Identification of Tissue Factor in Two Human Pancreatic Cancer Cell Lines

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ABSTRACT

We have studied the effects of two human pancreatic cancer and two human small cell lung cancer cell lines on clotting and platelet aggregation. Both pancreatic lines markedly shortened recalcification times and induced platelet aggregation. The lung cancer lines produced little shortening of recalcification times and no platelet aggregation. The clotting and aggregation activities of the pancreatic lines were further characterized. Recalcification times following the addition of cancer cell line material to plasmas deficient in factors VII and X were markedly prolonged, suggesting that the activity is due to tissue factor. Hirudin, an inhibitor of thrombin from the saliva of leeches, and rabbit polyclonal immunoglobulin G anti-brain tissue factor inhibited both procoagulant and aggregation activities. Apyrase (an enzyme degrading ADP), diisopropylfluorophosphate (a serine protease inhibitor) and t-trans-epoxysuccinylleucylamido(4-guanidino)butane (a cysteine protease inhibitor) failed to inhibit these activities. Increasing concentrations of heparin inhibited platelet aggregation. Subcellular fractionation studies showed these activities to be localized to the plasma membrane. The association between mucin and the acceleration of clotting has been well described. The absence of mucin in electron micrographs of these pancreatic whole cells, membrane fractions, and shed microvesicles, as well as the failure of chaotropic agents (i.e., agents stripping material extrinsic to the cell membrane such as mucin) to abrogate this activity support these activities being intrinsic to the plasma membrane. These data strongly suggest that these activities are due to tissue factor which appears to be released as microvesicles in vitro. The release of tissue factor via microvesicles in vitro is one possible mechanism for the coagulopathy sometimes seen in patients with pancreatic carcinoma.

INTRODUCTION

Coagulation abnormalities in patients with cancer represent a common and potentially catastrophic complication of malignancy. Disseminated intravascular coagulation is a relatively common coagulopathy of neoplasms and has been commonly reported in patients with adenocarcinomas and acute promyelocytic leukemia (1-3). The process of DIC may result from a variety of underlying disease processes which complicate malignancy such as liver disease, sepsis, and hypotension. Disseminated intravascular coagulation may be caused by activators of coagulation found in the tumor cells themselves such as tissue factor (4, 5) and cancer procoagulant A (6-8). Finally, activated nonneoplastic monocytes/macrophages containing increased tissue factor activity have been found in some neoplasms and may contribute to the process of DIC (9-11).

Pancreatic carcinoma has been strongly linked to chronic DIC presenting as a migratory thrombophlebitis or Trousseau's syndrome (12, 13). The strength of this association has been questioned by others (14). In order to better understand the pathogenesis of DIC in patients with pancreatic carcinoma, we studied the in vitro effects of two human pancreatic ductal adenocarcinoma cell lines (15) on clotting and platelet aggregation. As a control, we looked at two small cell lung cancer lines (16). The coagulopathy of small cell lung cancer, although clearly demonstrable by laboratory evaluation, is typically less clinically overt than that seen in pancreatic carcinoma in the absence of an additional inciting event (17-19).

MATERIALS AND METHODS

Reagents. The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO: ADP, apyrase, DFP, EDTA, hirudin, and EP64. All factor-deficient plasmas and rabbit thromboplastin (Simplastin) were obtained from General Diagnostics, Morris Plains, NJ. Heparin sodium (Liquaemin Sodium) at 1000 USP units/ml was obtained from Organon, Inc., West Orange, NJ. Firefly extract (luciferase; lucifere) was obtained from Chrono-log Corp., Havertown, PA. Bacto-lysosome buffer (pH 6.2), Bacto-lysosome substrate, and Bacto-lysylzyme was obtained from Difco Laboratories, Detroit, MI. The IgG fraction of polyclonal antibodies (rabbit) raised against bovine tissue factor was kindly supplied by Dr. Ron Bach.

Cell Culture. Both pancreatic cancer cell lines (RWP1 and RWP2) were kindly supplied by Dr. D. L. Dexter (15). These lines were derived from liver metastases of two different patients. At the time of the study, both lines had been maintained continuously in culture in our laboratory without any period of heterotransplantation into athymic mice for a minimum of 1 year.

The human small cell lung cancer lines NCI-H69 and NCI-H82 were kindly provided by Dr. J. D. Minna of the Bethesda Naval Hospital, National Cancer Institute, Bethesda, MD, and have been previously described (16).

All lines were grown in an atmosphere of 5% CO2 either as monolayers (RWP1, RWP2) or spheroids (NCI-H69, NCI-H82) in polycarbonate flasks (Falcon Plastics, Oxnard, CA), using RPMI 1640 with supplements of 10% fetal calf serum as well as penicillin and streptomycin.

Platelet Aggregation and Release. Aggregometry with and without measurements of ATP release was done in a standard manner by using a Lumi-Aggregometer 460 (Chrono-log Corp.). Both aggregation and release were done at 37°C with constant stirring at 1000 rpm (20). Platelets were obtained from normal donors who had not taken any aspirin-containing medications for the preceding 10 days. Ten ml of blood were collected in plastic tubes containing heparin, usually at a final concentration of 4 units/ml. These tubes were centrifuged immediately at room temperature at 160 × g for 10 min. PRP was removed with siliconized Pasteur pipets into a plastic tube. PPP was then prepared by recentrifuging the remaining specimen at 4000 × g for 10 min. A platelet count was then done on PRP and adjusted as necessary with PPP to a final platelet count of approximately 300,000 ± 50,000/ mm3. All tests were performed between 30 min and 3 h after phlebotomy.

Platelet aggregometry measures the change in light transmission of PRP relative to PPP when an agonist is added. Initially, 0.45 μl of PRP was simply allowed to equilibrate in the aggregometer for at least 5 min to observe for any spontaneous aggregations. As a control, all runs had 27 μl of 20 μM ADP added to one cuvet of PRP to demonstrate normal platelet aggregation. Most runs with pancreatic cells also used rabbit tissue thromboplastin diluted to a similar recalcification time as a control. Generally, 50 μl of the material to be tested were added with base lines reset if necessary. At least 5 min were allowed before assuming that the material used was not aggregating platelets.

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3 The abbreviations used are: DIC, disseminated intravascular coagulation; DFP, diisopropylfluorophosphate; EP64, t-trans-epoxysuccinylleucylamido(4-guanidino)butane; PRP, platelet rich plasma; PPP, platelet poor plasma.
that, ADP was added to document that the platelets were still active.

To document that aggregation was active and causing release, 50 µl of luciferase-luciferin were added to PRP prior to adding RWP1 or RWP2 material. This was done twice for each cell line.

Clotting Assays. The recalcification times were performed in the standard manner with a fibrometer (Bio-Quest, Baltimore, MD). In brief, procoagulant activity was measured by using a one-stage assay in which 0.1 ml of pooled normal citrated human plasma was incubated with 0.1 ml of sample at 37°C for 1 min. Following this, 0.1 ml of CaCl₂ (concentration, 25 mmol/liter) was added and the clotting time was recorded. A minimum of 2 assays were performed for each sample tested. If the results of these 2 assays were within 10% of each other, no additional assays were done. If not, an additional 2 assays were performed with obvious outlying points excluded and the average of the 3 assays taken. As almost all the data reported is a result of only 2 assays, means are reported but standard deviations are not. In some experiments, procoagulant activity was assayed with congenitally deficient human plasma (factors VII, VIII, IX, X, XI, or XII). The controls used for these assays were 0.9% sterile saline (i.e., negative control) and rabbit tissue thromboplastin or Simplastin (i.e., positive control). Rabbit thromboplastin was diluted with 0.9% sterile saline to an approximately equivalent recalcification time to that of the tumor material being examined.

Inhibition Studies. A number of chemical agents and physical means were used to treat RWP1 and RWP2 materials. Microvesicles obtained by differential centrifugation were boiled for either 15 or 60 min or frozen at -70°C for 24 h. These treated microvesicles were then studied by recalcification times for loss of activity.

The rest of the inhibition assays were performed on combined membrane fractions (see "Preparation of Material Tested"). Heparin was added to the initial blood drawn to concentrations as high as 50 units/ml. The PRP was prepared in the standard manner. RWP1 and RWP2 material as well as controls were added as outlined earlier. Apyrase was added to PRP immediately prior to the addition of tumor material at final concentrations of 1.25-125 units/ml. Hirudin was added to PRP 30 min prior to the addition of tumor material at final concentrations of 0-100 units/ml. All other inhibitor assays, including DFP (final concentration, 10 mmol/liter), EDTA (final concentration, 2 mmol/liter), EP64 (final concentration, 3 x 10⁻⁷M), and polyclonal antibody to tissue factor (50 µA of various dilutions added to 50 µl RWP1 or RWP2) were incubated with RWP1 or RWP2 at 37°C for 2 h prior to being added to PRP or PPP for aggregations or recalcifications, respectively. Apyrase (final concentration, 1.25-125 units/ml) and hirudin (final concentration, 0-50 units/ml) were similarly incubated with RWP1 and RWP2 combined membrane fractions prior to recalcification time assays.

All inhibition studies had a positive (i.e., Simplastin) and a negative (i.e., 0.9% saline) control.

Electron Microscopy. Samples for electron microscopy were fixed with 3% sodium cacodylate-buffered glutaraldehyde. A 0.2 M cacodylate buffer wash was followed by postfixation with 1% buffered osmium tetroxide, block staining with uranyl acetate, and subsequent dehydration in 50-100% alcohol solutions. Propylene oxide treatment was replaced by overnight infiltration by using a solution of 50% propylene oxide—50% Epon-Araldite epoxy resin. The pellet was embedded in 100% epoxy resin and polymerized at 28°C for 16 to 20 h. One-mm sections were cut for light microscopy with a LKB ultramicrotome. Thin sections, 20 to 30 nm, were collected on 200-mesh copper grids and were examined by a Hitachi HU-12 electron microscope.

Extraction of Nonintegral Proteins from Tumor Plasma Membrane. RWP1 and RWP2 combined membrane fractions were treated with chaotropic agent 2 M potassium chloride in buffer at 4°C to remove nonintegral membrane proteins (21). After the 30-min incubation, the material was centrifuged at 100,000 x g for 3 h and resuspended in 0.9% saline at the original volume. Specimens of both treated and untreated material were tested in both the recalcification and platelet aggregation assays.

Preparation of Material Tested. Experiments on the pancreatic tumor cells were performed by using whole cells (either fresh or freeze-thawed); microvesicles shed into spent media from cells incubated 2 to 4 days and then differentially centrifuged (initially at 1,000 x g for 15 min to bring down whole cells and large fragments followed by 100,000 x g ultracentrifugation of the remaining supernatant for 3 h to obtain a pellet enriched with microvesicles); and subcellular fractions prepared by nitrogen cavitation and sucrose density gradient centrifugation, as previously described (22, 23).

Experiments on small cell lung cancer cells were performed by using either fresh whole cells or cells freeze-thawed three times.

All cell culture material was washed with 0.9% sterile saline to remove any media contamination prior to testing. Whole cells were washed three times. They were spun down at 1,000 x g for 15 min after each wash. Microvesicles were spun at 100,000 x g for 3 h and then washed and respun twice.

Muramidase Assay. Muramidase levels were measured as described (24).

RESULTS

Electron Microscopy. Fig. 1 shows representative electron micrographs of ultracentrifuged RWP1 tumor preparations. Note the similarity between MEM 1, the most highly purified plasma membrane preparation, and the differentially centrifuged spent RWP1 medium. The latter preparation, however, was contaminated with other cell organelles and debris. Preparations of RWP2 tumor material were similar in appearance,
although the differentially centrifuged spent medium was somewhat less enriched in plasma membranes.

Coagulation and Aggregation Studies. RWP1 and RWP2 coagulation and aggregation study results were quantitatively similar regardless of whether the materials used were whole cells, microvesicles, or membrane preparations. Both small cell lung cancer lines shortened recalcification times minimally and failed to aggregate platelets in either intact form or when freeze-thawed. Therefore, membrane preparations and microvesicles were not examined in these lines.

Recalcification times done on pancreatic and small cell lung cancer lines are shown in Table 1. The pancreatic lines (i.e., whole cells, freeze-thawed cells, microvesicles, and membrane preparations) showed marked shortening of recalcification times as well as the ability to aggregate platelets after a lag period. Fig. 2 shows the corresponding platelet aggregations for RWP1 and RWP2. This was not seen in the small cell lung cancer lines (i.e., whole cells, freeze-thawed cells) studied in which little or no shortening of the recalcification times were seen and platelet aggregation was not observed even when cell concentrations were increased 10-fold or greater. The release studies done with luciferase-luciferin showed ATP release (data not shown) with all the various RWP1 and RWP2 preparations used. This demonstrates active aggregation as opposed to some nonspecific binding or clumping to the tumor material.

Recalcification times using RWP1 and RWP2 material with plasmas deficient in factors VII, VIII, IX, X, XI, or XII were performed (results not shown here). Prolongation of recalcification times to levels near those seen with control saline were seen with factor VII- and X-deficient plasmas only. The addition of RWP material to factors VIII-, IX-, XI-, and XII-deficient plasmas resulted in clotting times within 10% of comparable studies with normal plasma. Parallel studies done with Simplastin showed comparable results.

Inhibition Studies. Hirudin, an antithrombin, inhibited both procoagulant activity and platelet aggregations. For example, a representative study of inhibition of RWP1 combined membrane fractions with this polyclonal antibody had a base-line recalcification of 27.3 s (i.e., without antibody). After incubation of various titers of antibody with this material at 37°C for 2 h, recalcification times were measured. Antibody titers done included undiluted, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. Corresponding recalcification times were 137.3, 104.0, 79.7, 66.2, 50.0, 31.2, 29.2, 28.4, and 28.0 s, respectively. The inhibition of coagulant activity was paralleled by the inhibition of platelet aggregation as seen in Fig. 4. Similar data were found when RWP2 coagulant material was used. Of note, when rabbit thromboplastin was used, the antibody to tissue factor showed minimal or no inhibition of either coagulant activity or aggregations (data not shown), suggesting that this antibody is specific for human tissue factor.

A variety of chemical agents and physical means were evaluated for inhibition of aggregation and/or coagulant activities of the pancreatic cancer lines. Freezing as well as boiling for 15 min had little or no effect on recalcification times, while boiling for 60 min resulted in marked prolongation of recalcification times (i.e., RWP1 went from 53.9 to 200 s; RWP2 went from 27.5 to 52.5 s). Dithiopropylfluorophosphate, a serine protease inhibitor, failed to inhibit either coagulant or aggregation activity as did ETP64 (a cysteine protease inhibitor), apyrase (an enzyme degrading ADP), and EDTA. Minimal increases of heparin concentration completely inhibited platelet aggregation by RWP1 and RWP2. Chaotropic agents (2 M KCl) failed to remove any activity from RWP1 or RWP2 combined membrane fractions.

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Times (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RWP1</td>
<td>22.5</td>
</tr>
<tr>
<td>RWP2</td>
<td>23.0</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>100.5</td>
</tr>
<tr>
<td>NCI-H82</td>
<td>91.5</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>&gt;200.0</td>
</tr>
</tbody>
</table>

Subcellular Fraction Studies. Table 2 shows the recalcification and platelet aggregation studies done with RWP1 subcellular fractions. The 50,000 x g supernatant, which contained minimal amounts of cell membranes, had the least activity despite having the highest protein concentration. This indicates that the coagulant and aggregation activity is primarily cell membrane related. The enriched plasma membrane preparations had the highest coagulant activity per µg protein. MEM 1 has

Fig. 2. Platelet aggregations for RWP1 and RWP2 combined membrane fractions corresponding to those in Table 1. The upward deflection of the pen in platelet aggregometry indicates an increase in light transmission due to platelet aggregation. Note the characteristic lag period always seen with aggregation studies performed on either RWP1 or RWP2. The slopes of these platelet aggregations may also vary.

Fig. 3. Inhibition of platelet aggregations by hirudin for RWP2 corresponding to text. Final concentration of hirudin: a, 0 units/ml; b, 1 unit/ml; c, 10 units/ml; d, 100 units/ml.
**DISCUSSION**

This study clearly demonstrated that human pancreatic RWP1 and RWP2 ductal adenocarcinoma cell lines have significant tissue factor activity in the plasma membrane which is released into the media in the form of microvesicles. There was little or no tissue factor activity demonstrable in two small cell lung cancer lines, a tumor type not associated with clinically significant DIC. The evidence that RWP1 and RWP2 contain tissue factor includes the fact that the tumor procoagulant activity could not be demonstrated in plasmas that were deficient in factors VII and X, whereas the procoagulant activity was observed in plasmas that were deficient in factors VIII, IX, XI, and XII. Inhibition of pancreatic tumor procoagulant and tumor induced platelet aggregation by hirudin and anti-tissue factor antibody is also consistent with the presence of tissue factor in these cell lines as is the fact that increasing concentrations of heparin inhibited platelet aggregation.

Tissue factor requires both an apoprotein and lipid for activity (25, 26). Our subcellular fraction experiments demonstrate that the plasma membrane is the principal site of the procoagulant and platelet aggregation activities. The parallel nature of the results obtained in the recalcification times and in the platelet aggregations suggests that both phenomena are induced by the same mechanism (i.e., thrombin formation). Bastida et al. (27) also failed to show inhibition of tumor tissue factor with DFP.

Tumor shedding of microvesicles containing procoagulant material is a well-described laboratory phenomenon (27-29). Contact of the membraneous material or whole tumor cells with plasma is a possible mechanism for the DIC described in some patients with pancreatic adenocarcinoma. Rickles and Edwards (17) reviewed potential factors of activation of blood coagulation in cancer patients. These included mucin, monocyte tissue factor, platelet aggregating factors, proteases, cancer procoagulant A, and cancer tissue factor. To rule out the possibility that monocytes were contributing to the procoagulant activity in our assay, only cell cultures maintained in vitro without xenografting for at least 1 year were used. Also, muramidase levels were measured in spent media and were not detectable (<0.156 μg/ml for both RWP1 and RWP2 cell lines). Finally, even if mouse monocytes were present in the cell cultures, it is highly unlikely that mouse tissue factor would contribute significantly to the shortening of human plasma recalcification times (30).

Our data suggest that tissue factor activity in RWP1 and RWP2 pancreatic cancer cells does not reside in mucin. Mucin appears to be a source of cancer procoagulant A (6) and not tissue factor. Also, electron micrographs of our membrane fractions show no evidence of mucin. Furthermore, chaotropic agents failed to strip the microvesicles of tissue factor activity, suggesting that the activity demonstrated is intrinsic to the membrane. Finally, the possibility of tumor proteases activating blood coagulation was ruled out by showing the ineffectiveness of serine and cysteine protease inhibitors in abrogating the coagulation in cancer patients. These included mucin, monocyte tissue factor, platelet aggregating factors, proteases, cancer procoagulant A, and cancer tissue factor. To rule out the possibility that monocytes were contributing to the procoagulant activity in our assay, only cell cultures maintained in vitro without xenografting for at least 1 year were used. Also, muramidase levels were measured in spent media and were not detectable (<0.156 μg/ml for both RWP1 and RWP2 cell lines). Finally, even if mouse monocytes were present in the cell cultures, it is highly unlikely that mouse tissue factor would contribute significantly to the shortening of human plasma recalcification times (30).

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Tissue factor activity has been described in a variety of neoplastic and normal cell cultures (5, 31). These findings in nonneoplastic cells as well as the fact that these are long-standing cell cultures whose characteristics may differ from the progenitor tumors make the interpretation of our data difficult in terms of clinical significance. Further study of other human pancreatic adenocarcinomas are needed to see whether our

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**Table 2** RWP1 subcellular fraction recalcifications and aggregation lag times

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein concentration (μg/ml)</th>
<th>Recalcification time (s)</th>
<th>Aggregation lag time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCH</td>
<td>2700</td>
<td>17.3</td>
<td>1</td>
</tr>
<tr>
<td>50K Sup</td>
<td>4000</td>
<td>44.3</td>
<td>1</td>
</tr>
<tr>
<td>50K Pellet</td>
<td>1500</td>
<td>16.5</td>
<td>1</td>
</tr>
<tr>
<td>MEM 1</td>
<td>20</td>
<td>33.3</td>
<td>5</td>
</tr>
<tr>
<td>MEM 2</td>
<td>260</td>
<td>20.0</td>
<td>2.5</td>
</tr>
<tr>
<td>MEM 3</td>
<td>280</td>
<td>25.0</td>
<td>6</td>
</tr>
<tr>
<td>MEM 4</td>
<td>190</td>
<td>36.0</td>
<td>8</td>
</tr>
</tbody>
</table>

* Small primary aggregation wave which completely and rapidly disaggregated (presumed intracellular ADP).

* No aggregation seen after 9 min.

been previously demonstrated to be the most enriched in plasma membrane, although MEM 2-4 are also plasma membrane enriched (22).

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**Fig. 4. Inhibition of platelet aggregations by anti-bovine brain tissue factor rabbit polyclonal IgG for RWP1 corresponding to text.** Note that, like hirudin, the inhibition of aggregation may be complete or may be demonstrated as prolongation of the lag period and/or a decreased slope. a, undiluted antibody; b, 1:2, c, 1:4; d, 1:8; e, 1:16; f, 1:32; g, 1:64; h, 1:128; i, 1:256; j, control.
findings can be generalized for this tumor type. A hamster pancreatic ductal adenocarcinoma model did not demonstrate tissue factor activity but rather showed platelet aggregation without clot formation (32).

Despite the aforementioned limitations on the conclusions that can be drawn, a hypothesis can be constructed for DIC in patients with pancreatic cancer. It is well known that tumors have abnormal vessels feeding them (33). These vessels have increased permeability that may permit access of coagulation factors to these tumor cells and/or access of tissue factor-containing microvesicles into the circulation. This would result in activation of clotting with the DIC picture seen in these patients.

Finally, the possible role of cancer tissue factor as well as other cancer procoagulants and platelet aggregation agonists in the formation of metastasis in patients such as those from which these lines were derived is of continuing clinical and research interest (34, 35).

ACKNOWLEDGMENTS

We would like to thank Dr. Ron Bach for kindly providing the rabbit polyclonal IgG anti-bovine brain tissue factor, and Dr. Bernard Lane for providing and interpreting the electron micrographs of our specimens.

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