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Early Presence of Activated ('Exhausted') Platelets in Malignant Tumors (Breast Adenocarcinoma and Malignant Melanoma)

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Abstract—*To evaluate whether or not the finding of platelet activation in patients with tumors is related to the stage of malignancy, a study of biochemical markers indicative of the presence of circulating activated ('exhausted') platelets was carried out in* 95 *untreated patients with breast adenocarcinoma or malignant melanoma, localized or spread to regional lymph nodes with no detectable distant metastasis. These tumors were chosen as examples of tumors which can be accurately staged for localization or spread, and as examples of mucin-secreting tumors (breast adenocarcinoma) or neuroectodermic tumors (malignant melanoma). Results were compared with those for* 26 *patients with benign breast disease,* 23 *blood donors and* 50 *hospital workers. The most frequent abnormalities were low levels of intraplatelet ADP and* 5*-hydroxytryptamine and high ATP/ADP ratios. Although these abnormalities occurred with both types of tumor, they were more frequent and marked for melanomas and breast carcinomas spread to regional lymph nodes. Our data indicate that the presence of exhausted platelets is an early finding in patients with malignant tumors.*

INTRODUCTION

IN THE LAST 10 years there has been increasing interest in studies of platelet behavior in patients with malignant tumors. This interest was triggered by several *in vitro* and animal studies indicating that certain tumor lines activate and aggregate platelets, that activated platelets favor the growth and spread of certain tumors, and that these processes might be controlled or interrupted by antiplatelet drugs (for reviews, see [1–6]). Recently, the biochemical defects that characterize increased platelet activation have been defined more precisely. Platelets from a number of patients with malignant tumors contain low levels of substances normally stored in the platelet dense (delta) granules [7]. It is thought that these substances are released from dense granules into plasma when platelets are activated *in vivo*, so that platelets are depleted totally or partially of them and circulate 'exhausted'. In turn, substances released from activated platelets might favor tumor growth and spread because they increase vasodilation and vascular permeability, which would facilitate tumor cell dissemination and extravasation [8,9].

There are, however, a number of unresolved problems about the interpretation of these findings, which make it difficult to understand their significance and role in the process of tumor growth and spread, and to translate them into the design of therapeutic trials of antiplatelet agents. For instance, exhausted platelets were demonstrated in unselected series of patients with a variety of tumor types [7], so that it is not known whether or not this pattern of platelet behavior varies with the tumor type. Most importantly, patients with early and widespread malignancy were mixed [7], so that it is not clear to what extent exhausted platelets are due to the direct effects of tumor cells or to secondary diseases occurring in advanced malignancy, such as, for instance, liver or renal disease.

This study was planned to address these issues by measuring biochemical markers indicative of the presence of exhausted platelets in a series of newly diagnosed and untreated patients with two types of tumors (breast adenocarcinoma or malignant melanoma) in their early stages (localized or spread to regional lymph nodes only). We chose to measure the intraplatelet levels of ADP and 5-hydroxytrypta-

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mine (5HT), markers of substances contained in the dense granules; the ATP/ADP ratio, which is high when platelets circulate depleted in their dense granules; and the intraplatelet levels of two substances specifically contained in alpha granules, beta-thromboglobulin and platelet factor 4.

MATERIALS AND METHODS

Patients

Patients were selected from those consecutively referred to the Surgical Division B of the National Cancer Institute of Milan between 1985 and 1987. The first selection was on the basis of tumor type: breast adenocarcinoma or malignant melanoma. For patients with breast carcinoma, the diagnosis was made by mammography and fine needle aspiration cytology with or without needle biopsy. For patients with malignant melanoma, the diagnosis was made by biopsy of cutaneous lesions. Patients were entered in the study providing they were surgically and medically untreated and had local tumor or tumor spread to regional lymph nodes only. Local tumor was defined as a single tumor mass with no detectable regional or distant metastasis. Regionally spread tumor was defined by the presence of regional lymph node metastases but no distant macroscopic metastasis. The presence or absence of lymph node metastases was determined microscopically in pathological specimens of regional lymph nodes obtained during surgical removal of the tumor. The presence of lung metastases was excluded by negative chest films and CT scans; that of brain metastases by CT scans; of liver metastases by liver ultrasound scans; of bone metastases by skeletal X-ray survey and isotope bone scans. Patients with clinical or laboratory signs indicative of liver or renal disease, with a clinical history or symptoms indicative of past or ongoing arterial or venous thrombosis, with decompensated intravascular coagulation and consumption coagulopathy and those taking oral anticoagulants or any drug affecting platelet behavior were also excluded from the study. Using these criteria, we selected a total of 95 patients: 22 with local and 26 with regionally spread breast adenocarcinoma, 24 with local and 23 with regionally spread malignant melanoma.

Controls

Three control populations were studied in parallel. The first included 23 healthy blood donors at the Blood Bank of the National Cancer Institute; the second, 26 patients with confirmatory bioptic diagnosis of benign breast disease; the third, 50 age- and sex-matched hospital workers whose laboratory values were used to generate the reference intervals (±2 standard deviations about the means).

Laboratory tests

Forty ml of venous blood were drawn into a plastic syringe from an anterior cubital vein and added to one-tenth volume of trisodium citrate (0.109 M) in plastic tubes. Platelet-rich plasma (PRP) was prepared by centrifugation at 200 ***g*** at room temperature for 15 min, platelet-poor plasma by centrifugation at 2000 ***g*** for 15 min.

Platelet adenine nucleotides. Platelet ADP and ATP were measured by a firefly-luciferase method [10]. The firefly-luciferase reagent was obtained from LKB-Wallace (Turku, Finland). Results were expressed as nmoles/10^8 platelets.

Platelet serotonin. Platelet 5HT was measured using the fluorometric method of Drummond and Gordon [11] and the results were expressed as nmoles/10^8 platelets.

Platelet beta-thromboglobulin (BTG). After PRP preparation and platelet counting, platelets were lyzed by adding 50 µl of 20% v/v Triton X-100 to 1 ml PRP. Platelet BTG content was measured by radioimmunoassay (Amersham, U.K.) and the results were expressed as ng/10^6 platelets.

Platelet factor 4 (PF4). After preparation of samples as described for BTG, platelet PF4 content was measured by radioimmunoassay (Abbott Laboratories, U.S.A.).

Statistical analysis. The measurements were not normally distributed, so we expressed results as medians and range. For each laboratory measurement, the significance of differences between groups was tested by one-way non-parametric analysis of variance according to Kruskal–Wallis. The Mann–Whitney *U* test was used to compare the pairs of groups and to define where the differences of medians between groups lay. The Fisher exact test was used to test the significance of differences for the between-group prevalences of storage-pool deficiency.

RESULTS

Table 1 shows the median values and ranges for platelet count and intraplatelet 5HT, ADP, ATP, ATP/ADP ratio, BTG and PF4 for the three control groups and the four tumor groups. By analysis of variance, significant between-group differences existed only for dense granule markers, i.e. 5HT, ADP and the ATP/ADP ratio. In average, patients with regionally spread malignant melanoma had significantly lower levels of ADP and 5HT and elevations of the ATP/ADP ratios as compared to all the control groups (laboratory workers, hospital blood donors and patients with benign breast

Table 1. Platelet biochemical markers related to tumor type and spread

	Platelets ($\times 10^{-9}$/l)	5HT (nmol/10^8 platelets)	ADP (nmol/10^8 platelets)	ATP (nmol/10^8 platelets)	ATP/ADP ratio	BTG (ng/10^6 platelets)	PF4 (ng/10^6 platelets)
Blood donors (n = 23)	249 (160–348)	0.28 (0.20–0.54)	2.6 (1.5–3.4)	5.0 (2.9–6.9)	1.96 (1.6–2.8)	66 (28–152)	22 (6–63)
Benign breast disease (n = 26)	212 (87–282)	0.35 (0.07–0.63)	2.1 (0.5–4.3)	4.8 (3.4–7.8)	2.35 (1.8–4.5)	76 (35–213)	36 (20–67)
Local breast carcinoma (n = 22)	247 (110–287)	0.30 (0.09–0.57)	2.4 (1.1–4.7)	5.2 (3.3–7.8)	2.1 (1.5–3.2)	99 (12–282)	27 (7–90)
Regionally spread breast carcinoma (n = 26)	225 (173–307)	0.27 (0.09–0.49)	1.9 (1.1–4.2)	4.4 (2.9–7.3)	2.2 (1.6–5.7)	79 (20–172)	23 (6–67)
Local malignant melanoma (n = 24)	200 (136–834)	0.26 (0.05–0.72)	2.1 (1.0–4.0)	4.8 (2.9–8.2)	2.2 (1.5–3.8)	71 (5–192)	31 (8–68)
Regionally spread malignant melanoma (n = 23)	226 (79–330)	0.23* (0.07–0.43)	1.7* (0.8–3.6)	4.2 (2.5–7.8)	2.6* (1.5–6.5)	88 (12–276)	18 (5–40)
Laboratory workers (n = 50)	280 (156–358)	0.31 (0.21–0.54)	2.8 (1.7–4.3)	5.6 (3.8–7.2)	2.04 (1.4–2.72)	56 (32–171)	18 (5–50)

*$P < 0.01$ vs. all control and patient subgroups. Values indicate medians, with ranges in parentheses.

tumors). No statistically significant difference could be found in multiple comparisons of tumor and control groups for BTG and PF4, platelet proteins contained in alpha granules only. The values of 5HT, ADP and ATP/ADP ratio obtained in patients and controls are shown in Figs. 1–3, which indicates that despite the statistical significance of differences there was considerable overlap of values between groups.

Since low levels of 5HT, ADP and high ATP/ADP ratios identify a state of delta storage pool deficiency, we evaluated the prevalence of this abnormality (defined as the concomitant presence in platelets of increased ATP/ADP ratio *and* of ADP *and* 5HT levels below the lower limit of the reference intervals established with the group of hospital workers) in the groups studied. There was no increase of delta storage pool deficiency in blood donors and low prevalences, which were not significantly different between each other, in patients with benign breast disease and with local breast adenocarcinoma or malignant melanoma (7%, 9% and 4%, Table 2 and Figs. 1–3). In regionally spread breast adenocarcinoma there was a higher prevalence of storage pool deficiency (19%) than in blood donors ($P < 0.05$). The prevalence was not significantly different from that found in benign breast disease and in either local tumor. In patients with regionally spread malignant melanoma the prevalence of storage pool deficiency was higher (35%) than in patients with local malignant melanoma (4%, $P < 0.01$) and in patients with regionally spread breast adenocarcinoma (19%). The latter difference, however, was not statistically significant.

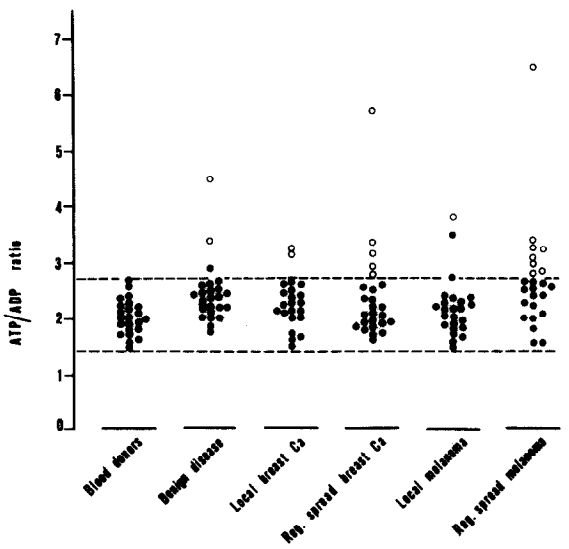

Fig. 1. ATP/ADP ratio in platelets of blood donors and of patients with benign breast disease, local or regionally spread breast carcinoma (Ca), local or regionally (reg.) spread malignant melanoma. The area between the horizontal broken lines is the normal range (from 50 normal hospital workers). Open symbols denote patients with acquired storage pool deficiency, i.e. high ATP/ADP ratio and low intraplatelet ADP and 5HT. Closed symbols denote all the remaining patients.

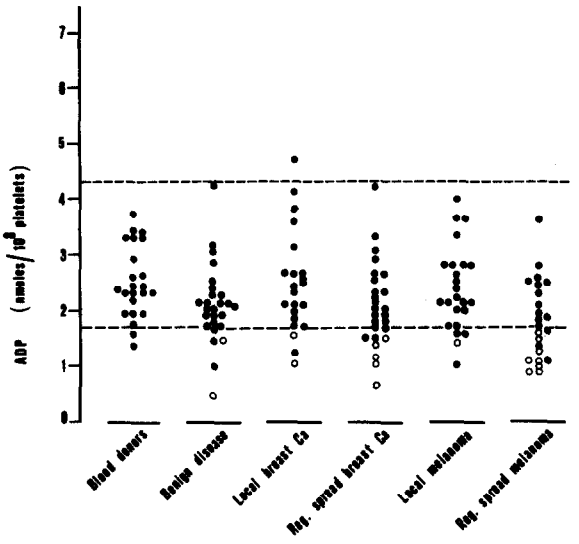

Fig. 2. Intraplatelet ADP concentration (as for Fig. 1).

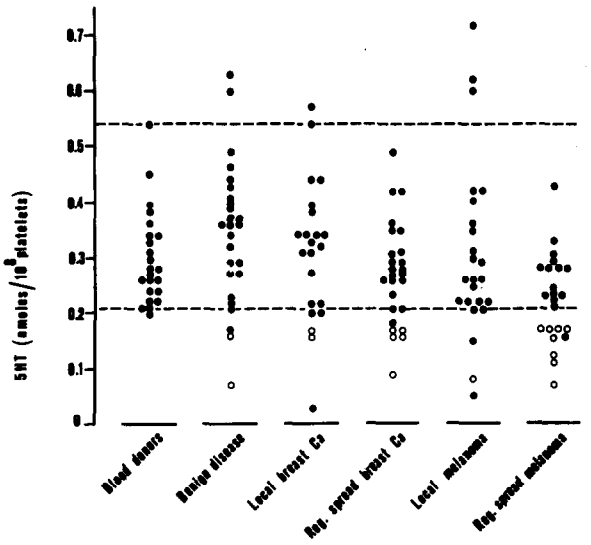

Fig. 3. Intraplatelet 5HT concentration (as for Fig. 1).

Table 2. Prevalence of acquired storage pool deficiency (SPD)

	Groups	*n* with SPD	% with SPD
1.	Blood donors ($n = 23$)	0	0%
2.	Benign breast disease ($n = 26$)	2	7%
3.	Local breast carcinoma ($n = 22$)	2	9%
4.	Regionally spread breast carcinoma ($n = 26$)	5	19%
5.	Local malignant melanoma ($n = 24$)	1	4%
6.	Regionally spread malignant melanoma ($n = 23$)	8	35%

Statistically significant differences between prevalences for different groups (Fisher exact test): 6 vs. 1: $P < 0.001$; 6 vs. 2: $P < 0.005$; 6 vs. 3: $P < 0.05$; 6 vs. 5: $P < 0.01$; 4 vs. 1: $P < 0.05$.

DISCUSSION

This study was planned to clarify the pathogenic and clinical significance of the findings of activated platelets in patients with tumors. At least two features of the design of this study are quite new. First, we chose to study highly selected patients with only two types of untreated malignant tumor, because we thought that the interpretation of previous studies was confounded by the heterogeneous composition of the case material in terms of tumor type and therapy. We chose these tumors because they can be accurately staged for localization and spread. We also chose breast adenocarcinoma as an example of mucin-secreting tumors (which are characterized by a particularly high prevalence of hemostatic abnormalities) [12–14], and malignant melanoma as an example of neuroectodermic tumors. The second peculiar feature of this study was the inclusion of only patients with early untreated cancer, either localized or spread only to regional lymph nodes. Patients with more advanced, widespread cancer were excluded because they may have severe thrombocytopenia, which makes it difficult to study platelet function; decompensated intravascular coagulation with consumption coagulopathy, during which platelet function may be altered by the presence of high circulating levels of proteolytic enzymes such as thrombin and plasmin [12, 13]; or other secondary diseases, such as renal and liver insufficiency, which affect platelet behavior *per se* [14].

A previous study [7] has shown that the biochemical platelet abnormalities observed in heterogeneous series of patients with tumors of different stages and types resemble those of patients with congenital or acquired delta storage-pool deficiency [15–17]. They are characterized by low platelet levels of delta granule constituents such as 5HT and ADP, high ATP/ADP ratios (a high ATP/ADP ratio is indicative of delta storage pool deficiency) [16, 17] and by normal levels of substances (such as beta-thromboglobulin and platelet factor 4) contained in platelet alpha granules. Our findings confirm and extend these observations. Storage pool deficiency is a rare event in patients with benign tumors and in those with localized malignant tumors, and is more frequent in regionally spread malignant tumors, suggesting that the processes that induce the platelet release reaction and lead to the circu-

lation of exhausted platelets are proportional to the tumoral mass. Moreover, we found that the biochemical markers indicative of the presence of storage pool deficiency were more frequently and markedly altered in malignant melanoma than in breast adenocarcinoma of comparable extension. Although we do not know the reason for the differences between the two tumors, these findings are consistent with our previous observations that abnormalities of coagulation tests are more severe in malignant melanoma than in breast adenocarcinoma [18]. They also emphasize again the need to study patients with well-selected and well-defined types of tumors instead of mixing all patients together whatever the tumor type.

The conclusion that stems from this study is that the biochemical abnormalities indicative of the presence of circulating exhausted platelets are present even in patients with early tumors, spread only to regional lymph nodes. Since these patients have only mild signs of low-grade activation of the coagulation system with no sign of decompensated intravascular coagulation (consumption coagulopathy) [18, 19] nor of renal or hepatic dysfunction, the abnormalities are more likely to be due to the presence of tumor cells *per se* rather than to the contributory intervention of secondary disease, although a role for thrombin forming *in vivo* cannot be ruled out. Whether the early use of antiplatelet agents aimed at inhibiting the release reaction and preventing the formation of exhausted platelets might be useful adjuvant in the management of these patients remains to be established by clinical trials.

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