Enrichment of Collagen and Gelatin Degrading Activities in the Plasma Membranes of Human Cancer Cells

Stanley Zucker, Janine M. Wieman, Rita M. Lysik, Dean Wilkie, Nungavaram S. Ramamurthy, Lorne M. Golub, and Bernard Lane

Department of Medicine and Research [S. Z. J. M. W., R. M. L., D. W. J., Veterans Administration Medical Center, Northport, New York 11768 and Schools of Dental Medicine [N. S. R., L. M. G.] and Medicine [S. Z., B. L.], Health Science Center, State University of New York at Stony Brook, Stony Brook, New York 11794

ABSTRACT

Interactions between connective tissue substrates and proteinases localized to the surface of cancer cells are implicated in cancer invasion. In this report, we have compared the enrichment of collagen and gelatin degrading activities and cysteine proteinase(s) in well-characterized (enzyme markers and electron microscopy) subcellular membrane fractions isolated from human small cell lung cancer lines (NCI-H69 and NCI-H82) and the RWP-1 pancreatic cancer line. With each cell line collagenolytic, gelatinolytic, and cysteine proteinase activities were enriched 5- to 128-fold in the plasma membrane fractions with differences noted between microvilli versus smooth membrane profiles. Incubation of tumor plasma membranes with methyl-3H-labeled collagen resulted in extensive degradation of the γ, β, α1, and α2 chains, suggesting the combined action of metalloproteinases. Treatment of tumor plasma membranes with the chaotrophic agent, 2 M KCl, did not diminish membrane collagen or gelatin-degrading activity, but extensively leached out the cysteine proteinase, suggesting that the latter enzyme is not an integral membrane protein. Enzyme inhibitors specific for metalloproteinases and cysteine proteinase were used to corroborate enzymatic classification. In conclusion, we have demonstrated variations in the localization of proteinases in the plasma membrane domains of different human cancer cells.

INTRODUCTION

The secretion of proteinases (collagenase, cathepsin B, plasminogen activator) and glycosidases by cancer cells has been proposed to play an essential role in cancer invasion and metastasis (1–7). We and others have emphasized the important function that cancer cell surface-localized proteinases exert in the degradation of connective tissue and cellular components during the process of cancer invasion in animal models (8–11). Using a nitrogen cavitation procedure to rupture tumor cells (10, 11) followed by sucrose density gradient ultracentrifugation, we have been able to isolate plasma membrane fractions which are highly enriched in proteolytic enzyme activity. The purpose of this report is to describe and compare the enrichment of collagenolytic, gelatinolytic, and a cathepsin B-like cysteine proteinase in the plasma membranes of highly metastatic human small cell lung and pancreatic cancer cells. The presence of these proteinases in the plasma membranes of cancer cells appears to localize these enzymes in the optimal environment to facilitate local cancer invasion.

MATERIALS AND METHODS

Athymic nude mice were obtained from the Charles River Laboratory under the auspices of an interagency agreement with the Frederick Cancer Research Center, National Cancer Institute, Frederick, MD.

Reagents. Fetal calf serum, nonessential amino acids, sodium pyruvate, vitamin solution, and L-glutamine were obtained from Flow Laboratories, Walkersville, MD. Trypsin-N-tosyl-L-phenylalaninechloromethyl ketone, DFP, 5-α-tosyl-l-lysine chloromethyl ketone (TLCK), soybean trypsin inhibitor, diithiothreitol, EDTA, N-ethyl maleimide, (Z-Phe-Arg-N-Mec), 4-amino-7-methyl coumarin, leupeptin, phenyl-methanesulfonyl fluoride, iodoacetate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were obtained from Sigma Chemical Co., St. Louis, MO. RPMI 1640 was obtained from Gibco, Grand Island, NY. Hydroyfluor was obtained from National Diagnostics, South Somerville, NJ. 1, 4-Dioxane and methanol were obtained from Fisher Scientific Products, Springfield, NJ. The human NCI-H69 and NCI-H82 small cell lung cancer lines were kindly provided by Dr. J. D. Minna and propagated in vitro as floating aggregates of cells in 90% RPMI 1640 and 10% heat-inactivated fetal calf serum in 95% air and 5% CO2 at 37°C as described previously (12). The human RWP-1 pancreatic cancer line was kindly provided by Dr. D. L. Dexter and propagated as adherent cells in 75-cm2 tissue culture flasks (13). Tumor cells were shown to be free of Mycoplasma and viral contamination. When cell numbers reached 1–2 x 107/flask the cells were collected nonenzymatically, washed by centrifugation, disaggregated by passage through a 25-gauge needle, and resuspended at 2 x 107 cells/ml in cold hypotonic lysis media containing 25 mM sucrose, 5 mM CaCl2, and 5 mM Tris-HCl, pH 7.4 (11). The cell suspension was then placed in a Parr bomb (Parr Instruments, Moline, IL) and exposed to 500 psi (for lung cancer cells) or 200 psi (for pancreatic cells) of N2 at 4°C for 30 and 20 min, respectively (11). The crude cell fragments (terming whole cell homogenate) were collected and centrifuged at 770 x g for 15 min at 4°C. The nuclear-enriched pellet was discarded and the supernatant was centrifuged at 50,000 x g for 20 min, resulting in a pellet enriched in cell organelles and a supernatant containing cytosol proteins. The resuspended 50,000 x g pellet was layered on a discontinuous sucrose density gradient consisting of 20, 25, 30, 35, 40, 45, and 50% sucrose. The gradient was centrifuged at 100,000 x g for 3 h at 4°C in a Beckman L5B ultracentrifuge with an SW28 rotor (Beckman Instruments Corporation, Palo Alto, CA). Visible bands were individually removed and the cell homogenates, crude cell organelles, and membrane bands washed by centrifugation. Pellets were resuspended in buffer or fixed on agar for electron microscopy (11). The cell suspensions were then placed in a Parr bomb (Parr Instruments, Moline, IL) and exposed to 500 psi (for lung cancer cells) or 200 psi (for pancreatic cells) of N2 at 4°C for 30 and 20 min, respectively (11). The crude cell fragments (terming whole cell homogenate) were collected and centrifuged at 770 x g for 15 min at 4°C. The nuclear-enriched pellet was discarded and the supernatant was centrifuged at 50,000 x g for 20 min, resulting in a pellet enriched in cell organelles and a supernatant containing cytosol proteins. The resuspended 50,000 x g pellet was layered on a discontinuous sucrose density gradient consisting of 20, 25, 30, 35, 40, 45, and 50% sucrose. The gradient was centrifuged at 100,000 x g for 3 h at 4°C in a Beckman L5B ultracentrifuge with an SW28 rotor (Beckman Instruments Corporation, Palo Alto, CA). Visible bands were individually removed and the cell homogenates, crude cell organelles, and membrane bands washed by centrifugation. Pellets were resuspended in buffer or fixed on agar for electron microscopy (11). The cell membranes were analyzed for cell organelle enzyme markers as we have previously described (10). Tumor cytosol from NCI-H69 cells was partially purified by 25–60% ammonium sulfate precipitation (1 h at 4°C), dialyzed against 25 mM cacodylate buffer, pH 7.2 containing 0.05% Brij 35 and 5 mM CaCl2, and applied to a Mono Q anion exchange column (Pharmacia Fine Chemicals, Piscataway, NJ). A linear gradient of 0–1.0 mM NaCl in cacodylate buffer was used to elute the bound proteins (procedural details will be published separately). Fractions active in collagenase and gelatinase activity were pooled and tested with pharmacological inhibitors.

Collagenolysis Assay. The degradation of collagen was measured using acid soluble lathyritic rat skin type I collagen labeled in vitro with [3H]Formaldehyde using our modification (10, 11) of the technique of Bhatnager and Decker (14). The suitability of the methylated lathyritic rat skin collagen used in our assay as a substrate for collagenase has been examined by examination of the conformation of methyl-3H-labeled collagen and random coiled gelatin substrates using circular dichroism. The helical form of methyl-3H-labeled collagen at 4, 24, and 2 The abbreviations used are: DFP, diisopropylfluorophosphatase; Z-Phe-Arg-N-Mec, benzoyloxycarbonyl-phenylalanyl-l-arginyl-7-(4-methyl)coumarylamide; TLCK, 5-α-tosyl-l-lysine chloromethyl ketone; TC, slow tropocollagen degradation product A (length of chain; TC, small tropocollagen degradation product B (length of chain).
29°C showed a band of positive ellipticity at 221 nm with a (θ) value of 6700 which is comparable to previous findings for native collagen [θ] of 7200 as described by Piez and Sherman (15). The methyl-3H-labeled gelatin had a weak band of negative ellipticity [θ] of ~1420 around 225 nm as previously described. Further confirmation of the suitability of our methyl-3H-labeled collagen substrate was the observation that this substrate was resistant to attack by gelatinase isolated from human polymorphonuclear leukocytes but was degraded by classical polymorphonuclear collagenase.4

Tumor cell fractions were activated with trypsin-G5-tosyl-L-phenylalaninylchloromethyl ketone and then inactivated with an excess of soybean trypsin inhibitor. Samples were then incubated with methyl-3H-labeled collagen and maintained in solution at 27°C for 18 h. The reaction was stopped and the undigested collagen was precipitated by the addition of dioxane. The reaction mixture was then centrifuged and an aliquot containing the collagen degradation products was added to Hydrofluor and counted in a liquid scintillation spectrometer. Activity was expressed as the percentage of degradation of 1 μg of methyl-3H-labeled collagen Type I. As previously described (11), trypsin (100 ng) alone did not raise the background degradation of collagen (<3%) as compared to 70% degradation by 2 ng bacterial collagenase. Enzyme inhibitor studies were performed as previously described (10). Fluorograms showing the digestion of methyl-3H-labeled collagen by tumor subcellular fractions were prepared by the method of Sodek et al. (16). After incubation at 22°C for 18 h, samples were thermally denatured at 60°C after which the collagen subunits and degradation products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were then processed for fluorography and exposed to Kodak XAR-5 film for 2 days.

Gelatinolytic Assay. The degradation of gelatin was measured over 2 h as we have previously (10) described using methyl-3H-labeled collagen Type I heated to 45°C for 20 min to produce the gelatin substrate. Data were analyzed as described in the collagenolysis assay.

Cysteine Proteinase Assay. Cysteine proteinase activity was determined using Z-phe-Arg-N-Mec, which is degraded by cathepsins B and L (10).

Miscellaneous. Protein determinations were done by the method of Markwell et al. (17) using bovine serum albumin as standard. Significant differences between groups were calculated using Student's t test.

Extraction of Nonintegral Proteinase Activity From Tumor Plasma Membranes. Lung cancer plasma membrane and crude cell organelle fractions isolated from NCI-H82 and NCI-H69 cells were washed extensively in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM) buffered NaCl (150 mM), pH 7.4, and then treated with the chaotropic agent 2 M potassium chloride in buffer at 4°C to remove nonintegral membrane proteins (18). After the 30-min incubation with 2 M KCl, or buffer (as a control) the subcellular fractions were centrifuged at 50,000 x g for 30 min. Collagen and gelatin degradation and cysteine proteinase activity residing in the pellets were measured as described above and compared to activity in the starting materials.

RESULTS

Microscopic Examination of Cancer Cells. On light microscopy NCI-H69 lung cancer cells are small cells with a nucleus:cytoplasmic ratio of 1. At the ultrastructural level, the cytoplasm is rich in ribosomes and dispersed filaments. There are few mitochondria and clusters of dense core granules. There is very little endoplasmic reticulum and Golgi complexes are small, single, and without dense contents. Of note is the scarcity of intracellular membranes which might contaminate a plasma membrane preparation (Fig. 1).

Intact RWP-1 pancreatic ductal cancer cells contained few lysosomes or secretory granules, small Golgi complexes, and extensive surface microvilli with alternating areas of glycolcalyx-free smooth zones.

Cancer Subcellular Fractionation and Marker Enzyme Assays. The NCI-H69 whole cell homogenate contained all of the cytoplasmic components including nuclei, secretory components, mitochondria, filaments, and small segments of rough endoplasmic reticulum. Disrupted membrane profiles represented less than 5% of the whole cell homogenate pellet by area. The 50,000 x g pellet consisted primarily of 10-nm diameter filaments in bundles, mitochondria, and membrane profiles representing less than 5% by volume of the specimen.

Ultracentrifugation of the 50,000 x g NCI-H69 pellet on a sucrose density gradient resulted in the production of three distinct bands at sucrose densities of 16 (band 1), 25 (band 2), and 35% (band 3), respectively. Membrane band 1 consisted of greater than 95% large, round, single membrane profiles ranging up to 2 μm in total circumference with an average of 0.5 μm (Fig. 2). Most of the membranes were approximately 10 nm thick and were consistent with plasma membranes. Less than 5% of the membranes were associated with fine filaments. Membrane band 2 was composed of 50% membrane profiles measuring approximately 0.5 μm in circumference and 30% smaller and flattened membrane profiles with associated filaments approximately 6 nm diameter (Fig. 3). Another class of filaments of 10 nm diameter represented 10% of the profiles. Scattered secretory granules and rare mitochondria were noted. Membrane band 3 was composed of 50% membrane profiles of approximately 0.5 μm diameter, 20–40% filaments of approximately 10 nm diameter, and occasional mitochondria and secretory granules (Fig. 4). Some of the membranes had associated glycolcalyx.

Comparison of enzyme markers in each H69 cell fraction revealed 16- to 18-fold enrichment in the plasma membrane enzyme, 5'-nucleotidase and a lesser degree of enrichment of
HUMAN CANCER CELL MEMBRANE PROTEINASES

Fig. 2. Membrane band 1 (isolated from NCI-H69 cells), highly enriched in plasma membranes, which are large round single membrane profiles. x 20,000.

Fig. 3. Membrane band 2 (isolated from NCI-H69 cells), composed of plasma membrane profiles and associated filaments of approximately 10 nm diameter. Scattered secretory granules are noted. x 20,000.

Fig. 4. Membrane band 3 (isolated from NCI-H69 cells), composed of approximately equal amounts of plasma membrane profiles and filaments of approximately 10 nm diameter. x 20,000.

the golgi marker, thiamine pyrophosphatase and the lysosomal marker, N-acetyl-β-glucosaminidase in membrane bands 1 and 2 (Table 1A). Band 2 had more of the mitochondrial marker, NADH oxidase, than did band 1. Of the membrane fractions, band 3 was least enriched in each of the cell organelle markers. The 50,000 x g pellet was somewhat enriched in 5'-nucleotidase and NADH oxidase and henceforth will be referred to as the crude organelle fraction. The 50,000 x g supernatant had lower levels of the above enzymes and will be referred to as the cytosol fraction. Characterization of NCI-H82 subcellular fractions gave results that were similar to NCI-H69 cells.

As we have previously described with RWP-2 pancreatic cancer cells (11), RWP-1 membrane band 1 demonstrated a preponderance of plasma membranes recognized at greater than 1 μm perimeter profiles. Membrane band 3 consisted largely of linear arrays of microvillous cell surface with glycocalyx coat and associated microvilli. The other major components were round membrane profiles consistent with plasma membranes of the microvillus-free regions of cells. Band 2 contained components present in both bands 1 and 3 (see Ref. 11 for comparable photomicrographs of RWP-2). Bands 1–3 isolated from RWP-1 cells were also highly enriched in 5'-nucleotidase (Table 1B).

Collagen and Gelatin Degrading Activities. The digestion of methyl-3H-labeled collagen by fresh human cancer subcellular fractions was minimal in the absence of trypsin activation. With trypsin activation, each of the NCI-H69, NCI-H82, and RWP-1 subcellular fractions demonstrated collagenolytic activity with significant (P < 0.01) enrichment (5- to 128-fold) in the plasma membrane-enriched fractions (Fig. 5; Table 2). After 2 months of storage at 4°C, tumor subcellular fractions became autoactivated and no longer required trypsin for activation. Evaluation
Glucosaminidase, NADH oxidase, and thiamine pyrophosphatase are most enriched in membrane band 2. Pancreatic cancer membrane fractions 1 and 3 are both highly enriched in 5′-nucleotidase.

Relative specific activities and thiamine pyrophosphatase using standard techniques (10). Specific enzyme activities (μM per mg per min) are listed for each fraction. Each enzyme assay was performed on three dilutions of the specimen, and the relative specific activities were determined from the linear portion of each curve. Relative specific activities (numbers in parentheses) are the specific activity of subcellular fraction/specific activity of cell homogenate. The relative specific enzyme enrichments for the membrane bands were reproduced on three separate occasions. Lung cancer cell membrane fractions 1 and 2 are highly enriched in the plasma membrane marker 5′-nucleotidase. Glucosaminidase, NADH oxidase, and thiamine pyrophosphatase are most enriched in membrane band 2. Pancreatic cancer membrane fractions 1 and 3 are both highly enriched in 5′-nucleotidase.

Table 1 Marker enzyme characterization of NCI-H69 small cell lung cancer and RWP-1 pancreatic cancer subcellular fractions

Lung cancer and pancreatic cancer subcellular fractions, isolated following nitrogen cavitation, were assayed for 5′-nucleotidase, NADH oxidase, glucosaminidase, and thiamine pyrophosphatase using standard techniques (10). Specific enzyme activities (μM per mg per min) are listed for each fraction. Each enzyme assay was performed on three dilutions of the specimen, and the relative specific activities were determined from the linear portion of each curve. Relative specific activities (numbers in parentheses) are the specific activity of subcellular fraction/specific activity of cell homogenate. The relative specific enzyme enrichments for the membrane bands were reproduced on three separate occasions. Lung cancer cell membrane fractions 1 and 2 are highly enriched in the plasma membrane marker 5′-nucleotidase. Glucosaminidase, NADH oxidase, and thiamine pyrophosphatase are most enriched in membrane band 2. Pancreatic cancer membrane fractions 1 and 3 are both highly enriched in 5′-nucleotidase.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cell homogenate</th>
<th>Cytosol</th>
<th>Crude organelles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Membrane bands</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A. Lung cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′-Nucleotidase (μM Pi/mg/min)</td>
<td>6.4</td>
<td>4.4 (0.7)</td>
<td>28.5 (4.4)</td>
</tr>
<tr>
<td>NADH oxidase (μM/mg/min)</td>
<td>980</td>
<td>920 (0.9)</td>
<td>2810 (2.9)</td>
</tr>
<tr>
<td>Glucosaminidase (μM/mg/30 min)</td>
<td>170</td>
<td>330 (1.9)</td>
<td>316 (1.9)</td>
</tr>
<tr>
<td>Thiamine pyrophosphatase (μM Pi/mg/min)</td>
<td>3.2</td>
<td>6.8 (2.1)</td>
<td>5.0 (1.6)</td>
</tr>
<tr>
<td>B. Pancreatic cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′-Nucleotidase (μM Pi/mg/min)</td>
<td>29</td>
<td>10 (0.3)*</td>
<td>35 (1.2)</td>
</tr>
<tr>
<td>NADH oxidase (μM/mg/min)</td>
<td>89</td>
<td>124 (1.4)</td>
<td>364 (4.1)</td>
</tr>
<tr>
<td>Glucosaminidase (μM/mg/30 min)</td>
<td>72</td>
<td>140 (1.9)</td>
<td>97 (1.3)</td>
</tr>
<tr>
<td>Thiamine pyrophosphatase (μM/mg/min)</td>
<td>2.1</td>
<td>3.6 (1.7)</td>
<td>1.8 (0.9)</td>
</tr>
</tbody>
</table>

Fluorographic studies of 3- to 18-hour incubations of trypsin-activated or autoactivated tumor cell membranes or crude organelles with methyl-3H-labeled collagen revealed extensive degradation of the γ, β, α1, and α2 chains. The collagen degradation products were multiple and varied between M, 60,000 and 20,000 (Figs. 6A and 7). TC^A products of approximately M, 75,000 were not visualized even at lower tumor enzyme concentrations or at shorter time intervals. By contrast, human granulocyte collagenase (purified by gel filtration and anion exchange chromatography) produced the typical TC^A degradation products (Fig. 6B).

Collagen degradation by NIH-69C or RWP-1 membrane bands 1 and 3 was inhibited (76–100%) by either 1,10-phenanthroline (1 mM), EDTA (3 mM), or dilute human serum (1:10). Diisopropyl fluorophosphate inhibited collagen degradation by 30–42%. Gelatin degradation by NIH-69C or RWP-1 membranes was likewise inhibited 54–100% by 1,10-phenanthroline, EDTA, or dilute human serum. Diisopropyl fluorophosphate inhibited gelatin degradation by 15–35%. Iodoacetate (2 mM) had no inhibitory effect on either tumor collagen or gelatin degrading activity.

Treatment of NCI-H82 plasma membranes and crude cell organelles for 30 min with the chaotropic agent, 2 M potassium chloride, resulted in no significant loss of tumor plasma membrane collagenolytic and gelatinolytic activities (Table 2). In fact, 2 washes of the cell membranes in buffered NaCl prior to the KCl treatment often led to an increase in metalloproteinase activities of the pellet fractions suggesting that an enzyme inhibitor may have been released or the enzyme enhanced in the washing process. In contrast, the NaCl washing and KCl treatment of the plasma membranes (bands 1 and 2 combined) and membrane band 3 resulted in a considerable decrease of residual cysteine proteinase activity (Table 2).

A 1300-fold enrichment in collagenolytic and gelatinolytic activity of the NCI-H69 tumor cytosol was achieved by ammonium sulfate precipitation followed by anion exchange chro-
Small cell lung cancer cell (NCI-H82) organelles were isolated by nitrogen cavitation and sucrose density gradient centrifugation. Aliquots of whole cell homogenate crude organelles, combined plasma membrane bands 1 and 2 and band 3 were washed three times in buffered 150 mM NaCl, pH 7.4, by centrifugation at 50,000 x g for 30 min. After washing, aliquots were treated with 2 mM KCl or buffered NaCl (as control) at 4°C for 30 min followed by an additional wash with buffered NaCl to remove the KCl. Collagenolytic, gelatinolytic, and cysteine proteinase in the pellets after centrifugation were assayed as described in the text. Collagen- and gelatin-degrading activities in the plasma membranes were not significantly decreased (P > 0.1) by extensive washing or treatment with a chaotropic agent. In contrast, extensive washing and KCl treatment resulted in a significant decrease in cysteine proteinase activity of NCI-H82 plasma membrane fractions. Similar results were achieved with NCI-H69 cancer cells (data not shown).

**Table 2: Extraction of non-integral proteinase activity from plasma membranes of NCI-H82 cancer cells by the chaotropic agent, potassium chloride**

<table>
<thead>
<tr>
<th>Sample (µg protein)</th>
<th>Collagen degradation (%/18 h)</th>
<th>Gelatin degradation (%/2 h)</th>
<th>Cysteine proteinase (nm Z-Phe-Arg-N-Mec/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Buffer</td>
<td>2 M KCl</td>
</tr>
<tr>
<td>Cell homogenate (8 µg)</td>
<td>6.5 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.8 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.6 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude organelles (37 µg)</td>
<td>15.2 ± 3.2</td>
<td>9.9 ± 1.9</td>
<td>10.6 ± 0.2</td>
</tr>
<tr>
<td>Plasma membranes (2 µg)</td>
<td>11.1 ± 0.1</td>
<td>10.3 ± 0.9</td>
<td>11.9 ± 1.6</td>
</tr>
<tr>
<td>Membrane band 3 (3 µg)</td>
<td>12.6 ± 0.5</td>
<td>13.0 ± 0.5</td>
<td>12.3 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SD of three determinations.

<sup>b</sup>Significantly different from enzyme determinations on the Initial sample; P < 0.05 (Student’s t test).

---

**Fig. 6. A, Fluorogram showing digestion of Type I methyl-<sup>3</sup>H-labeled collagen by trypsin-activated RWP-1 pancreatic cancer subcellular fractions. Lane 1, no enzyme added; lane 2, trypsin blank (buffer activated by trypsin and then inactivated by soybean trypsin inhibitor); lane 3, membrane 1 (2 µg); lane 4, whole cell homogenate (35 µg); lane 5, cytosol (10 µg); lane 6, membrane 3 (5 µg); lane 7, membrane 2 (10 µg); lane 8, crude organelles (60 µg). All incubations included 70 µl of sample activation by trypsin (except lane 1) and then inactivated with a 5-fold excess of soybean trypsin inhibitor; methyl-<sup>3</sup>H-labeled collagen (10 µg) was then added and the incubations at 22°C were terminated after 18 h. The α, β, α1, and α2 chains of collagen Type I are identified along with the diffuse digestion products found with the cancer samples (–•) and the buffer front (B.F.). B, Fluorogram showing the digestion of Type I methyl-<sup>3</sup>H-labeled collagen by trypsin-activated human granulocyte collagenase and the production of the typical TC<sup>a</sup> degradation products. Lane 1, buffer without added collagenase; lane 2, partially purified human granulocyte collagenase. Incubation conditions were identical to those of A and included trypsin activation of each sample followed by soybean trypsin inhibitor inactivation. The same batch of methyl-<sup>3</sup>H-labeled collagen substrate was used as in A. These chromatograms show that the cancer cell membrane collagenolytic activity differs from the effect of a classical collagenase which produces three-fourths and one-fourth degradation products.**
TLCK (0.3 mM) also inhibited cysteine proteinase activity by control containing buffer activated by trypsin and then inactivated by soybean trypsin inhibitor: lane 2. membrane band 1 (6 µg) that had become autoactivated after 3 months of storage at 4°C; lane 3. trypsin-activated membrane band 1 (5 µg). Incubation conditions were the same as in Fig. 6.

Fig. 7. Fluorogram showing the digestion of Type I methyl-3H-labeled collagen by auto- and trypsin-activated NCI-H69 lung cancer plasma membranes. Lane 1, control containing buffer activated by trypsin and then inactivated by soybean trypsin inhibitor; lane 2, membrane band 1 (6 µg) that had become autoactivated after 3 months of storage at 4°C; lane 3, trypsin-activated membrane band 1 (5 µg). Incubation conditions were the same as in Fig. 6.

a requirement for enzymatic activity. The cysteine proteinase inhibitors, N-ethyl maleimide (5 mM) and iodoacetate (2 mM) totally inhibited enzyme activity. Leupeptin (0.3 mM) and TLCK (0.3 mM) also inhibited cysteine proteinase activity by more than 90%. Minimal inhibitory activity was noted with DFP (1 mM) or soybean trypsin inhibitor (500 µg/ml). Cysteine proteinase activity of RWP-1 pancreatic cancer cells was more enriched in membrane band 3 than band 1 as we previously described for RWP-2 cells.

DISCUSSION

The pathophysiological role that proteinases play in cancer invasion has been the subject of intensive investigation. Most studies have used animal cancer cell lines as models of human disease and have emphasized the secretion of proteolytic enzymes by these tumors (1–10). Correlations have been made between tumor collagenase, cathepsin B, or plasminogen activator and the metastatic behavior of cancer cells (1–5). Recent emphasis has been on the secretion by tumor cells of collagenases with specificity for either interstitial collagens types I–III (6, 7, 19–24) or basement membrane collagen type IV (2, 22).

In this study, we have examined proteolytic enzyme activity from recently isolated human small cell lung and pancreatic ductal cancer cell lines. A comparison of these two tumors is of biological interest because (a) each tumor is representative of a different type of highly invasive and metastatic human cancer, (b) on xenogenic transplantation in nude mice, these tumors maintain morphological characteristics similar to the original tumors noted in humans, and (c) these tumors have markedly different growth characteristics in vitro (12, 13). In these experiments we have identified collagenolytic and gelatinolytic activities, and cathepsin B-like cysteine proteinase in the cell homogenates of both small cell lung (NCI-H69 and NCI-H82) and pancreatic cancer cell lines (RWP-1). The plasma membranes, isolated from cultivated tumor cells, were highly enriched in these proteolytic activities. While cysteine proteinase and metalloproteinase activity were noted in the smooth plasma membrane fractions of NCI-H69 and NCI-H82 lung cancer cells, the highest proteinase activities in RWP-1 and RWP-2 pancreatic cancer cells (11) were noted consistently in the microvillous surface membranes which were also enriched in 5′-nucleotidase activity. These observations are consistent with the concept of different specificities of plasma membrane domains on various types of tumor cells (11). Attempts to correlate proteinase activity with other biological characteristics of small cell lung cancer lines such as c-myc oncogenes (25) would be of considerable interest.

The subcellular localization of proteinases to the cancer cell surface membrane has been previously demonstrated in animal melanoma and breast cancer cells (9, 10). In this study, we have documented that the membrane-associated metalloproteinases appear to function as integral membrane proteins (18) since they were not extracted from the plasma membrane of human lung cancer cells following treatment with the chaotropic agent, potassium chloride. In contrast, cysteine proteinase activity was extensively leached out of the tumor plasma membrane by buffered saline washes even before treatment with KCl, indicating that the cysteine proteinase(s) are only loosely associated with the lung cancer plasma membrane. As characterized by fluorograms, the tumor cell fractions incubated with 3H-labeled collagen substrates, produced multiple digestion products smaller than the TCα and TCβ fragments which are characteristic of mammalian collagenase (6). This extensive collagen degradation, which was seen within 30–180 min incubation, is consistent with the activity of gelatinase or under special circumstances, cathepsin B, followed upon the initial split in native collagen Type I produced by classic interstitial collagenase (6, 26, 27). An alternative explanation is that this extensive degradation of collagen is the result of a single, broad spectrum metalloproteinase such as we have recently isolated from highly metastatic mouse B16-BL6 melanoma cells (28). Inhibition of the collagenase and gelatinase activities of partially purified NCI-H69 enzyme by EDTA or human serum and the lack of an effect with TLCK confirms the concept that the tumor enzyme is a metalloproteinase. The partial inhibitory effect of DFP on tumor gelatinase suggests that the partially purified metalloproteinase is contaminated by a serine proteinase.

Human cancer cells, especially of epithelial origin, have been previously reported to release metalloproteinases, serine proteinases, cysteine proteinases, and glycosidases during short-term incubations (2, 4, 19–21). Human tumors originating in the oral cavity, larynx, and bladder, containing the highest levels of collagenase have been reported to be more aggressive clinically than were tumors with lower enzymatic activity (19–20). Collagenase production in some tumors, however, appears to originate primarily from the stroma and not from the tumor.
elements (29). Our use of tumor cell lines lacking connective tissue components excludes a contribution by host tissues in enzyme production.

The secretion of cathepsin B-like cysteine proteinases by animal cancer cells as well as viable human breast cancer explants is well described (5, 30). In studies of B16 melanoma cell lines, Sloane et al. (5) reported significantly higher levels of lysosomal cathepsin B activity in tumor variants with greater metastatic potential. They suggested that cathepsin B may contribute to cancer invasion and dissemination by degrading extracellular matrix collagen and proteoglycans and by converting latent collagenase to its active form. Cysteine proteinase activity has also recently been described in the plasma membranes isolated from animal cancer cell lines (10) and human neoplastic cervical epithelial cells, but not in normal tissues (31).

In conclusion, we have demonstrated variations in the localization and activities of proteinases in the plasma membrane domains of different types of human cancer cells which might be of importance in the further characterization of factors involved in human cancer invasion and metastasis.

ACKNOWLEDGMENTS

The authors would like to thank Clara Krause and Hsi-Ming Lee for technical assistance, Nancy Mauceri for typing the manuscript, Joanne Ducato for preparing the illustrations, and Drs. J. Minna and D. Dexter for providing the cancer cell lines.

REFERENCES


HUMAN CANCER CELL MEMBRANE PROTEINASES
Enrichment of Collagen and Gelatin Degrading Activities in the Plasma Membranes of Human Cancer Cells


Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/47/6/1608

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.