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Changes in Fibrinolysis in Patients with Localized Tumors

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Abstract—*An array of fibrinolysis tests was applied to the plasmas of 125 untreated patients with breast carcinoma and malignant melanoma, localized or spread to regional lymph nodes with no detectable distant metastases, to see whether or not there may be changes related to the type or to the stage of malignancy. Breast carcinoma (a mucin secreting tumor) and melanoma (a neuroectodermal tumor) were chosen as examples of tumors that can be accurately staged for localization or spread. Forty healthy subjects matched for age served as controls. The most marked differences between malignant tumors and controls were elevated plasma levels of tissue plasminogen activator antigen* ($P < 0.005$), *plasminogen activator inhibitor* ($P < 0.01$), *cross-linked fibrin degradation products* ($P < 0.001$), *fragment Bβ 15-42* ($P < 0.001$) *and histidine-rich glycoprotein* ($P < 0.005$). *For no fibrinolysis test were results significantly different between patients with localized and spread tumors. Our data indicate that in these tumors fibrinolytic alterations are an early phenomenon unrelated to spreading.*

INTRODUCTION

FIBRINOLYSIS, caused by the activation of the plasma zymogen plasminogen to the proteolytic enzyme plasmin, plays an important role in many biological processes. The importance of fibrinolysis in the metastatic spread of solid tumors is supported by several experimental studies (for review, see [1–5]). These studies indicate that high fibrinolytic activity of the primary tumor facilitates local invasion and the release of tumor cells into the circulation [6], and that such release can be prevented by synthetic fibrinolysis inhibitors [7, 8]. When the fibrinolytic system was studied *ex vivo* in plasma from patients with tumors, the main general findings were high levels of plasminogen activator inhibitor activity [9, 10], of tissue-type plasminogen activator antigen [9] and, in some types of carcinoma, of plasma urokinase-type plasminogen activator [10, 11]. However, interpretation of the results of these studies and their translation into the design of clinical trials of antifibrinolytic drugs aimed at decreasing tumor spread are rendered difficult by the heteogeneity of the case material, because tumors of different types and stages were studied. The fibrinolytic system may be affected not only by the presence of tumor cells *per se* but also by secondary diseases occurring in advanced malignancy (such as, for instance, liver disease, venous thromboembolism or disseminated intravascular coagulation), by chemotherapy and by surgical treatment. With this as background, an array of fibrinolysis tests was applied to plasmas from patients with only two types of untreated tumors (breast carcinoma or malignant melanoma) in their early stages (localized or spread to regional lymph nodes only) to see if there are changes of fibrinolysis related to the type or stage of malignancy.

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MATERIALS AND METHODS

Patients

One hundred and twenty-five untreated patients were selected from those referred to the Surgical Division B of the National Cancer Institute of Milan between 1985 and 1987 on the basis of tumor type: breast carcinoma or malignant melanoma. Breast carcinoma was diagnosed by mammography and fine needle aspiration cytology and sometimes by needle biopsy. Malignant melanoma was diagnosed by biopsy of cutaneous lesions. Patients who entered the study had had no surgical or medical treatment and had local tumors or tumors spread only to

regional lymph nodes. Tumor spread was determined by physical examination and appropriate laboratory and radiological tests. A local tumor was defined as a single tumor mass with no detectable regional or distant metastases. A tumor was 'spread' when there was regional lymph node metastasis in pathological specimens of regional lymph nodes obtained during surgical removal of the tumor. Liver metastases were excluded by CT scan or ultrasound tomography, pulmonary metastases by chest films and CT scans, brain metastases by CT scan and bone metastases by X-ray survey and technetium scintiscan. Patients who had clinical or laboratory signs of liver or renal disease, arterial or venous thrombosis, decompensated intravascular coagulation or were taking oral anticoagulants, oral contraceptives or antiplatelet drugs were also excluded from the study.

Thirty women with localized breast carcinoma (median age 59 years, range 26–80 years), 32 women with regionally spread breast carcinoma (median age 53 years, range 36–85 years), 30 patients with localized malignant melanoma (20 women and 10 men, median age 59 years, range 32–86 years) and 33 patients with regionally spread malignant melanoma (17 women and 16 men, median age 57 years, range 32–86 years) were selected. Finally, 40 healthy subjects (23 women and 17 men, median age 51 years, range 22–86 years) were the controls. The differences in median ages between the five groups of patients and controls were not statistically significant (Mann–Whitney U test).

Samples

Blood samples were collected in the morning before 10.00, after an overnight fast, and the patients and controls had rested for 15 min. After discarding the first 2 ml of blood, the next 9 ml, to be used for the assay of the fibrinogen fragment Bβ 15-42, was drawn into a precooled syringe containing 1 ml of 0.15 M NaCl, 1000 IU heparin and 1000 KIU aprotinin. Then, 9 ml of whole blood were collected into a plastic tube containing 1 ml of 0.129 M trisodium citrate. Platelet-poor plasma was obtained by centrifuging the blood within 30 min at 4°C and 2000 ***g*** for 30 min. Aliquots (0.05 ml) of plasma were snap frozen in plastic tubes in a mixture of solid carbon dioxide and methanol and stored at −80°C until use.

Laboratory tests

Fibrinolytic activity in the normal euglobulin fraction of plasma was measured by the fibrin plate method [12]. Purified C1-inhibitor (C1-INH, Immuno, Vienna, Austria) was added to a portion of the euglobulin fraction to inhibit intrinsic fibrinolysis activator and to provide a more specific measurement of tissue plasminogen activator activity [13]. *Tissue-plasminogen activator antigen* was measured with a commercial enzyme linked immunosorbent assay (American Diagnostica, New York, NY). *Urokinase-type plasminogen activator*, contained in plasma as well as in urine, was determined in plasma by an immunosorbent activity assay measuring both activable single-chain urokinase and two-chain urokinase (Chromolise U-PA, Biopool AB, Umea, Sweden). *Plasminogen activator inhibitor activity* was measured by the titration method of Verheijen *et al.* [14] with a chromogenic substrate. *Plasminogen*, the zymogen of the fibrinolytic system, was measured by the streptokinase activation procedure with a chromogenic substrate [15]. *Histidine-rich glycoprotein (HRG)*, a protein that binds to plasminogen decreasing its binding to fibrin, was measured by the electroimmunoassay of Laurell, using a specific antiserum [16]. *Free plasminogen* was calculated from the equilibrium between plasminogen and histidine-rich glycoprotein [17]. *Alpha 2 antiplasmin*, the main fast-acting inhibitor of the activity of plasmin in the circulation, was measured with an indirect spectrophotometric assay [18] *Fibrin(ogen) degradation products* were measured in serum by a commercial semi-quantitative method with latex (Thrombo-Wellcotest, Dartford, U.K.). *Cross-linked fibrin degradation products (XDP)*, which derive from fibrin stabilized by factor XIIIa, were measured in plasma with a commercial enzyme-linked immunosorbent assay (American Diagnostica, New York, NY). The *fibrinogen fragment* Bβ 15-42, a product of the action of plasmin on fibrin II, fibrin I and fibrinogen, was measured in plasma by radioimmunoassay, using a commercial kit (IMCO, Stockholm, Sweden).

Statistical tests

The results were expressed as medians and ranges. Kruskal–Wallis analysis of variance was performed and the significance of differences between groups was assessed by the non-parametric Mann–Whitney test. Since multiple comparisons provide weaker statistical tests, $P < 0.01$ was accepted as the minimum level for statistical significance.

RESULTS

Fibrinolysis tests for all tumor patients (Table 1)

When normal controls were compared with all tumor patients, whatever the tumor type and spread, plasminogen activator inhibitor, tissue-plasminogen activator antigen, cross-linked fibrin degradation products, fragment Bβ 15-42 and histidine-rich glycoprotein were all significantly higher in tumor patients than in controls (P values <0.01, <0.005, <0.001, <0.001 and <0.005). None of

Table 1. *Fibrinolytic tests related to tumor type*

Fibrinolytic tests	Normal controls $n = 40$		All tumors $n = 125$	Breast $n = 62$		Melanoma $n = 63$
Plasminogen activator inhibitor (IU/ml)	6.0 (0.6–11.4)	$P < 0.01$	7.5 (0.6–48.8)	7.0 (0.6–48.8)		7.6 2.0–25.0)
Tissue plasminogen activator antigen (ng/ml)	6.8 (2.5–14.2)	$P < 0.005$	8.8 (3.7–23.9)	8.0 (4.3–18.1)		9.7 (3.7–23.9)
Urokinase plasminogen activator activity (ng/ml)	0.41 (0.17–0.68)		0.40 (0.16–0.75)	0.40 (0.16–0.70)		0.41 (0.16–0.75)
Fibrin(ogen) degradation products (μg/ml)	4.0 (4.0–16.0)		4.0 (4.0–32.0)	4.0 (4.0–32.0)		4.0 (4.0–32.0)
Cross-linked fibrin degradation products (ng/ml)	60 (28–150)	$P < 0.001$	90 (50–999)	98 (52–800)		85 (50–999)
Fibrinogen fragment Bβ 15-42 (pm/ml)	3.2 (1.3–4.2)	$P < 0.001$	5.3 (3.2–31.6)	5.5 (4.1–14.5)		5.0 (3.2–31.6)
Fibrin plates (mm^2)						
Normal euglobulin fraction	171 (61–462)		165 (55–670)	172 (61–670)		155 (55–386)
Normal euglobulin fraction +C1-INH	46 (16–159)		62 (21–271)	69 (24–271)		56 (21–192)
Plasminogen activity (%)	96 (76–111)		96 (61–130)	95 (61–130)		98 (64–129)
Histidine-rich glycoprotein (%)	85 (65–120)	$P < 0.005$	100 (54–200)	106 (54–190)	$P < 0.005$	90 (54–200)
Free plasminogen (%)	49 (38–64)		46 (20–77)	42 (20–67)		50 (21–77)
Alpha 2 antiplasmin (%)	92 (70–112)		94 (57–115)	93 (63–115)		94 (57–115)

Values are expressed as median and (range).
% = percentage of normal human pooled plasma.

the remaining fibrinolysis tests gave significantly different results.

Fibrinolysis tests and tumor type (*Table* 1)

When all patients with breast carcinoma were compared with those with melanoma whatever the tumor stage, the only significant difference in any fibrinolysis test was for histidine-rich glycoprotein (higher in breast carcinoma, $P < 0.005$).

Fibrinolysis tests and tumor spread (*Table* 2)

When all patients with local tumors were compared with those with spread tumors whatever the tumor type, no fibrinolysis test gave significantly different results. There were also no differences between patients with local breast carcinoma or melanoma when compared with patients with the corresponding spread tumor.

DISCUSSION

This study demonstrates that, notwithstanding a high degree of overlap with normal controls, fibrinolysis was altered in patients with solid tumors, whatever the type and the stage of tumor. There was a modest enhancement of fibrinolysis, which was revealed mainly by such sensitive indexes as the presence of high levels of cross-linked fibrin degradation products and of the fibrinogen fragment Bβ 15-42. The latter is a specific index of the action of circulating plasmin (the proteolytic enzyme resulting from the activation of fibrinolysis) on early fibrin, and the former is an index of the action of plasmin on stabilized fibrin. Acute phase reactions to tissue injury and neoplastic growth are the most plausible causes of the high levels of plasminogen activator inhibitor [19]. The parallel increases in plasminogen activator inhibitor and of tissue-plasminogen activator antigen, both produced in endothelial cells, found in this study are consistent with previous studies that reported concordant changes of the two measurements in tumors [19–21]. Perhaps they have a common regulatory mechanism. Other, less sensitive, more global tests of fibrinolytic activity that give an overall indication of the balance between plasminogen activation and inhibition were substantially unchanged. Perhaps plasminogen activator activity, as measured by the fibrin plate assays with or without C1-INH, was hindered by the high plasma levels of plasminogen activator inhibitor activity.

Table 2. Fibrinolytic tests related to tumor spread

Fibrinolytic tests	All tumors		Breast		Melanoma	
	Local $n = 60$	Spread $n = 65$	Local $n = 30$	Spread $n = 32$	Local $n = 30$	Spread $n = 33$
Plasminogen activator inhibitor activity (IU/ml)	7.2 (0.8–48.8)	8.3 (0.6–21.5)	7.0 (0.8–48.8)	7.0 (0.6–20.0)	7.3 (2.0–25.0)	9.5 (3.5–21.5)
Tissue plasminogen activator antigen (ng/ml)	8.2 (3.7–19.6)	9.3 (3.7–23.9)	8.4 (4.3–17.9)	7.7 (4.3–18.1)	8.1 (3.7–19.6)	11.0 (3.7–23.9)
Urokinase plasminogen activator activity (ng/ml)	0.41 (0.16–0.75)	0.39 (0.16–0.70)	0.41 (0.28–0.68)	0.38 (0.16–0.70)	0.41 (0.16–0.75)	0.40 (0.20–0.67)
Fibrin(ogen) degradation products (μg/ml)	4.0 (4.0–32.0)	4.0 (4.0–32.0)	4.0 (4.0–16.0)	4.0 (4.0–32.0)	4.0 (4.0–32.0)	4.0 (4.0–32.0)
Cross-linked fibrin degradation products (ng/ml)	95 (52–800)	90 (50–999)	100 (53–800)	91 (52–550)	85 (52–300)	85 (50–999)
Fibrogen fragment Bβ 15-42 (pm/ml)	5.4 (3.4–31.6)	5.2 (3.2–14.5)	5.5 (4.1–14.5)	5.5 (4.2–14.5)	5.1 (3.4–31.6)	4.8 (3.2–9.4)
Fibrin plates (mm^2)						
Normal euglobulin fraction	156 (61–367)	167 (55–670)	153 (72–376)	184 (61–670)	179 (61–348)	153 (55–386)
Normal euglobulin fraction +C1-INH	62 (26–174)	64 (21–271)	61 (26–174)	99 (24–271)	65 (27–160)	46 (21–192)
Plasminogen activity (%)	95 (61–130)	97 (64–129)	94 (61–130)	96 (72–117)	98 (68–119)	99 (64–129)
Histidine-rich glycoprotein (%)	103 (54–168)	98 (54–200)	104 (54–168)	108 (64–190)	99 (62–164)	84 (54–200)
Free plasminogen (%)	44 (21–64)	48 (20–77)	43 (32–62)	42 (20–67)	46 (21–64)	53 (25–77)
Alpha 2 antiplasmin (%)	95 (74–115)	93 (57–112)	95 (74–115)	93 (63–110)	95 (80–115)	94 (57–112)

Values are expressed as median and (range).
% = percentage of normal human pooled plasma.

The main goal of this study was not to establish which fibrinolysis test is altered in solid malignancies and to what degree, because in this respect our results substantially confirm previous findings. Its specific character is the choice of studying only patients with two well-defined types of untreated solid tumors with well-defined stages of spread. Breast carcinoma and malignant melanoma were chosen from the types of tumors seen at the National Cancer Institute because they have different tissue origins (neuroectodermic for melanoma and mucin-secreting for breast carcinoma) and, more important for our purposes, localization and spread can be precisely staged. Only patients with local tumor or tumor spread to regional lymph nodes were included in an attempt to ascertain to what extent alterations of fibrinolysis are related to the presence of the tumor *per se* or are secondary to changes in general homeostasis accompanying widespread malignancy. Since the liver, for instance, plays a central role in the regulation of the fibrinolytic system, alterations in patients with liver metastases and liver damage would be difficult to interpret. Our findings of hyperfibrinolysis in not only patients with tumors spread to regional lymph nodes but also in patients with local tumors indicate that the enhancement of fibrinolysis in early tumor stages is probably related to the tumor cells *per se* rather than to secondary organ involvement. Very recently, McCulloch *et al.* [22] independently found, like us, that women with breast cancer had elevated levels of Bβ 15-42 whatever the stage of their malignancy, whereas other tests that measure global fibrinolytic activity gave normal results.

With this study of the fibrinolytic system, we have completed a series of studies of the behavior of the three main sub-systems of hemostasis (coagulation, platelets and fibrinolysis) in untreated patients with two well staged forms of tumors. The studies gave consistent results, indicating that alterations of coagulation, platelets and fibrinolysis develop early, being already present in patients with local tumors and more marked in those with spread tumors ([23, 24], this study). These results indicate that tumors affect hemostasis even when the mass of neoplastic cells is small, there is little or no spread and there is no evidence of generalized diseases, such as thromboembolism, liver disease or disseminated intravascular coagulation. These findings might have general implication because of the purported

role of the hemostatic system in facilitating tumor growth and dissemination [1–5]. Perhaps, in the design of controlled clinical trials of drugs affecting the hemostatic system, the drugs to be investigated to see whether or not they modify tumor progression and spread should be chosen on the basis of the results of laboratory tests of the hemostatic system.

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REFERENCES

1. Bick RL. Alterations of hemostasis associated with malignancy: etiology, pathophysiology, diagnosis and management. *Semin Thromb Hemostas* 1978, **5**, 1–26.
2. Donati MB, Poggi A, Semeraro N. Coagulation and malignancy. In: Poller L, ed. *Recent Advances in Blood Coagulation*. Edinburgh, Churchilll Livingstone, 1981, Vol. 3, 227–254.
3. Rickles FR, Edwards RL. Activation of blood coagulation in cancer; Trousseau's syndrome revisited. *Blood* 1983, **62**, 14–31.
4. Markus G. The role of hemostasis and fibrinolysis in the metastatic spread of cancer. *Semin Thromb Hemostas* 1984, **10**, 61–70.
5. Dano K, Andreasen PA, Grondahl-Hansen J, Kinstensen P, Nielsen LS, Shriver L. Plasminogen activators, tissue degradation and cancer. *Adv Cancer Res* 1985, **44**, 139–255.
6. Peterson HI, Kjartansson I, Korsan-Bengtsen K, Rudenstam CM, Zettergren L. Fibrinolysis in human malignant tumours. *Acta Chir Scand* 1973, **139**, 219–223.
7. Tanaka N, Ogawa H, Tanaka K, Kinjo M, Kohga S. Effects of tranexamic acid and urokinase on hematogenous metastases of Lewis lung carcinoma in mice. *Invasion Metast* 1981, **1**, 149–157.
8. Tanaka N, Ogawa H, Kinjo M, Kohga S, Tanaka K. Ultrastructural study of effects of tranexamic acid and urokinase on metastasis of Lewis lung carcinoma. *Br J Cancer* 1982, **46**, 428–435.
9. de Jong E, Knot EAR, Piket D *et al.* Increased plasminogen activator inhibition levels in malignancy. *Thromb Haemostas* 1987, **57**, 140–143.
10. Kirchheimer JC, Huber K, Wagner O, Binder BR. Pattern of fibrinolytic parameters in patients with gastrointestinal carcinomas. *Br J Haematol* 1987, **66**, 85–89.
11. Grondahl-Hansen J, Agerlin N, Munkholm-Larsen P *et al.* Sensitive and specific enzyme-linked immunosorbent assay for urokinase type plasminogen activator: its application to plasma from patients with breast cancer. *J Lab Clin Med* 1988, **111**, 42–51.
12. Haverkate F, Brakman P. Fibrin plate assay. In: Davidson JF, Samama MM, Desnoyers PC, eds. *Progress in Chemical Fibrinolysis and Thrombolysis*. New York, Raven Press, 1975, 151–159.
13. Kluft C. C1-inactivator-resistant fibrinolytic activity in plasma euglobulin fractions: its relation to vascular activator in blood and its role in euglobulin fibrinolysis. *Thromb Haemostas* 1978, **41**, 365–383.
14. Verheijen JH, Chang GTG, Kluft C. Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. *Thromb Haemostas* 1984, **51**, 392–395.
15. Friberger P, Knos M. Plasminogen determination in human plasma. In: Scully M, Kakkar VV, eds. *Chromogenic Peptide Substrate*. Edinburgh, Churchill Livingstone, 1979, 128–139.
16. Lijnen HR, Jacobs G, Collen D. Histidine rich glycoprotein in a normal and a clinical population. *Thromb Res* 1981, *22*, 519–523.
17. Kluft C, Preston FE, Malia RG *et al.* Stanozolol-induced changes in fibrinolysis and coagulation in healthy adults. *Thromb Haemostas* 1984, **51**, 157–164.
18. Teger-Nilsson AC, Friberger P, Gyzander E. Determination of a new rapid plasmin inhibitor in human blood by means of a plasmin specific tripeptide substrate. *Scand J Lab Invest* 1977, **37**, 403–409.
19. Kluft C, Verheijen JH, Jie AFH *et al.* The postoperative fibrinolytic shutdown: a rapidly reverting acute phase pattern for the fast-acting inhibitor of tissue-type plasminogen activator after trauma. *Scand J Clin Lab Invest* 1985, **45**, 605–610.
20. Kudryk B, Robinson D, Netré C, Hessel B, Blomback M, Blomback B. Measurement in human blood of fibrinogen/fibrin fragments containing the Bβ 15-42 sequence. *Thromb Res* 1982, **25**, 277–291.
21. Kruithof EKO, Gudinchet A, Bachmann F. Plasminogen activator inhibitor 1 and plasminogen activator inhibitor 2 in various disease states. *Thromb Haemostas* 1988, **59**, 7.
22. McCulloch P, Douglas J, Lowe GDD, Murray G, George WD. *In vivo* measurements of fibrin formation and fibrinolysis in operable breast cancer. *Thromb Haemostas* 1989, **61**, 318–321.
23. Mannucci PM, Vaglini M, Maniezzo M, Magni E, Mari D, Cascinelli N. Hemostatic alterations are unrelated to the stage of tumor in untreated malignant melanoma and breast carcinoma. *Eur J Cancer Clin Oncol* 1985, **21**, 681–685.
24. Mannucci PM, Cattaneo M, Canciani MT, Maniezzo M, Vaglini M, Cascinelli N. Early presence of activated ('exhausted') platelets in malignant tumors (breast adenocarcinoma and malignant melanoma). *Eur J Cancer Clin Oncol* 1989, **25**, 1413–1417.