n =	Age range (years)	Median (years)	Sex distribution	
				Male	Female
Normal controls	15	20–67	42	6	9
Surgical inpatients	22	37–81	58	12	10
Benign breast	15	18–45	38	—	15
Breast cancer	39	30–91	62	—	39
Benign colorectal	11	54–85	66	3	8
Colorectal cancer	20	33–88	68	10	10

Table 2 Statistical analysis of whole blood recalcification times (seconds) with (+ LPS) and without (- LPS) endotoxin, together with the percentage shortening of the clotting time (% S)

		Median	IQR	Range
Normal controls (n = 15)	- LPS	313	271 - 381	208 - 473
	+ LPS	301	273 - 356	219 - 385
	% S	11.3	0.0 - 21.9	0 - 26.5
Surgical inpatients (n = 22)	- LPS	304	267 - 372	178 - 450
	+ LPS	272	238 - 308	156 - 370
	% S	13.6	8.2 - 18.8	0 - 33.5
Benign breast (n = 15)	- LPS	251	213 - 376	161 - 563
	+ LPS	236	225 - 333	163 - 505
	% S	7.2	0.0 - 13.2	0 - 17.0
Breast cancer (n = 39)	- LPS	294	254 - 359	199 - 535
	+ LPS	255	237 - 294	175 - 454
	% S	11.1	2.0 - 23.8	0 - 55.5
Benign colorectal (n = 11)	- LPS	310	236 - 333	156 - 432
	+ LPS	258	199 - 286	134 - 291
	% S	14.1	12.6 - 18.9	0 - 38.3
Colorectal cancer (n = 20)	- LPS	320	281 - 382	191 - 520
	+ LPS	263	254 - 287	141 - 377
	% S	14.5	8.0 - 25.6	0 - 47.7

the two cancer groups and the corresponding benign controls. Furthermore, there were no significant differences between the surgical inpatient control group and any other group.

With endotoxin

Table 2 also shows the median values, IQRs, and ranges of the clotting times for samples stimulated with endotoxin. The median clotting times of the breast ($p < 0.05$), colorectal cancer ($p < 0.05$), and benign colorectal ($p < 0.05$) groups were significantly shorter than those of normal controls. There were no differences between the cancer groups and the corresponding benign controls, or with the surgical inpatient control group.

Table 3 Statistical analysis of total white cell counts ($\times 10^9/l$), absolute monocyte counts ($\times 10^9/l$), and fibrinopeptide A concentrations (ng/ml)

Group	Test	n	Median	IQR
Normal controls	Total white cell count	14	6.86	6.16 - 7.43
	Monocytes	15	0.23	0.11 - 0.32
	Plasma fibrinopeptide A	14	2.9	2.2 - 5.4
Surgical inpatients	Total white cell count	22	7.54	6.42 - 8.69
	Monocytes	22	0.31	0.17 - 0.37
	Plasma fibrinopeptide A	22	6.8	2.4 - 10.9
Benign breast	Total white cell count	13	7.59	6.57 - 9.58
	Monocytes	15	0.25	0.12 - 0.33
	Plasma fibrinopeptide A	15	0	0.0 - 29.9
Breast cancer	Total white cell count	39	7.73	6.69 - 10.5
	Monocytes	39	0.25	0.15 - 0.37
	Plasma fibrinopeptide A	39	4.6	1.92 - 12.9
Benign colorectal	Total white cell count	11	8.4	6.13 - 11.02
	Monocytes	11	0.39	0.12 - 0.60
	Plasma fibrinopeptide A	11	4.5	0.0 - 8.3
Colorectal cancer	Total white cell count	20	8.14	5.81 - 9.50
	Monocytes	20	0.35	0.22 - 0.51
	Plasma fibrinopeptide A	20	6.95	0.0 - 19.4

Percentage reduction in recalcification time by endotoxin

The shortening of the clotting time in the presence of endotoxin was expressed as a percentage of the unstimulated clotting time for each subject. The results are summarised in table 2. Statistical analysis of these results showed no significant differences between any of the groups, with the exception of the benign breast control group and the surgical inpatient control group ($p < 0.05$).

Effect of endotoxin on clotting time within subject groups

To assess the effect of endotoxin on whole blood recalcification times the median clotting times with and without endotoxin were compared *within* each subject group. Only the clotting times in the groups with breast ($p < 0.005$) and colorectal ($p < 0.005$) cancer were significantly shortened by addition of endotoxin.

TOTAL WHITE CELL COUNT, ABSOLUTE MONOCYTE COUNTS, AND FIBRINOPEPTIDE A

The median values and interquartile ranges for total white cell count, absolute monocyte count, and plasma fibrinopeptide A are shown in table 3. There were no significant differences in these variables among any of the groups.

HISTOLOGICAL STAGING

The classification of breast cancer made after surgery and used in this study was the internationally recognised TNM classification. No patients recruited to this study had evidence of distant metastases (M), and statistical analysis was therefore confined to the primary tumour (T) and regional lymph node (N) disease.

Table 4 Statistical analysis of results in patients with breast cancer classified according to T stage*

Stage		Median	IQR
T1 (n = 8)			
Without LPS	(s)	293.5	270 - 402
With LPS	(s)	254.0	233 - 292
Shortening	(%)	10.5	1.0 - 27.3
Total white cell count	($\times 10^9/l$)	7.6	6.1 - 10.0
Monocytes	($\times 10^9/l$)	0.19	0.15 - 0.28
Plasma fibrinopeptide A	(ng/ml)	7.6	0 - 11.9
Age	(years)	60.0	44 - 64
T2 (n = 29)			
Without LPS	(s)	296.0	280 - 359
With LPS	(s)	262.0	239 - 309
Shortening	(%)	13.9	4.0 - 23.8
Total white cell count	($\times 10^9/l$)	7.7	6.7 - 10.5
Monocytes	($\times 10^9/l$)	0.27	0.16 - 0.40
Plasma fibrinopeptide A	(ng/ml)	4.3	2.0 - 10.1
Age	(years)	60.0	56 - 68

*Only one patient was allocated to each of groups T3 and T4 and these were therefore not included in the statistical analysis.

There were no significant differences in clotting times (with or without endotoxin), percentage shortening of clotting time, white cell count, absolute monocyte count, and plasma fibrinopeptide A concentrations of patients with breast cancer classified as T1 and T2 (table 4) or N0 and N1 (table 5). Only two patients were classified as T3 or T4 and were therefore omitted from statistical analysis.

The colorectal tumours were classified after surgery according to the Dukes' staging system. There were no differences in clotting times (with or without endotoxin), percentage shortening, white cell counts, absolute monocyte counts and plasma fibrinopeptide A of patients with colorectal cancer subdivided into Dukes' stages A-C (table 6).

EFFECT OF AGE ON PROCOAGULANT ACTIVITY

The median ages and age ranges of each subject group are shown in table 1. All subject groups were significantly

Table 5 Statistical analysis of results in patients with breast cancer classified according to N stage

Stage		Median	IQR
N0 (n = 26)			
Without LPS	(s)	286.5	254 - 337
With LPS	(s)	251.5	237 - 290
Shortening	(%)	10.6	0.0 - 17.8
Total white cell count	($\times 10^9/l$)	8.4	7.2 - 10.9
Monocytes	($\times 10^9/l$)	0.25	0.15 - 0.38
Plasma fibrinopeptide A	(ng/ml)	4.9	2.9 - 10.1
Age	(years)	60.5	49 - 66
N1 (n = 13)			
Without LPS	(s)	318.0	285 - 362
With LPS	(s)	266.0	239 - 309
Shortening	(%)	16.8	7.0 - 24.9
Total white cell count	($\times 10^9/l$)	6.9	5.9 - 8.1
Monocytes	($\times 10^9/l$)	0.27	0.16 - 0.37
Plasma fibrinopeptide A	(ng/ml)	3.9	1.8 - 19.9
Age	(years)	62.0	57 - 69

Table 6 Statistical analysis of results in patients with colorectal cancer classified according to Dukes' stage

Stage		Median	IQR
A (n = 4)			
Without LPS	(s)	285.5	257 - 357
With LPS	(s)	259.0	232 - 295
Shortening	(%)	11.1	9.2 - 16.9
Total white cell count	($\times 10^9/l$)	8.5	6.1 - 10.4
Monocytes	($\times 10^9/l$)	0.27	0.15 - 0.56
Plasma fibrinopeptide A	(ng/ml)	20.1	6.4 - 115.7
Age	(years)	78.0	71 - 84.5
B (n = 7)			
Without LPS	(s)	340.0	288 - 415
With LPS	(s)	263.0	255 - 287
Shortening	(%)	22.4	6.9 - 25.3
Total white cell count	($\times 10^9/l$)	8.3	7.5 - 12.5
Monocytes	($\times 10^9/l$)	0.38	0.12 - 0.49
Plasma fibrinopeptide A	(ng/ml)	4.2	0.7 - 13.4
Age	(years)	68.0	64 - 71
C (n = 9)			
Without LPS	(s)	312.0	282 - 350
With LPS	(s)	272.0	235 - 287
Shortening	(%)	16.6	8.0 - 26.2
Total white cell count	($\times 10^9/l$)	6.8	5.3 - 8.3
Monocytes	($\times 10^9/l$)	0.30	0.26 - 0.54
Plasma fibrinopeptide A	(ng/ml)	1.8	0.0 - 20.6
Age	(years)	63.0	58 - 77

cantly older than the normal controls and patients with benign breast disease ($p < 0.001$), although there was no difference between the ages of subjects with malignant and benign colorectal disease. Individual subject ages were poorly correlated with whole blood procoagulant activity expressed as percentage shortening of the clotting time ($r = 0.147$, $p = 0.105$, $n = 122$). Nevertheless, the median procoagulant activity of each group was significantly correlated with the median group age ($r = 0.83$, $p < 0.05$, $n = 6$).

Discussion

The shortening of the recalcification time of whole blood incubated with endotoxin is directly related to the increase in monocyte tissue factor synthesis.¹⁶ Thus Dasmahapatra and coworkers assessed monocyte tissue factor activity in whole blood from patients with solid tumours, and suggested that this test might distinguish cancer patients from normal controls and those with benign disease.¹⁷ To confirm these findings we measured monocyte tissue factor activity in whole blood from patients with breast and colorectal cancer and compared the results with histological staging, absolute monocyte counts, and plasma concentrations.

Dasmahapatra *et al* reported that the recalcification times of both stimulated and unstimulated whole blood from cancer patients were significantly shorter than normal.¹⁷ In the present study there were no

differences in the unstimulated recalcification times, although the median recalcification time of both breast and colorectal cancer patients were significantly shorter than normal controls after addition of endotoxin. Even after endotoxin stimulation, however, the median recalcification times of the cancer patients were not different from the corresponding benign or surgical inpatient controls. Furthermore, the median stimulated recalcification time of the benign colorectal control group was also significantly shorter than that of the normal controls. As monocyte tissue factor generation may be increased in active Crohn's disease, however,¹⁸ and colonic diverticula in the United Kingdom are common in persons over 60 years old,¹⁹ concurrent inflammatory processes, such as diverticulitis, may account for this finding.

Although there were no differences in unstimulated or stimulated clotting times between benign and malignant groups, it was noteworthy that the stimulated clotting times were significantly shorter than the corresponding unstimulated values in the patients with cancer alone. Thus despite the lack of difference among subject groups, significant amounts of monocyte tissue factor induced by endotoxin were produced only in subjects with malignant disease.

Taken together, therefore, these data show only partial agreement with the earlier study.¹⁷ Our inability to distinguish controls and cancer patients, however, may be partly due to the technical drawbacks of this method for assessing whole blood procoagulant activity. Firstly, unless contact activation is standardised, the whole blood clotting time²⁰ has a very wide normal range.²¹ The whole blood recalcification time is closely related to this test, and has similar inherent problems. Indeed, we encountered very wide variability with this technique; replicate clotting times sometimes varying by more than 100 seconds. The within group variation was even greater, particularly in patients with benign breast disease. Therefore, in addition to the wide variation in normal monocyte tissue factor expression *in vitro*^{14,22} the poor reproducibility of the technique and wide normal range may have contributed to our failure to show significant differences between benign and malignant breast disease. Secondly, all blood samples in this study were incubated in siliconised glass tubes, and only transferred to plastic tubes immediately before recalcification. In contrast, Dasmahapatra's group used plastic tubes throughout,¹⁷ and adherence of monocytes to plastic surfaces may have artefactually stimulated monocyte tissue factor generation.²³ Thirdly, the presence of contaminating bacterial endotoxin was not excluded in the previous study. Because endotoxin is a ubiquitous contaminant of laboratory reagents, it is important to consider this possibility when interpreting the results of such studies.

In the original study¹⁷ absolute monocyte numbers were not determined, and monocytosis, which often accompanies malignant disease,²⁴ may have contributed to the higher monocyte tissue factor activity in the cancer patients. In the present study, however, the absolute monocyte counts did not differ significantly among subject groups.

There were no significant differences in plasma fibrinopeptide A concentrations among any of the groups. The median values in the benign breast and normal control groups were normal (<3 ng/ml), although those in the other four groups were slightly raised. Despite a previous report of a correlation between plasma fibrinopeptide A and monocyte tissue factor generation in cancer patients,¹⁴ no relation between whole blood monocyte tissue factor and plasma fibrinopeptide A was apparent in the current study.

In agreement with an earlier report,²⁵ there was no correlation between monocyte tissue factor concentrations and TN staging in patients with breast cancer. Because advanced breast cancer is treated palliatively, however, only those patients with early disease are admitted for surgery and were included in this study. Similarly, there were no significant differences in monocyte tissue factor concentrations among colorectal cancer patients classified according to Dukes' stage.

Monocyte tissue factor activity may increase with age,²⁶ and, as patients with malignant disease are usually older than those with benign disorders, increased monocyte tissue factor expression in cancer may simply reflect this age difference. We found no correlation between whole blood monocyte tissue factor and age, however, although a significant correlation was noted between the median ages and monocyte tissue factor concentrations of the six subject groups.

In conclusion, the results of this study suggest that the whole blood recalcification time in cancer patients is more sensitive to bacterial endotoxin than in normal controls or patients with benign disease. In its present form, however, this test cannot be used to distinguish malignant and benign disease on an individual basis, although further studies, preferably with an improved method for measuring procoagulant activity in whole blood, are justified.

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