Monocyte tissue factor levels in cancer patients

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ABSTRACT

Objective: The association between cancer and thromboembolic disease has been known for over a century. Increased tissue factor expression by endothelial cells, monocytes or macrophages is implicated. Thus, monocyte tissue factor measurements may reflect disease presence or progression.

Methods: Using a 2 stage kinetic chromogenic assay, monocyte tissue factor levels were assessed in normal controls (n=60), patient controls (hernia or cholecystectomy, n=60) and in patients with benign and malignant disease of the bladder (n=73), prostate (n=81), breast (n=83) and colorectum (n=62). This was performed as baseline (resting cells) and after 6 hours incubation with (stimulated) and without (unstimulated) lipopolysaccharide. Each benign disease group was sub-divided into inflammatory and non-inflammatory categories.

Results: The relative operating characteristic curve for the lipopolysaccharide-stimulated monocyte tissue factor assay showed sensitivity and specificity for cancer, the area under the curve being 0.71. The control groups and the benign non-inflammatory groups gave similar results and were pooled for further analysis. Each malignant group showed higher monocyte tissue factor levels than the control groups for baseline ($P<0.05$) and lipopolysaccharide-stimulated cells ($P<0.05$). All benign inflammatory groups apart from breast, showed increased monocyte tissue factor levels over controls for baseline ($P<0.05$) and lipopolysaccharide-stimulated cells ($P<0.05$). In all cases there was no significant difference between the malignant and the benign inflammatory groups. Monocyte tissue factor levels were related to tumor grade or stage, patients' survival time, serum prostate specific antigen and static bone scan images. Levels were also higher in patients with bladder cancer recurrence and in those who subsequently died.

Conclusion: Lipopolysaccharide-stimulated monocyte tissue factor assay showed sensitivity and specificity for cancer compared to controls. Monocyte tissue factor levels are raised in malignant groups compared to controls and non-inflammatory diseases but not when compared with inflammatory conditions. Stimulated cells give better discrimination between the groups and may be useful in identifying high risk individuals. Monocyte tissue factor levels were related to tumor progression.

Keywords: Monocyte tissue factor, coagulation activation, solid tumors.

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Tissue thromboplastin (tissue factor, Cluster of Differentiation (CD) 142) is an integral plasma membrane glycoprotein.[1] It serves as a receptor and essential co-factor for blood coagulation factors VII and VIIa in the activation of factors X and IX.[2] Tissue factor (TF) is produced by cells that are normally separated from blood by the vascular endothelium.[3,4] Normal peripheral blood cells and endothelium do not usually have detectable TF, nor do the smooth muscle cells of most vessels.[5,6]

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Table 1 - Sample size.

Group	n	Median age (year)	Age range	Male (n)	Female (n)
Normal	60	35	19-77	26	34
Hernia or Cholecystectomy	60	61	30-86	43	17
Bladder					
Benign, non-inflammatory[a]	29	71	50-90	20	09
Benign, inflammatory[b]	08	78	64-95	05	03
Cancer	36	68	24-86	26	10
Prostate					
Benign, non-inflammatory[a]	57	72	44-95	57	-
Benign, inflammatory[b]	07	69	41-80	07	-
Cancer	17	76	60-90	17	-
Breast					
Benign, non-inflammatory[a]	21	42	20-74	-	21
Benign, inflammatory[b]	04	57	42-76	-	04
Cancer	58	63	35-87	-	58
Colorectal					
Benign, non-inflammatory[a]	31	66	39-92	16	15
Benign, inflammatory[b]	13	60	44-89	07	06
Cancer	18	73	46-85	08	10
TCC					
No recurrence[c]	14	74	53-89	14	-
With recurrence	16	72	63-85	14	02

a = negative cystoscopy following hematuria; histologically proven benign prostatic hypertrophy, known diverticular disease but not active, fibrocystic disease, fibroadenoma or intraductal papilloma.
b = cystitis, prostatitis, mastitis or mammary duct ectasia, ulcerative colitis or diverticulitis.
c = previous bladder malignancy.
TCC = transitional cell carcinoma
n = number

However, TF synthesis in these cells can be induced by several agonists.[5-8] Upon stimulation peripheral blood monocytes express increasing amounts of TF. Its procoagulant activity (PCA) in response to lipopolysaccharide (LPS) in whole blood might, therefore, be of clinical significance. In this respect, Dasmahapatra et al, showed an increased level of LPS-induced monocyte tissue factor (mTF) in patients with various cancers compared to those with benign non-inflammatory diseases.[9] Contrino et al, demonstrated that TF expression by endothelial cells is present only in the blood vessels of malignant breast tumors and not in the vessels of benign breast lesions.[10] Thus, both studies demonstrate an association between TF expression and the malignant phenotype. Mellor et al, reported higher whole blood PCA in breast and colorectal cancer patients compared to normal controls, but there was no distinction between malignant and benign surgical control groups.[11] This study however, may not have paid enough attention to the benign groups since inflammatory conditions may cause similar rises in mTF levels.[12]

Previous techniques available for mTF measurements involve laborious methods to isolate cells.[13,14] In the present study we used a 2 stage kinetic chromogenic assay (KCA) which measures mTF activity on intact cells isolated in a one-step procedure.[15] This assay is not significantly affected by age, gender or smoking habit. Using this assay we assessed mTF levels in patients with bladder, prostate, breast and colorectal cancer, and compared the results with normal controls, surgical controls and subjects with benign diseases. To study the effect of inflammatory conditions on mTF PCA the benign groups were sub-divided into inflammatory and non-inflammatory categories. mTF levels were also correlated with tumor grade or stage, malignant recurrence, conventional markers of tumor progression and with patients' survival time.

Methods.

Controls and patients. Ethical Committee approval was obtained for the study by the Southampton and South West Hampshire Health Commission Joint Ethical Sub-Committee and informed consent was sought from each patient. A total of 449 subjects were studied: patients were admitted into the surgical wards of Southampton University Hospitals (see Table 1 for details). Blood samples were taken prior to operation, except for

those taken at the time of malignant recurrence of the bladder. The surgical control groups consist of patients awaiting hernia repair or cholecystectomy (non-specific organ and non-inflammatory benign disease). These patients had no inflammatory symptoms and a normal erythrocyte sedimentation rate. Benign disease was sub-divided into non-inflammatory and inflammatory benign disease groups (specific organ non-inflammatory or inflammatory benign disease). All patients had been clinically diagnosed and confirmed by biopsy. Tumors were classified as follows; the modified classification of the World Health Organization (WHO) for the bladder,[16] the Gleason's system for the prostate,[17] WHO classification for breast cancer[18] and Dukes' staging for colorectal cancer.[19]

Monocyte tissue factor measurements. Monocyte tissue factor levels were assayed using a 2 stage KCA[15] which is described below. mTF activity was measured as baseline (fresh resting cells), after 6 hours incubation without LPS (unstimulated cells) and after 6 hours incubation with LPS (stimulated cells) at 37°C and 5% CO_2 in air. Tissue factor on the surface of the monocytes forms a complex with factor VII and Ca++. The complex activates factor X to factor Xa which then acts on a specific chromogenic substrate resulting in the generation of a yellow color that can then be measured spectrophotometrically.

Cell preparation. Ten mls of venous blood was collected into ethylene-diamine-tetra acetic acid (EDTA) tubes. The blood samples were then transferred to Leukoprep™ tubes (Becton Dickinson, Oxford, UK) and centrifuged (within 2 hours of sample collection) for 15 minutes at 1500-1800 x g at room temperature. After centrifugation the plasma layer was discarded; the buffy coat layer, which contains the mononuclear cells as well as platelets, was transferred to a flat bottomed polystyrene tube (Greiner Labortechnik, Germany). Cells were then washed 3 times using cooled sterilized Dulbecco's saline buffer (Sigma Chemical Company, Pool, Dorset, UK). The washed mononuclear cells were then resuspended in RPMI 1640 medium with L-Glutamine (Gibco, UK) and adjusted to 2 x 10^6 cells/ml and platelet to monocyte ratio of between 4:1 and 6:1.

Assay procedure. One hundred ul of cell suspension was placed into duplicate wells in a 96 well flat bottomed microtitre plate and cells were allowed to adhere for 10 minutes. The non adherent cells and the pre-set ratio of contaminating platelets (see above) were then washed off using cold saline. To the adherent cells the following reagents were then added: 60 ul of the assay buffer (0.05 Tris (hydroxymethyl) aminomethane) (Tris), NaCl, pH 7.8), 20 ul of $CaCl_2$ (0.025 M) and 15 ul (0.75 unit/ml) of a commercially-available clotting factor concentrate (Prothromplex TIM4 30 U/ml, Immuno Ltd, Dunton Green, UK) as a source of factors VII and X. Control wells contained the above reagents, but with 15 ul of factor X buffer instead of prothromplex for background substraction. The plate was then incubated at 37°C for 10 minutes. The reaction was then stopped with 100 ul of assay buffer containing 7.5 mM EDTA. Forty ul of spectrozyme Xa was added and the rate of factor Xa generation, which is proportional to the amount of TF expressed by monocytes, was determined by measuring the increase in the absorbance of the free chromophore p-Nitroaniline (pNA) generated in comparison to the original substrate. Absorbance values were automatically calculated by the Kinetic-Calc™ package based on the standard curve constructed from serial dilutions of recombinant relipidated TF (0.75-83.0 ng/ml) and converted to TF (ng/10^6 cells). The recombinant TF was obtained from the American Diagnostica Inc, Greenwich, CT, USA. Relipidation was performed in our laboratory according to the method of Carson and Konigsberg.[20] The standard curve is set out on each microplate for each set of measurements. Results were then multiplied by 5 and expressed as ng TF/10^6 mononuclear cells/well (each well contained 2x10^6 cells).

Sensitivity and specificity of the monocyte tissue factor assays. The assays' validity for cancer patients was assessed by plotting the true positive (sensitivity) vs false positive (1-specificity) results on the relative operating characteristic curve (ROC), providing a graphical comparison of test performance. The area under the curve (AUC) of the ROC-curve is directly proportional to the reliability of a given test. A test with 100% specificity and sensitivity will display an AUC of 1.0; values above 0.5 are indicative of an acceptable sensitivity and specificity. The ROC-curve permits the measurements of sensitivity and specificity for all possible cut-off points of any given assay and avoids the need for the selection of an arbitrary cut off point. When more than one laboratory test is available for the same clinical parameter one can compare ROC-curves, by plotting both on the same figure.[21]

Serum prostate specific antigen. Prostate specific antigen (PSA) was measured using the IMx® PSA assay system (Abbott Laboratories, USA) in patients with benign prostatic hypertrophy (BPH) and patients with prostate cancer.

Static bone scan imaging. Single phase static bone scan imaging was assessed as described by McKillop and Fogelman.[22]

Statistical analysis. Data was included in a database and analyzed by the Statgraphics™ statistical software system (Statistical Graphics Corporation, Englewood Cliffs, NJ, USA). The sensitivity and specificity were determined by measuring the AUC of the ROC-curves. Data was not normally distributed, and summary statistics were

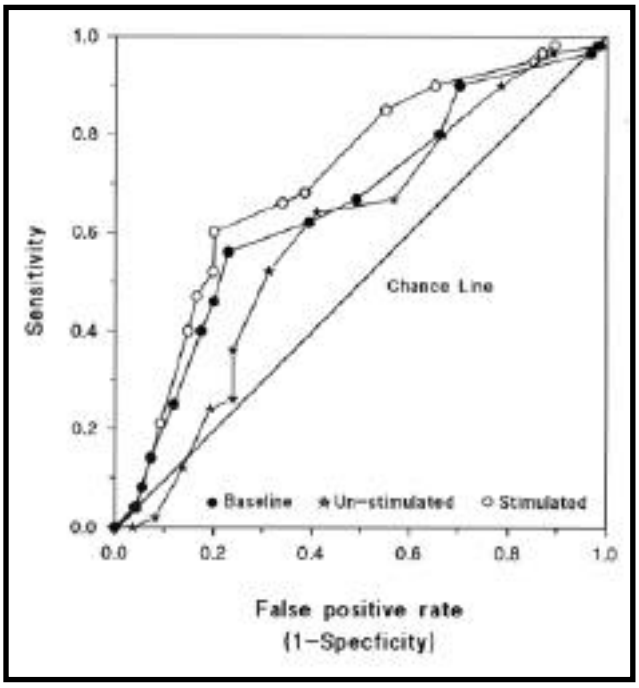

Figure 1 - Relative operating characteristic curves for baseline, unstimulated, and LPS-stimulated mTF activity in patients with cancer.

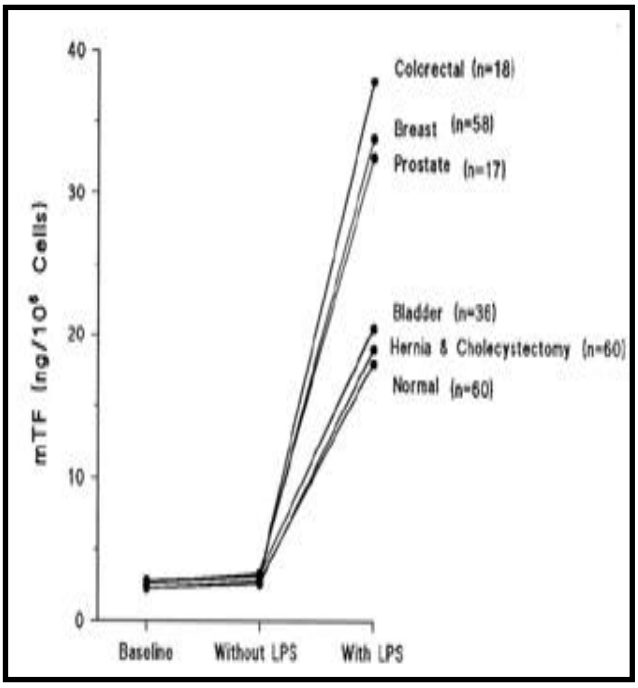

Figure 2 - Increased in mTF activity (ng/10^6 cells) after LPS-stimulation in normal controls, patients undergoing hernia repair or cholecystectomy, and patients with bladder, prostate, breast and colorectal malignancy.

expressed as medians and interquartile ranges (IQR). Differences between 2 groups were assessed by the Mann-Whitney U-test. Differences in tumor grading or staging were tested by Kruskal-Wallis One-Way Analysis by Ranks. Correlations were determined using Spearman's rank correlation test.

Results. ***The reliability of the mTF assays.*** The ROC-curves plotted for the mTF assays are shown in Figure 1. The corresponding AUC were; baseline (0.66), the unstimulated (0.60) and the LPS-stimulated cells (0.71).

Assessment of monocyte tissue factor activity in hospital patients. The median and IQR of mTF activity for bladder, prostate, breast and colorectal diseases are shown in Table 2.

Bladder disease. There was no significant difference between the normal controls, the patients control group (hernia or cholecystectomy) and the benign non-inflammatory bladder disease. The malignant group showed significantly higher levels than each control group for the baseline ($P<0.05$) and the stimulated readings ($P<0.05$). For the un-stimulated cells, the malignant group differed only from the hernia or cholecystectomy controls ($P<0.01$) and the benign non-inflammatory group ($P<0.05$). The benign inflammatory group showed a significant increase compared with each control group for the baseline cells ($P<0.01$), with the hernia or cholecystectomy and benign non-inflammatory groups for the unstimulated levels ($P<0.05$), and only with the benign non-inflammatory group for the stimulated cells ($P<0.001$). There was no significant difference between the benign inflammatory group and the malignant group for the baseline, un-stimulated and LPS-stimulated mTF levels.

Prostate disease. In prostate disease, the normal controls and the patient control groups displayed no significant differences when tested against each other. The inflammatory benign group showed a significant increase when compared with controls for the baseline levels ($P<0.05$). For the stimulated cells, the inflammatory benign group, was significantly higher than normal ($P<0.05$) and the benign non-inflammatory group ($P<0.01$). The malignant group showed significantly higher levels than each control group for the baseline ($P<0.01$) and the stimulated cells ($P<0.001$), but only with the non-inflammatory group for the unstimulated cells ($P<0.05$). No significant difference was observed between benign inflammatory and malignant patients for the 3 parameters measured.

Breast disease. There was no significant difference between the normal controls and the patient control group (hernia or cholecystectomy) and benign non-inflammatory breast disease. Inflammatory benign disease showed an elevation in

Table 2 - Baseline, unstimulated and LPS-stimulated mTF levels (ng/10^6 cells) in normal controls, patients awaiting hernia repair or cholecystectomy, non-inflammatory benign, inflammatory benign and malignant disease of the bladder, prostate, breast and colorectum.

Group	n	Baseline		Without LPS		With LPS	
		Median	IQR	Median	IQR	Median	IQR
Normal	60	2.3	2.1-2.7	2.8	2.5-3.4	17.4	11.8-23.3
Hernia or Cholecystectomy	60	2.4	2.1-2.6	2.7	2.4-3.2	19.0	17.8-24.7
Bladder							
Benign non-inflammatory	29	2.3	2.0-2.5	2.7	2.5-3.0	15.0	11.0-19.7
Benign inflammatory	08	3.2	2.6-3.3	3.5	2.6-3.8	20.2	18.9-38.8
Cancer	36	2.6	2.2-2.8	3.2	2.7-3.5	20.2	13.8-34.6
Prostate							
Benign non-inflammatory	57	2.4	2.1-2.7	2.5	2.3-3.0	18.5	12.5-22.0
Benign inflammatory	07	2.7	2.4-3.5	2.9	2.5-4.8	19.8	19.5-53.0
Cancer	17	2.8	2.5-3.2	3.1	2.8-3.3	37.9	28.9-45.5
Breast							
Benign non-inflammatory	21	2.3	2.1-2.5	2.7	2.3-3.3	22.0	12.1-26.0
Benign inflammatory	04	2.6	3.4-3.0	2.8	2.6-3.1	38.4	28.2-48.3
Cancer	58	2.6	2.3-2.9	3.1	2.6-3.7	33.9	19.0-41.2
Colorectal							
Benign non-inflammatory	31	2.4	2.2-2.5	2.6	2.5-2.7	18.6	10.5-25.3
Benign inflammatory	13	2.6	2.5-3.0	3.6	2.8-4.2	33.4	28.6-37.0
Cancer	18	2.6	2.4-2.9	3.3	2.8-3.5	32.5	18.7-39.5

n = number
LPS = lipopolysaccharide
IQR = interquartile ranges

mTF levels compared with each control group for the stimulated cells only. Although the numbers in the inflammatory group were small the statistical test gave $P<0.01$. On the other hand, the malignant groups showed variable significant increases over the control groups for the baseline ($P<0.05$), un-stimulated ($P<0.05$) and the stimulated cells ($P<0.001$). No significant differences were observed between the benign inflammatory group and the malignant group for all mTF levels studied.

Colorectal disease. For colorectal disease, the 3 control groups (normals, hernia or cholecystectomy, and benign non-inflammatory disease of the colorectum) displayed no significant differences when tested against each other. Malignancy conferred a significant increase over each control group for all mTF parameters measured; baseline ($P<0.05$), unstimulated ($P<0.05$) and stimulated cells ($P<0.01$). The inflammatory benign disease group showed significantly raised mTF over all controls for baseline ($P<0.05$), unstimulated ($P<0.05$) and stimulated cells ($P<0.01$). There was no significant difference between the benign inflammatory and the malignant disease group for the 3 mTF levels.

Percentages of increased monocyte tissue factor levels in patients with malignant disease. Monocyte tissue factor in patients with bladder malignancy gave results above the upper quartile range of the normal controls for baseline 47%, un-stimulated 25% and LPS-stimulated cells 42%. For patients with prostate cancer these were 59% for baseline, 12% unstimulated and 82% for stimulated cells. By the same criteria patients with breast malignancy gave 47% for baseline, unstimulated 33% and stimulated cells 62%, and finally, patients with colorectal cancer showed 34% for baseline, 33% unstimulated and 67% for stimulated cells. The stimulated mTF activity of the colorectal group showed the highest increase compared with controls or patients with bladder, prostate or breast malignancy (Figure 2).

Tumor grade or stage. Tumor classification was carried out according to tumor grade or stage. There was an increase in mTF levels corresponding to a higher tumor grade or stage. For all cancers studied the trend did not reach statistical significance for baseline, unstimulated or LPS-stimulated cells (Kruskal-Wallis).

Patient survival. There was a trend towards decreased monocyte tissue factor activity in longer surviving patients with malignancy. Again, the differences were not statistically significant (Mann-Whitney).

Patients with and without recurrent bladder cancer. mTF levels were studied in; A) patients without recurrent tumor and B) patients with recurrent tumor. Analysis of mTF levels in the 2 groups showed that in group B there was a wide range and a higher median compared to group A. The

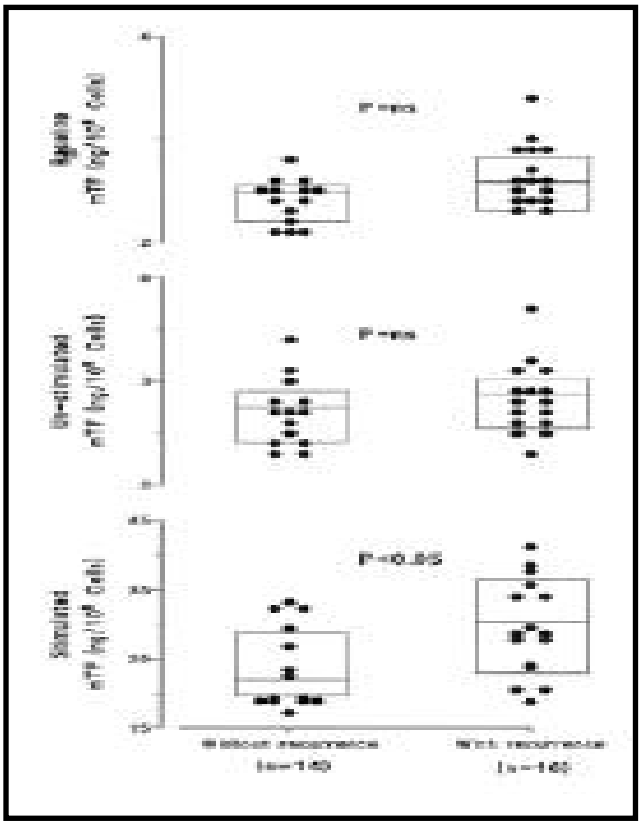

Figure 3 - mTF levels in patients with and without recurrent bladder cancer.

difference between the 2 groups was statistically significant (P<0.01; Figure 3).

Serum prostate specific antigen. Monocyte tissue factor correlated weakly with PSA in baseline, unstimulated and LPS-stimulated cells. This association was higher and statistically significant after LPS-stimulation, for the malignant groups only (r=0.5; P<0.01).

Static bone scan imaging. Patients with positive bone scans showed slightly higher mTF levels then those with negative bone scans for all mTF levels measured. However, the results were statistically significant for the LPS-stimulated levels only (P=0.05).

Discussion. Although mTF measurements are technically demanding, the results obtained remain potentially a good indicator of intravascular clotting activation and disease status. Our mTF assays demonstrated reasonable sensitivity and specificity in detecting malignant disease, with a peak performance using the LPS-stimulated cells (AUC=0.71). The ROC-curve results are usually expressed in a way that does not require the users to define a cut-off point, thus potential users do not have to determine which cut-off point should be used in which context, and at what level of efficacy. Tests with continuous values give different information associated with different cut-off points.[23] A choice of the cut-off point between a positive and negative test result will affect both sensitivity and specificity, and often in opposite directions; i.e., sensitivity can be improved only at the expense of specificity and vice versa.[24] Furthermore, assessment of the sensitivity and specificity of an assay with a given threshold will not generally allow the comparison of 2 or several assays with respect to their overall discriminating ability. All these shortcomings however, can be met by the use of the ROC-curve, which is thus a most useful analytical test for certain assays.[21]

Patients with malignant tumors of the bladder, prostate, breast and colon showed significant increases in mTF levels when compared with controls or the relevant benign non-inflammatory conditions. Similarly, patients with inflammatory conditions (cystitis, mastitis, mammary duct ectasia, ulcerative colitis and diverticulitis) showed significantly increased mTF levels when compared with the controls or the relevant benign non-inflammatory disease groups. However, for all tumors studied no significant differences were observed between the appropriate benign inflammatory and malignant disease groups.

Inflammatory conditions enhance mTF expression.[12] When the benign groups here were sub-divided into organ-specific non-inflammatory and inflammatory diseases, the former showed comparable levels with the normal controls. Patients having a hernia repair or a cholecystectomy (with a normal erythrocyte sedimentation rate) also showed a low variation in mTF levels similar to that of the normal controls. Similar results have been reported[9,15] and are supported by immunohistochemical and in situ detection studies.[10] mTF assays respond with equal sensitivity to benign inflammatory and malignant conditions. The link may be immunologically mediated,[12] or could be related to the precancerous nature of some inflammatory conditions.[25] However, there are markers of inflammation (e.g C-reactive protein, serum amyloid protein A or mannose binding protein) which could potentially combine with mTF assays to give greater cancer specificity.

Although the malignant and inflammatory conditions showed increased levels of mTF as compared with the controls, there was considerable overlap between the groups. This may have stemmed from factors such as the heterogeneity of the various groups, including the stage of evolution of the disease or treatment schedules. All patients on anticoagulants or steroids, or both were excluded from the study. However, some patients with Crohn's disease were treated with prednisolone. These were excluded from the main study, but when examined

separately few showed normal mTF levels.

A comparison of the 3 parameters of mTF activity measured (baseline, unstimulated and LPS-stimulated cells) shows that the LPS-stimulated cells has better discriminating power between controls and patient groups, consistent with the previous reports.[9,15] The fact that stimulated mTF levels gave the best clinical correlation perhaps not only reflects in vivo conditions, but possibly changes in the cellular phospholipid compositions caused by LPS in vitro.[26] Lipopolysaccharide also stimulates monocytes to secrete pharmacological mediators.[27] Many cancer patients show thrombocytosis[28] and increased platelet numbers enhance LPS-stimulated mTF activity.[15] Platelets from patients with cancer may be activated and weakly activated platelets secrete PF4 and P selectin which greatly enhances mTF activity in the presence of LPS.[29] In that respect, baseline mTF activity may be more physiologically relevant. However, both levels may be required in assessing the PCA of mTF in disease states.[30]

Previous studies on mTF activity have failed to demonstrate a relationship between tumor grade or stage and mTF levels[15] and it was suggested that elevated mTF levels are present right from the initial growth of the primary tumor.[15] While it is true that mTF levels are increased at an early stage of the disease perhaps enough attention was not paid to factors such as treatment which may influence measurements. Hence, the incidence and severity of clotting abnormalities rise as the disease progresses.[31] The present study, however, demonstrated an increase in the mTF levels associated with increasing tumor grade or stage.

An unexplained greater increase in mTF levels was observed for colorectal cancer as compared with the other tumors. Colorectal cancer is often associated with ulceration and bacterial infection which can stimulate mTF expression.[8] Inflammation is another condition that significantly enhances mTF production and bowel cancer is often accompanied by such a condition. Another important factor which may have contributed to this finding is that most colon cancer patients present at a later stage of disease progression and the tumors are physically larger, particularly in relation to breast cancer which is subject to screening.

Patients with recurrent bladder disease showed a significant increase in mTF levels compared to those with a normal check cystoscopy. Even though this increase was statistically significant there was an overlap between the 2 groups, which may be due to several factors; i) No biopsies were taken in any of those patients with a normal cystoscopy. It is possible therefore that patients with urothelial atypia (e.g., carcinoma in situ) which was not detectable during cystoscopy, were erroneously classified as normal.[32] ii) Treatment (i.e., intravesical chemotherapy) is an additional factor that may have contributed to the observed overlap. iii) The possible presence of undetected urothelial tumor higher in the urothelial tract (e.g., ureter) or missed tumor due to technical difficulty in viewing (e.g., small tumors in diverticulae or near the dome). Despite this overlap between the 2 groups, mTF levels appear to be useful in indicating disease activity in patients undergoing check cystoscopy.

Monocyte tissue factor assays showed a moderate association with PSA with better association in the malignant groups. This apparent lack of a strong correlation between mTF assays and PSA may have several origins as they measure different aspects of the disease process. Another measure, which truly reflects tumor stage, is the bone scans. This was correlated with mTF levels. Of the 3 mTF parameters measured, only LPS-stimulated activity was significantly different between the positive and negative bone scan groups. These results agree with the previous finding in that mTF activity tended to be increased with increasing tumor grade.

In conclusion, the ROC-curve displayed satisfactory sensitivity and specificity for LPS-stimulated mTF levels. Monocyte tissue factor levels are significantly raised in patients with cancer and inflammatory conditions. Lipopolysaccharide enhances mTF activity and stimulated cells can distinguish, better than baseline or unstimulated levels, patients with cancer from normal controls and benign diseases in the absence of inflammation. Thus, LPS-stimulated mTF levels may be helpful in the preliminary investigation of patients that are at risk of inflammatory disease or malignancy. Monocyte tissue factor levels were also related to tumor grade or stage and to markers of tumor progression, which suggest that mTF levels may play a role in assessing tumor progression and response to treatments.

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