Cancer Procoagulant in Human Tumor Cells: Evidence from Melanoma Patients

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ABSTRACT

It has repeatedly been proposed that fibrin plays a role in tumor growth and metastasis. Among tumor cell products or activities which may promote clot formation, cancer procoagulant (CP), a direct activator of coagulation factor X, has been suggested to be selectively associated with the malignant phenotype.

We report here the enzymatic and immunological identification of this cysteine proteinase procoagulant in extracts and cells from human melanoma. CP activity was independent of both the intrinsic and extrinsic pathways of blood coagulation, using factor IX and factor VII deficient plasmas, and was inhibited by the cysteine proteinase inhibitors iodoacetamide and HgCl2. CP activity was detectable in extracts and cell suspensions from all 32 patients studied and was higher in extracts from metastases (14.8 ± 3.9 units/mg protein) than from the primary tumors (3.7 ± 1.0 units/mg protein). CP activity was not affected by an anti-apoptotein III antibody or by concanavalin A, a known inhibitor of thromboplastin.

In contrast, no CP activity or antigen was detected in extracts from six benign melanocytic lesions. The procoagulant activity was dependent on factor VII and was inhibited by anti-apoptotein III antibody and by concanavalin A, properties that suggest that the procoagulant was tissue thromboplastin.

These data indicate that CP can be expressed by human tumor cells and that, among melanotic lesions, its presence is associated with the malignant phenotype and its activity is particularly high in metastatic cells.

INTRODUCTION

Fibrin formation within the tumor microenvironment has been suggested to play a role in tumor growth and the development of metastasis (1-3). Cancer cells may contribute to clot formation through availability of their procoagulant activities (1-5). Several types of such activities have been described, the most important of which are: (a) tissue factor (TF) or tissue thromboplastin, a phospholipid-protein complex that activates factor X by a direct mechanism; (b) CP, a cysteine proteinase that directly activates factor X, bypassing both the extrinsic and intrinsic pathways of the coagulation cascade. While tissue factor is produced by normal cells, cancer procoagulant has thus far been described only in neoplastic and fetal cells and may be a "marker" for malignancy (6, 7). CP was first characterized in extracts of animal and human tumors (7, 8). In rabbit V2 carcinoma, CP is a Mr = 68,000 protein with an isoelectric point of 4.9 (9, 10). Enzymatically it behaves like a cysteine proteinase; it is sensitive to cysteine proteinase inhibitors (i.e., iodoacetamide and mercury) and activators (i.e., dithiothreitol, KCN, EDTA) and it binds to p-chloromercuribenzoate agaroase. Its amino acid composition differs from other known factor X activators (including factor IX, factor VII, tissue factor) and from cathepsin B, a mammalian cysteine proteinase. A procoagulant activity which directly activates factor X has been described in cells isolated from murine tumors such as the mouse Lewis lung carcinoma, Ehrlich carcinoma, JW sarcoma, and B16 melanoma (11, 12). In these experimental tumors, the involvement of CP in metastasis formation is suggested by the following two observations: (a) concomitant depression of CP activity and lung metastasis development by treatment with warfarin or vitamin K deficient diet in Lewis lung carcinoma, B16 melanoma, and JW sarcoma (4, 5, 13); (b) close relationship between CP activity and metastatic capacity of B16 melanoma variants (12).

The presence of CP in isolated cells from human tumors has not been documented. We report here that tumor cells from primary and metastatic human melanomas, but not benign melanocytic nevi, possess CP activity that directly activates coagulation factor X and is sensitive to cysteine proteinase inhibitors.

PATIENTS AND METHODS

Patients. Thirty-two patients (17 males and 15 females), ages 17-84 years, admitted to the Istituto Nazionale per lo Studio e la Cura dei Tumori for primary or metastatic melanoma entered the study. Ten samples were from primary lesions and 23 from s.c. (3) or lymph node (20) metastases. From one patient, tissue samples were obtained from both primary melanoma and lymph node metastasis.

According to tumor-nodes-metastasis classification, 7 patients were at clinical stage I, 21 were at stage II, and 4 were at stage IV. Benign nevi were obtained from 6 otherwise healthy subjects; histological diagnosis was "functional melanocytic nevi" without evidence of dysplasia.

Tumor Extracts. Surgical specimens were cleaned, washed twice in RPMI 1640, and immediately frozen at −80°C. After thawing, the tissue was minced into 0.2-0.4-cm pieces, in order to increase the exposed surface, and then incubated for 3 h in 20 mm Veronal buffer (pH 8 at 5°C), which was replaced every hour (8, 10).

The tissue extract was concentrated 10-15 times by membrane ultrafiltration with Amicon PM 10 (Amicon Corp., Danvers, MA). In the resulting sample the protein concentration was analyzed by the method of Bradford (14) and the procoagulant activity tested.

Tumor Cells. Surgical specimens were mechanically disaggregated by mincing in RPMI 1640 (RPMI Gibco, Glasgow, Scotland); tissue debris and cell aggregates were removed by filtration through three layers of sterile gauze, and the cell suspension was washed twice and resuspended in the same medium. This preparation contained 80% tumor cells or more (as judged by routine staining procedure) and less than 10% of normal blood cells and debris. CP activity was independent of both the intrinsic and extrinsic pathways of blood coagulation, using factor IX and factor VII deficient plasmas, and was inhibited by the cysteine proteinase inhibitors iodoacetamide and HgCl2. CP activity was detectable in extracts and cell suspensions from all 32 patients studied and was higher in extracts from metastases (14.8 ± 3.9 units/mg protein) than from the primary tumors (3.7 ± 1.0 units/mg protein). CP activity was not affected by an anti-apoptotein III antibody or by concanavalin A, a known inhibitor of thromboplastin.

These data indicate that CP can be expressed by human tumor cells and that, among melanotic lesions, its presence is associated with the malignant phenotype and its activity is particularly high in metastatic cells.

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3 The abbreviations used are: CP, cancer procoagulant (direct activator of coagulation factor X); Con A, concanavalin A; RVV, Russell's viper venom; PCA, procoagulant activity.

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thromboplastin (tissue factor), giving a clotting time of 39.7 s, or RVV, (0.5 μg/ml; Wellcome Research Laboratories, Beckenham, England), giving a clotting time of 39.1 s; the procoagulant activity of these concentrations was arbitrarily considered to represent 100 units. Procoagulant activity in the tissue extracts or in the cell preparations were expressed in seconds or as units of either RVV or tissue factor per mg protein. There was a linear relationship from 0.2 to 100 units of either thromboplastin or RVV and clotting time. The slopes of the curves obtained with thromboplastin and RVV were similar and the curves obtained with RVV in normal and factor VII deficient plasmas coincided.

The type of CPA was first identified using human plasmas selectively deficient in factor II, VII, IX, or X (Merz-Dade, Duing, Switzerland). For further characterization, known inhibitors of cell procoagulants were used, namely the cysteine protease inhibitors HgCl₂ and iodoacetamide (Sigma Chemical Co., St. Louis, MO) (15) and the tissue factor inhibitor, Con A, (Sigma) (16). Samples of the tissue extracts or of the cells were incubated with HgCl₂ (0.1 mM, final concentration) or iodoacetamide (2 mM) at 37°C for 30 min (9) and the procoagulant activity was then measured. Control enzymes for procoagulant activity were the cysteine protease papain and the serine protease RVV. Reactivation of CPA after HgCl₂ treatment was obtained by incubation of the cells with 5 mM DL-dithiothreitol (Sigma), 2 mM EDTA (Merck, Darmstadt, West Germany), and 10 mM KCN (Sigma) at 5°C for 30 min, followed by overnight dialysis against 20 mM Veronal buffer, pH 8.0 (10). To assess the effect of Con A, 20 mg of tissues extracts were incubated with the inhibitor (100 μg/ml, final concentration) for 1 h at 37°C, and then CPA was determined. To verify the specificity of the reactivation of Con A, the sample to be tested in the presence of Con A was preincubated for 15 min at 37°C with α-methyl-D-glucoside (Sigma) (17).

Immunological characterization of the melanoma procoagulant was performed by the use of a goat antiserum against the apoprotein III of human brain tissue factor (kind gift of Professor H. Prydz, Tromso, Norway). The samples were incubated at 37°C with the antiserum or goat nonimmune serum for 15 min and then tested for CPA, as described above.

Polyclonal goat antibody was developed to pure CP from rabbit V2 carcinoma (10). This antibody was cross-reactive with purified antigen from human amnio-chorion tissue (18, 19). Crossed immunodiffusion was used to study the immunoreactivity of the goat antibody with CP antigen from the melanoma extracts. The immunodiffusion was performed in 1% agar in low-salt Veronal buffer (ionic strength, 0.025; pH 8.2). Samples were allowed to react at 5°C for 24 h. After washing and drying the plate was stained with crocein scarlet and Coomassie blue, as described (10).

Statistical Analysis. Data were expressed as mean ± SE. The statistical evaluation was performed by Student's t test and by Duncan's test for multiple comparisons.

RESULTS
Tumor extracts and freshly harvested cells from the tumors of the first three melanoma patients were tested. Both types of tumor samples significantly shortened the recalcification clotting time of normal human plasma. A similar shortening was observed in plasma selectively deficient in coagulation factor VII or factor IX whereas the level of activity was substantially less in factor X or factor II deficient plasmas (Table 1). The independence from factors of both the intrinsic and extrinsic pathways suggested the activity was a direct activator of factor X. In contrast, the extracts of the benign melanocytic lesions did not express any activity in factor VII deficient plasma, suggesting the presence of tissue factor. Therefore, in subsequent experiments, the presence of CPA was tested by the shortening of the recalcification clotting time of both normal and factor VII deficient plasmas and the results were expressed as specific procoagulant activity (units of procoagulant per mg of protein) (Table 2).

Table 1 Dependence of procoagulant activity of human malignant melanoma and benign melanocytic nevi on coagulation factor VII, factor X, factor IX, and factor II

| Tissue extracts and isolated cells from lymph nodal metastases of human melanoma and tissue extracts from benign melanocytic nevi were assayed for procoagulant activity by measuring the recalcification clotting time of normal human plasma (NHP) and of human plasmas selectively deficient of factor VII (VII-D), factor X (X-D), factor IX (IX-D), or factor II (II-D). The clotting time results are means ± SE of 3 samples.

<table>
<thead>
<tr>
<th>Clotting time (s)</th>
<th>NHP</th>
<th>VII-D</th>
<th>X-D</th>
<th>IX-D</th>
<th>II-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracts (n = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>49 ± 1</td>
<td>51 ± 3</td>
<td>282 ± 14</td>
<td>50 ± 4</td>
<td>313 ± 16</td>
</tr>
<tr>
<td>Blank</td>
<td>93 ± 4</td>
<td>99 ± 5</td>
<td>290 ± 12</td>
<td>103 ± 3</td>
<td>299 ± 18</td>
</tr>
<tr>
<td>Melanocytic nevi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracts (n = 3)</td>
<td>143 ± 8</td>
<td>207 ± 9</td>
<td>315 ± 11</td>
<td>308 ± 9</td>
<td>320 ± 15</td>
</tr>
<tr>
<td>Blank</td>
<td>&gt;360</td>
<td>210 ± 11</td>
<td>325 ± 14</td>
<td>340 ± 15</td>
<td>318 ± 18</td>
</tr>
</tbody>
</table>

Table 2 Specific activity and factor VII dependence of procoagulants from malignant and benign tissue

Procoagulant activities of samples from malignant melanomas and benign nevi were quantitated in the presence and absence of factor VII, to distinguish the type of procoagulants (CP or tissue factor) and to determine the extent of their expression. The activity (clotting times) of malignant melanoma samples was largely independent of factor VII and was calculated from a calibration curve with different concentrations of RVV (see text), the standard enzyme equally active in normal (NHP) and factor VII deficient (VII-D) plasma. The percentage of factor VII independent CPA compared to the total activity in NHP was computed for each group of samples. On the other hand the activity of benign nevi extracts, totally factor VII dependent, like tissue factor, was computed as arbitrary units from a calibration curve of standard thromboplastin (see text). Results are mean ± SE.

<table>
<thead>
<tr>
<th>% of factor VII independent</th>
<th>NHP</th>
<th>VII-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary extracts</td>
<td>10</td>
<td>5.4 ± 1.6°</td>
</tr>
<tr>
<td>Metastasis extracts</td>
<td>23</td>
<td>19.1 ± 4.8°</td>
</tr>
<tr>
<td>Metastasis cells</td>
<td>13</td>
<td>3.8 ± 0.9°</td>
</tr>
<tr>
<td>Melanocytic nevi extracts</td>
<td>6</td>
<td>3.2 ± 0.4°</td>
</tr>
</tbody>
</table>

Approximately 75% of the procoagulant activity (ranging from 69 to 84%) in both the extracts and disaggregated cells from the primary and metastatic tumors was CP (factor VII independent) and the remaining 25% was tissue factor (factor VII dependent) (Table 2). There was almost 4-fold more activity per mg protein in extracts of the metastatic tumors than in the primary tumors (P < 0.05). In the only patient from whom specimens of primary melanoma and lymph node metastasis were obtained, the activity from the metastasis (59.2 units/mg protein) was 8 times that of the primary tumor (7.6 units/mg protein). There was about 5 times greater specific procoagulant activity in the extracts of metastatic tumors than in their corresponding disaggregated cell suspensions.

Extracts from the six melanocytic nevi contained much less procoagulant activity than melanoma extracts; as mentioned above, such activity was identified as tissue factor (Table 2). It is worth mentioning that tissue extracts from normal skin (three samples) failed to show any kind of CPA (data not shown).

A unique procoagulant capable of directly activating factor X has been described in some experimental tumors and was found to be associated with a cysteine protease in V2 carcinoma tissue (9). We tested the hypothesis that human melanoma procoagulant was also a cysteine protease. As shown in Table 3, the procoagulant activity of extracts and cells from primary or metastatic tumors was significantly inhibited by iodoacetamide and HgCl₂ (P < 0.01 at Duncan's test for multiple comparisons). Control experiments showed that these chemicals
CANCER PROCOAGULANT IN HUMAN MELANOMA

Table 3 Effect of cysteine protease inhibitors on the procoagulant activity of human melanoma tissue

RVV* units derived from recalcification times of normal human platelet poor plasma. Results are mean + SE of 4 values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RVV activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Primary tumor extract</td>
<td></td>
</tr>
<tr>
<td>HgCl₂</td>
<td>12.7 ± 1.2</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>11.9 ± 1.1</td>
</tr>
<tr>
<td>Metastasis extract</td>
<td></td>
</tr>
<tr>
<td>HgCl₂</td>
<td>13.5 ± 1.4</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>17.6 ± 1.3</td>
</tr>
<tr>
<td>Metastasis cells</td>
<td></td>
</tr>
<tr>
<td>HgCl₂</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>RVV</td>
<td></td>
</tr>
<tr>
<td>HgCl₂</td>
<td>8.5 ± 0.5</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>Papain</td>
<td></td>
</tr>
<tr>
<td>HgCl₂</td>
<td>12.4 ± 0.4</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>10.8 ± 1.0</td>
</tr>
</tbody>
</table>

* RVV is the serine protease control, a direct activator of factor X; papain is the cysteine protease control.

significantly (P < 0.01) inhibited the cysteine protease papain but not the serine protease RVV. The inhibition of CP and papain by HgCl₂, a reversible inhibitor of cysteine proteases, could be completely reversed by the appropriate reactivators (Table 3).

Fig. 1 shows that the activity of melanoma extracts was not significantly affected by Con A, a known inhibitor of tissue factor. The slight inhibition observed was most probably due to the presence in the extracts of small amounts of tissue factor. In contrast, the PCA of extracts from melanocytic nevi was almost completely inhibited by Con A (Fig. 1). Preincubation of the extracts with α-methyl-D-glucoside partially prevented the inhibitory effect of Con A. In a parallel experiment, preincubation with an anti-apolipoprotein III antibody completely inhibited the PCA of benign nevi, but did not affect the activity of malignant tissue extract.

Crossed immunodiffusion of extracts from three tumors and one benign nevus are shown in Fig. 2. The immunoprecipitin band that corresponds to the tumor samples but is absent between the antibody and nevus extract wells confirms the presence of the antigen in these melanoma samples and its absence in the benign nevus sample.

DISCUSSION

This study shows that human melanoma expresses a procoagulant activity with unique enzymatic and immunological characteristics. In all the melanoma patients studied, tumor specimens, processed as extracts or as disaggregated cell suspensions, promoted fibrin formation by direct intervention on the common pathway of blood clotting. The identification of human melanoma procoagulant activity as a direct factor X activator was based on clotting assays in human plasmas selectively deficient in one clotting factor. These experiments showed that both tumor extracts and cells from human melanoma accelerated thrombin formation independently from the presence of factors of either the extrinsic or intrinsic pathway of blood clotting. However, the higher procoagulant activity in normal than in factor VII deficient plasma suggests that contamination of tumor material with tissue factor cannot be excluded.

Tissue extracts showed markedly higher activity than the corresponding tumor cell preparation. There could be several reasons for this: (a) host cells could contribute their activity to tumor extracts and raise their procoagulant content. However, thus far no normal cell has been reported to produce this type of procoagulant, with the exception of amnion-chorion tissue (18,19); (b) the extraction procedure could result in more stable and active procoagulant enzyme(s), whereas cell disaggregation could involve unpredictable cell damage with the release of proteinase inhibitors and the subsequent loss of activity.

This observation is of interest in view of previous reports that a direct activator of factor X was present in the mucus of mucus producing tumors (20) or of cancers from large intestine, breast, lung, and kidney (7). The latter study reported qualitative differences between the types of procoagulant in paired normal and malignant tissue extracts, since only the tumor tissue contained cancer procoagulant whereas different amounts of tissue factor were present in the normal tissue extracts.
Similarly, the six benign lesions we tested were completely devoid of cancer procoagulant.

Despite these reports of cancer procoagulant in extracts of several human tumor tissues, no evidence of this activity has been documented in cells isolated from human tumors. We examined both the isolated cells and the tissue extracts for each patient's specimen; the observed procoagulant activity is a specific property of cancer cells and the activity that was measured in the extracts came from the corresponding tumor cells.

In rabbit V2 carcinoma, cancer procoagulant was purified and was found to be a cysteine protease with unique properties (9, 10). It is a proteinase distinct from the other serine proteinase clotting enzymes. We explored the possibility that the procoagulant from the human melanoma might also be a cysteine protease. Experiments were carried out with two cysteine protease inhibitors which depressed the procoagulant activity of the protein purified from V2 carcinoma (9). Both iodoacetamide and HgCl₂ significantly prolonged the clotting time of human melanoma cells or extracts. The inhibition by HgCl₂ (a reversible inhibitor of cysteine proteases) could be reversed by appropriate reactivators, suggesting that the observed loss of activity of human melanoma procoagulant was due not to a nonspecific effect of the HgCl₂ treatment but to reversible inhibition of the enzyme. Moreover the activity of melanoma extracts was not affected by an anti-apoptoprotein III antibody and by Con A, an inhibitor of thromboplastin. In contrast, the procoagulant expressed by benign pigmented lesions was of tissue factor type, as also shown by its inhibition with anti-apoptoprotein III antibody and by Con A.

Moreover, using polyclonal goat antibodies raised against pure CP from rabbit V2 carcinoma, we showed the presence of the antigen in the melanoma samples and its absence in the benign pigmented lesions.

Thus, we have provided here evidence that human melanoma tissues possess a PCA with the enzymatic and immunological characteristics of the cancer procoagulant purified from some animal tumors.

Whether this potent procoagulant plays a role in fibrin deposition around tumor cells and influences the metastatic behavior of these cells is a question which merits appropriate studies. The preliminary results of histochemical studies with antifibrinogen/fibrin antibody suggest that fibrin is deposited mainly in association with high levels of CP, i.e., in metastatic lesions of human melanoma more than in primary tumors.

This study makes a novel contribution to our knowledge of the biology of melanoma, and possibly to its better characterization. It is of interest in this respect that specimens from all the patients studied were found to express procoagulant activity although to different degrees. While no simple correlation was found in this study between procoagulant activity and clinical stage, it is remarkable that the activity was significantly higher in metastasis that in primary melanomas. In the only patient in whom primary tumor and metastasis could be studied, procoagulant activity was 8 times higher in the metastasis.

Many hypotheses have been proposed to explain the biological significance of tumor cell procoagulant activity (1–4), but one is particularly interesting since it links this activity to another important field of cancer research, tumor immunology. In fact, fibrin deposition on tumor cell membranes might prevent recognition and lytic interaction with cytotoxic lymphocytes. It has been shown that the antimitastatic effect of warfarin and other anticoagulant drugs depends, at least in part, on integrity of the immune system, being abrogated in animals with depressed natural killer cell activity (21). Procoagulant activity of human melanoma cells could, therefore, inhibit tumor lysis by cytotoxic lymphocytes and contribute, together with other mechanisms (22, 23), to the growth and metastatization of the tumor.

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