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-apoprotein 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-apoprotein 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 promotion 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 clotting in association with factor VII in the extrinsic pathway; and (b) CP, a proteolytic enzyme 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 4.8 (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 agarose. 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 tumor was chopped 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 5% macrophages (by esterase staining). Viability was about 50%. Cells were adjusted to 1 × 10⁶/ml and frozen at −80°C. Immediately before testing for procoagulant activity, the samples were thawed and frozen twice.

Procoagulant Activity. The PCA of isolated cells and of tissue extracts was measured visually by a one-stage plasma recalcification assay using a test system containing 0.1 ml of test material or buffer, 0.1 ml of human platelet poor plasma, and 0.1 ml of 0.025 mM CaCl₂. The standards for the coagulation assay were a 1:10 dilution of rabbit brain.
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 PCA was first identified using human plasmas selectively deficient in factor II, VII, IX, or X (Merz-Dade, Duding, 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 PCA after HgCl2 treatment was obtained by incubation of the cells with 5 mM DL-dithiothreitol (Sigma), 2 mM EDTA (Merek, Darmstadt, West Germany), and 10 mM KCl (Sigma) at 3°C for 30 min, followed by overnight dialysis against 20 mM Veronal buffer, pH 8.0 (10). To assess the effect of Con A, portions of tumor extracts were incubated with the inhibitor (100 µg/ml, final concentration) for 1 h at 37°C, and then PCA was determined. To verify the specificity of the effect 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 non-immune serum for 15 min and then tested for PCA, 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-chorionic 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 CP 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>
<thead>
<tr>
<th>Metastasis</th>
<th>VII-D</th>
<th>X-D</th>
<th>IX-D</th>
<th>II-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracts (n = 3)</td>
<td>49 ± 1</td>
<td>51 ± 3</td>
<td>282 ± 14</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>Cells</td>
<td>93 ± 4</td>
<td>99 ± 5</td>
<td>290 ± 12</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>Blank</td>
<td>143 ± 8</td>
<td>207 ± 9</td>
<td>315 ± 11</td>
<td>308 ± 9</td>
</tr>
<tr>
<td>% of factor VII independent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>68.5</td>
<td>97.5</td>
<td>77.5</td>
<td>84.7</td>
</tr>
<tr>
<td>Metastatic cells</td>
<td>10</td>
<td>23</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Metastatic extracts</td>
<td>5.4 ± 1.6</td>
<td>19.1 ± 4.8</td>
<td>3.8 ± 0.9</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Metastatic neutrophils</td>
<td>&lt;0.2</td>
<td></td>
<td></td>
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</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 1). 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 PCA (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...
significant (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 proteinase, 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 for 15 min, at 37°C prior to preincubation with Con A. Untreated (A), Con A treated (B), and α-methyl-D-glucoside (C) plus-Con A treated samples were assayed for activity by the one stage clotting time of normal citrated human plasma. Results expressed as RVV units derived from recalcification times of normal human platelet poor plasma. Results are mean ± SE of 4 values.

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

<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><strong>Primary tumor extract</strong></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><strong>Metastasis extract</strong></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><strong>Metastasis cells</strong></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><strong>RVV</strong></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><strong>Papain</strong></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.

**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 protease 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 proteinases. 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 the anticoagulant. HgCl2 is a specific property of cancer cells and the activity that was measured in the extracts came from the corresponding tumor cells.

The preliminary results of histochemical studies with antibodies against fibrin deposition around tumor cells and influences the metastatic behavior of the tumor. It has been shown that the antimetastatic effect of warfarin and other anticoagulant drugs depends, at least in part, on the interaction 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.

ACKNOWLEDGMENTS

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