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ABSTRACT

A detailed understanding of the pathogenesis of colon cancer metastasis has been hindered by the lack of appropriate animal models which accurately reflect events in this complex process. An animal model for colon cancer metastasis is described in which spontaneously metastasizing colon tumors are formed after injection of murine colon cancer cells into the cecal wall of BALB/c mice. Using this model, tumor cells with different liver-metastasizing potential were selected and shown to possess several properties known to be associated with other metastatic cell lines. The ability of tumor cells to invade a reconstituted basement membrane and to secrete type IV collagenase was directly proportional to their metastatic ability. In addition, liver-metastasizing cells preferentially migrated toward liver extracts in a Boyden chamber assay, as compared to extracts of brain or lung, and adhered rapidly to highly purified hepatic sinusoidal endothelial cells versus hepatic parenchymal cells in vitro. This model may thus be useful for studying many aspects of the pathogenesis of colon cancer metastasis.

INTRODUCTION

Most cancers are potentially lethal because of the ability of malignant cells to disseminate from the primary tumor and proliferate at distant sites (1, 2). Tumor cell subpopulations with different metastatic abilities exist within the same primary tumor (3–5), and metastases result from the nonrandom, selective dissemination of those tumor cells possessing the ability to invade lymphatic and vascular structures, embolize in the bloodstream, survive interactions with blood components and the immune system, and be transported to distant organs where they adhere to target structures, extravasate, and establish secondary tumor foci (1–5). Methods for identifying those cells most likely to metastasize and understanding the biological properties which allow these cells to complete the process is a high priority of cancer research. Suitable models for cancer metastasis are necessary for such detailed analysis of this complex multistep process (1). Several experimental models using a number of metastatic variants and clones have been studied involving a variety of established cell lines and even primary tumors, particularly sarcomas (1, 6, 7). Metastatic models for carcinomas have been more limited and most often utilize murine carcinomas injected s.c. or by i.v. tail vein injection into syngeneic animals (1, 8–10). Some human tumors can metastasize in athymic nude mice after s.c. injection, footpad, or i.v. injection (11–13), with metastases often limited to lymph nodes or lungs. While these models may be appropriate for studying tumors such as melanoma, lymphoma, various sarcomas, and perhaps even mammary carcinoma, in the case of colon cancer these systems may bypass many of the complex processes of the metastatic cascade.

While hepatic metastases are a frequent event in patients with colon cancer, they rarely occur in existing animal models of colon cancer. Melanoma cell lines have been developed which preferentially metastasize to the liver after intraperitoneal or i.v. injection (14), and hepatic and pulmonary nodules have been reported in syngeneic C57BL/6 mice or in preconditioned nude mice given injections intrasplenically of murine (15) or human (16, 17) cell lines, respectively. It has been suggested that the establishment of experimental primary tumors in the colon after colonic wall injection may provide an accurate model for studying colon cancer metastasis after both syngeneic (18–20) and heterotopic (20, 21) implantation. In this paper we describe our experience with an animal model in which murine colon cancer cells reproductively metastasize to the liver of syngeneic animals via normal routes in a defined period of time. This model was used to establish colon cancer cell lines with high liver-metastasizing abilities, and these cells have been partially characterized with regard to biological properties known to be associated with the invasiveness of other metastatic cell lines.

MATERIALS AND METHODS

Cell Lines

Parental cell line 51B was established in this laboratory from transplantable murine colon carcinoma 51. The original transplantable tumor, established by Dr. T. H. Corbett (22) by repeated s.c. injection with 1,2-dimethylhydrazine, forms grade 11–111 carcinomas when passed into syngeneic BALB/c mice. It has been suggested that the establishment of experimental primary tumors in the colon after colonic wall injection may provide an accurate model for studying colon cancer metastasis after both syngeneic (18–20) and heterotopic (20, 21) implantation. In this paper we describe our experience with an animal model in which murine colon cancer cells reproductively metastasize to the liver of syngeneic animals via normal routes in a defined period of time. This model was used to establish colon cancer cell lines with high liver-metastasizing abilities, and these cells have been partially characterized with regard to biological properties known to be associated with the invasiveness of other metastatic cell lines.

Preparation of Cell Lines for Injection

All cell lines were grown and maintained in DMEM1 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and a 7% CO2 environment. Early passage cell lines (passaged fewer than 15 times) were used for all studies unless otherwise noted. Confluent cultures were harvested by brief trypsinization (0.05% trypsin-0.02% EDTA in Hanks' balanced salt solution without calcium and magnesium), washed several times with CMF, and resuspended at a final concentration of 5 × 106 cells/ml in serum-free medium. The presence of single cell suspensions was confirmed by phase contrast microscopy, and cell viability was determined by trypan blue exclusion.

Cecal Injection

BALB/c mice (15–18 g females obtained from Simonsen Laboratories, Gilroy, CA) were anaesthetized with methoxyflurane by

1The abbreviations used are: DMEM, Dulbecco's modified Eagle's minimal essential medium; CMF, calcium-magnesium-free phosphate-buffered saline; BSA, bovine serum albumin.

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inhalation, and the abdomen was prepared in a sterile fashion. A small abdominal incision was made, and the cecum was identified and isolated between sterile gauze; 5 x 10^6 viable tumor cells in 0.1 ml serum-free medium were injected into the cecal wall from the serosal side using a sterile tuberculin syringe and slightly curved 27-gauge needle. Frequent trituration of cells was performed in a sterile syringe in order to maintain uniform cell suspensions and to prevent clumping. Cells were injected so as to visibly infiltrate between the submucosal and subserosal tissues. The serosal surface at the injection site was dabbed gently with sterile gauze dampened with 95% ethanol in an attempt to kill free tumor cells and the organs replaced in situ. The abdomen was closed with continuous nylon sutures, and the animal was returned to its cage. After 4 weeks, animals were sacrificed by cervical dislocation and the abdominal organs and thorax examined for the presence of macroscopic "primary" cecal tumors and metastases. Pilot studies demonstrated that at this time cecal tumors of approximately 0.5 cm were present without peritoneal spread of tumor. Organs including cecum, liver, mesenteric lymph nodes, and lungs were divided, and paired specimens were processed for histological examination and tissue culture.

Histological Examination and Scoring of Metastases

Specimens for histological examination were fixed in 10% formalin for 24 h. The entire liver of each animal was sectioned at 0.2-cm intervals, the cut surfaces examined, and the specimens were embedded in paraffin. Representative sections of cecum, lymph nodes, and lungs were also cut and embedded in paraffin. Five-µm sections were then cut and stained with hematoxylin and eosin and periodic acid-Schiff to confirm the presence of tumor and examined for microscopic metastases by light microscopy.

Macroscopic metastases were defined as those visible to the naked eye. All macroscopic metastases were verified by histological examination. Microscopic metastases were those visible on histological examination by light microscopy of serial hematoxylin-eosin-stained tissue sections.

Electron Microscopy

Tissue sections of liver were immersed for 2 h in a fixature consisting of 2.5% glutaraldehyde-0.8% paraformaldehyde in 0.2 M sodium bicarbonate buffer. Tissues were osmicated with 1% osmium tetroxide containing 1.5% potassium cyanide for 90 min, washed in 0.2 M sodium bicarbonate buffer for 10 min, and then were dehydrated in ethanol and embedded in Epon. Sections were stained with lead citrate and uranyl acetate and examined in a Philips EM 300 microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Culture of Metastatic Foci and Establishment of Cell Lines with Enhanced Metastatic Activity

Specimens of macroscopic hepatic metastases and liver potentially containing metastases were washed several times in tissue culture medium containing 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone. The tissue was minced finely with a sterile scalpel blade and resuspended in DMEM supplemented as above and with 10% fetal calf serum. Various amounts of tissue were plated into 25-cm² plastic tissue culture flasks which were gassed to contain 7% CO₂ and 2.5 µg/ml fungizone. The tissue was minced finely with a sterile scalpel blade and resuspended in DMEM supplemented as above and then were dehydrated in ethanol and embedded in Epon. Sections were stained with lead citrate and uranyl acetate and examined in a Philips EM 300 microscope (Phillips Electronic Instruments, Inc., Mahwah, NJ).

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Invasion Assay

The invasive potential of tumor cells was determined with an in vitro invasion assay (24). Briefly, cells were tested for their ability to penetrate through a reconstituted basement membrane matrix which was composed of purified bovine tendon type I collagen (0.2 mg/matrix (25)), type IV collagen (0.2 mg/matrix (26)), and laminin (0.2 mg/matrix (27)) compressed to form a 200- to 600-µm thick mat (Heitlrex Corp., Princeton, NJ). The barrier was placed into a modified Boyden chamber directly on a type IV collagen-coated Nucleopore filter (5 µg protein/filter, pore size, 1.0 µm).

A suspension of tumor cells (7 x 10⁶) in DMEM containing 2% acid-treated NuSera (28) and 0.5% BSA was introduced into the upper compartment and DMEM containing 0.5% BSA was placed into the lower compartment of the chamber. For chemoinvasion assays (29), the medium in the lower compartment also contained partially purified organ-derive antitragents (prepared as in the chemotaxis assay below). Similar extracts have been used in previous studies of tumor cell chemoinsvasion and chemotaxis (24, 29). After 24–36 h of incubation at 37°C in 95% air and 5% CO₂ and the barrier was separated from the underlying Nucleopore filter. The cells having penetrated the matrix and attached to the upper surface of the Nucleopore filter were detached with trypsin/EDTA (0.05/0.1%) solution and counted using an electronic counter. The data are expressed as the percentage of cells which penetrated the matrix ±SD. All assays were performed at least in triplicate.

Chemotaxis Assay

To assess the tendency of tumor cells to be directionally attracted to organ-specific factors, they were studied using a modified Boyden chamber assay (30). Brain, liver, and lung tissues were removed b 8- to 10-week-old C57/BL female mice, and partially purified extracts were prepared as previously described (29), with slight modification. Tissue extracts were washed three times in DMEM containing 50 µg/ml gentamicin. After homogenization at 4°C in a buffer (1 ml buffer/g tissue) containing 0.05 M Tris-HCl, pH 7.4, 2 M guanidine HCl, 0.5 M NaCl, 5 mM N-ethylmaleimide, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 50 µg/ml gentamicin, homogenates were extracted by shaking with buffer for 24 h at 4°C. Extracts were then centrifuged at 100,000 x g for 4 h and the supernatant fractions dialyzed against phosphate-buffered saline. After an ammonium sulfate precipitation (0–60%), supernatants (dialyzed versus DMEM) were tested for their chemotactic activity. The protein level in the extracts was determined by the method of Lowry et al. (31).

For the chemotaxis assay, polynvinylpyrrolidone-free Nucleopore polycarbonate filters (pore diameter, 8 µm; Neuroprobe, Inc.) were coated with type IV collagen (5 µg/filter) and placed into a modified Boyden chamber to separate the upper and lower compartments. Tissue extracts (10% solutions) in DMEM containing 0.5% BSA were introduced into the lower compartment and tumor cells (5 x 10⁶, detached with 0.1% EDTA) in DMEM containing 0.5% BSA were placed into the upper compartment. After a 5-h incubation at 37°C, the filters were removed and stained in Hemacolor solutions 1, 2, and 3 (Hareleo, Gibbstown, NJ). Filters were then placed top side up on glass slides and cells that had not migrated were removed with cotton swabs. Migration of cells was quantitated by counting eight randomly selected high power (400) fields/filter. Results are expressed as the average number of migrated cells per high power field. All assays were done in triplicate.

Type IV Collagenase Assay

The type IV collagen-degrading metalloprotease activity secreted by selected tumor cells was measured as previously described (32, 33). Briefly, nearly confluent tumor cell monolayers were washed three times with DMEM and subsequently incubated in serum-free medium (DMEM) at 37°C with 95% air/5% CO₂. After a 24-h incubation, medium was collected and cell debris was removed by centrifugation at 2000 rpm for 5 min. Medium was concentrated 100-fold with ammonium sulfate precipitation (0–60%) followed by dialysis against 0.05 M Tris-HCl, pH 7.6-0.2 M NaCl, at 4°C (34). Type IV collagen degradation was assayed using soluble [³H]proline-labeled type IV procollagen as a substrate (35). The enzyme activity was expressed as the amount of type IV collagen degradation (cpm released/10⁶ cells).
**Cell-Cell Attachment**

Labeling of Tumor Cells. Tumor cells were labeled according to the method of Fidler and Nicolson (36). Cells were grown as described above in DMEM supplemented with penicillin, streptomycin, and 10% fetal calf serum. Actively growing subconfluent cultures were incubated for 24 h with medium containing 0.3 μCi [125I]iododeoxyuridine/ml medium (specific activity, 2000 Ci/mmol; ICN Radiochemicals, Irvine, CA) which labeled nearly all cells as determined by autoradiography.

Preparation of Target Cells. Purified hepatocytes and hepatic sinusoidal endothelial cells were prepared by a modification of that previously described (37, 38) from the livers of 200- to 250-g male Sprague-Dawley rats. Cells were purified by collagenase perfusion, Stractan density gradient centrifugation, and centrifugal elutriation. Equal numbers of the fractionated liver cells were plated into 35-mm culture dishes (Lux Scientific, New York, NY), and grown to confluency at 37°C in a humidified incubator under 2% CO₂ in air. The purity of endothelial cells prepared by this method has been verified by electron microscopy, specific uptake of modified lipoproteins (acetooctayed low-density lipoprotein), and the presence of cytochemical and morphological markers of vascular endothelia (37).

Attachment Assay. Tumor cells labeled as above were washed five times with CMF, harvested by brief trypsinization and gentle mechanical dissociation, suspended in “cold” tissue culture medium, and the number of cells determined using an electronic cell counter (Coulter Electronics, Hialeah, FL). Cell viability was greater than 95% as tested by trypan blue dye exclusion and single-cell suspensions documented by phase microscopy. Target cells were grown to confluent monolayers in 35-mm culture dishes as described above, washed with CMF, and overlayed with single-cell suspensions of 5 x 10⁵ labeled tumor cells in 2 ml DMEM/dish. Duplicate dishes not containing target cells were used as controls (“passive”). Dishes were incubated for 10 or 30 min at 37°C in a humidified incubator under 7% CO₂ in air. At specified time points cultures were observed by phase microscopy for attachment of tumor to target cells, and the overlying media and unattached cells were aspirated and collected. Cultures were washed x 3 with CMF to remove unbound cells, and washes were added to the previously aspirated media. Tumor cells remaining bound to target cells were collected separately by trypsinization and mechanical scraping. Collected material was then subjected to gamma counting (Beckman Instruments, San Jose, CA). The percentage of adhesion was defined as

Counts bound (cells)

Counts in medium + washes + counts bound (total counts) x 100

**Statistical Analysis**

The data were analyzed by an independent statistician. To take into account the multiple comparisons for chemoinvasion and invasion assays run for 24 h (between the three cell lines and between the two methods), pairwise comparisons between groups were done using the Tukey’s Studentized range test. Because of the inequality of variance for the two cell lines compared at 36 h, t tests for unequal variance were done for comparison between cell lines. For chemoinvasion versus invasion at 36 h, a t test for equal variance was done. We used Bonferroni-adjusted P values for these tests to allow for multiple comparisons. For the chemotaxis assay a comparison of means by one-way analysis of variance was shown to be significant at the 0.0001 level. Pairwise comparisons between groups were therefore performed using the Tukey’s Studentized range test.

**RESULTS**

Liver-metastasizing Potential of Tumor Cells. Murine colon cancer cell line 51B formed poorly to moderately differentiated carcinomas without liver-metastasizing ability when injected s.c. into BALB/c mice (0 of 20 animals). Similarly, this cell line did not form hepatic tumors when injected into the tail veins of additional mice (0 of 10 animals). On the other hand, cells derived from line 51B when implanted into the cecal wall developed into colonic tumors (Fig. 1) which spontaneously metastasized to the liver in a minority (4 of 21) of the animals. Metastases were demonstrated by light microscopy to invade the liver from portal tributaries, and to be present in subcapsular areas, or as intraparenchymal microfoci (Fig. 2). Electron microscopy revealed tumor cells in the hepatic sinusoids in apposition to sinusoidal endothelium and intercalated between sinusoidal endothelium and hepatic parenchymal cells (Fig. 3).

In order to determine whether tumor cell lines with progressively increased metastatic ability could be developed in our model, metastases from the original tumor line were cultured and used as a source of cells for successive cycling. Cells with progressively increased metastatic potential were isolated in successive cycles (Table 1). Thus parental cell line 51B formed visually apparent hepatic metastases in 2 of 21 and microscopic metastases in 4 of 21 animals tested, while all animals given injections of the cell lines derived from the fourth and fifth metastatic cycles developed macroscopic metastases. In addition the number of hepatic tumor nodules per animal increased with serial selection. This was most pronounced with cells from the fifth metastatic cycle. Mesenteric lymph node involvement by tumor could also be demonstrated (Fig. 4) in those animals developing liver metastases, but pulmonary metastases from the cecal site were only observed in one animal.

Although 51B appeared more heterogeneous in vitro (stellite cells with multiple processes, occasional round cells with abundant cytoplasm, triangular cells) than its most metastatic derivatives (predominantly triangular cells), s.c. or cecal homografts and metastases formed after injection of the selected lines did not differ histologically from those formed after injection of the parental cell line. Tumors were moderately to poorly differentiated with only sparse production of periodic acid-Schiff-positive mucin, and appeared similar histologically to those reported by Corbett for transplantable tumor 51 (22). The in vitro doubling times of all cell lines were similar, and s.c. and cecal homografts from the various cell lines grew with similar latency and growth rates, suggesting that doubling time variation did not explain the difference in metastatic ability. Rather, various studies as discussed below indicate that the metastases contain more invasive cells which show some specificity in their response to liver factors.

Metastases from Sites Other Than the Cecum. In a separate set of experiments additional paired groups of animals were given injections of cell line 51B or 51B LiM-5 either s.c. or i.v. via the tail vein. For s.c. injections, animals were given injections in the right posterior or anterior flank with 5 x 10⁶ tumor cells, sacrificed at 5 weeks, and organs examined for macroscopic and microscopic metastases as described above. Of the 10 animals given injections s.c. of parental cell line 51B, 2 developed spontaneous pulmonary parenchymal metastases (one macroscopic, one microscopic only), 2 additional animals had mediastinal lymph node metastases, and none was noted to have developed liver metastases. In contrast 0 of 7 animals given injections s.c. of cell line 51B LiM-5 developed pulmonary metastases, 2 of 7 developed mediastinal lymph node metastases and 4 of 7 had macroscopic liver metastases. Of the animals given injections i.v. of 51B (2.5 x 10⁶ cells) 2 of 12 had single microscopic foci of tumor present in the liver. In contrast, 7 of 10 animals given injections of 51B LiM-5 had multiple areas of macroscopic tumor present in this organ. Pulmonary metastases were present in both groups of animals given injections i.v.

Invasion. The ability of parental cell line 51B and its metastatic derivatives 51B LiM-4 and 51B LiM-5 to penetrate a reconstituted basement membrane was tested in the absence (invasion) and presence (chemoinvasion: chemotaxis plus in-
ANIMAL MODEL FOR COLON CANCER METASTASIS

Fig. 1. Experimental cecal “primary” tumor. BALB/c mice were sacrificed 4 weeks after injection of $5 \times 10^6$ tumor cells into the cecal wall. Tumor cells (7) are seen in relation to normal mouse colonic mucosa. Hematoxylin-eosin, x 100.

Invasion of partially purified liver extract in the lower compartment of the Boyden chamber (Fig. 5). Experiments were performed for 24 h with the three cell lines and in addition for 36 h with 51B and 51B LiM-4. More cells crossed the basement membrane barrier at the longer time and the more metastatic lines were also more invasive. This was particularly evident when a liver extract was added to the lower compartment. The liver extract increased the invasion of all tumor cell lines. Cell line 51B LiM-4 was 3-fold and 51B LiM-5 was 5.6-fold more invasive and chemoinvasive at 24 h than cell line 51B studied under the same conditions ($P < 0.001$; see Fig. 5). At 36 h, both invasion and chemoinvasion by cell line 51B LiM-4 were approximately 6-fold greater than that of 51B under the same conditions ($P < 0.005$ for invasion or chemoinvasion).

Chemotaxis. The possibility that these metastatic tumor cells were preferentially attracted to a liver factor(s) was tested in the Boyden chamber assay using filters which had been coated with type IV collagen and extracts from brain, lung, and liver (Fig. 6). Liver extract stimulated the movement of 51B LiM-5 tumor cells 12-fold compared to 0.5% bovine serum albumin alone, and 2.2- and 2.8-fold compared to extracts of lung or brain, respectively ($P < 0.001$ for migration to the presence of liver extract versus BSA, brain, or lung extract).

In order to further define the chemotactic movement of cells from line 51B LiM-5 in response to liver factors, a Zigmond-Hirsch checkerboard analysis was performed (39) (Fig. 7). Different concentrations of liver extract were placed on both sides of the Boyden chamber. Cells in the upper chamber migrated most actively and in a dose-dependent manner when the concentration of liver extract was greatest in the lower chamber (Fig. 7, data below the diagonal). This extract also stimulated the chemokinesis or random migration of 51B LiM-5 cells (Fig. 7, data on the diagonal), especially at high concentrations of extract. Little increase in cell migration was observed when a negative gradient of extract was established (Fig. 7, data above the diagonal).

Collagenase Type IV. The type IV collagen-degrading metalloprotease activity secreted by parental cell line 51B and metastatic cell lines 51B LiM-4 and 51B LiM-5 was compared using soluble [H]proline-labeled type IV procollagen as the substrate (Table 2). Cell line 51B LiM-4 produced 2.5-fold ($P < 0.05$) and 51B LiM-5 9-fold ($P < 0.005$) greater collagen degrading activity than did parental cell line 51B.

Tumor Cell Adhesion. The ability of tumor cells from metastatic line 51B LiM-3 to bind selectively to purified hepatocytes and hepatic sinusoidal endothelial cells was tested in vitro (Table 3). Tumor cells were found to bind rapidly and preferentially to hepatic sinusoidal endothelial cells when compared to hepatocytes or a plastic control. At 10 min tumor cell adhesion to endothelial cells was more than 5-fold greater than to hepatocytes ($P < 0.001$). Binding to hepatocytes differed little from that of control.

DISCUSSION

The study of cancer metastasis can be greatly facilitated by models which accurately reflect events in the metastatic process. We describe an animal model for colon cancer metastasis in which metastases arise from colonic tumors which are formed in a relatively short period after injection of tumor cells into the cecal wall of syngeneic BALB/c mice. By injecting a cell line derived from a transplantable murine colon carcinoma into the cecal wall, we found that tumor cells can disseminate from the resulting “primary” tumor in a minority of animals and...
form both micro- and macrometastases in the liver. Various observations discussed below suggest that this may represent a partially organ-specific metastasis.

Lines of more highly metastatic cells can be established by various selection procedures, including the sequential injection of cells derived from metastases in the classic method popularized by Fidler (23). This approach was attempted here by cultivating tumor cells which had spread to the liver, injecting them into additional animals, and reculturing subsequent metastases. This resulted in new lines of colon cancer cells which produced metastases from cecal sites in all animals given injections. Pulmonary lesions on the other hand were very rare, occurring in only one animal with extensive hepatic metastases.

At least some metastatic cells demonstrate a common phenotype in their interactions with basement membranes, binding to them, secreting proteases, and invading the extracellular matrix (33, 40-51). Some of these properties were examined using the colon cancer cells developed here to determine whether for these epithelial cancer cells invasiveness and metastatic potential were correlated. Indeed, when tested against a reconstituted basement membrane composed of collagen IV and laminin, their ability to penetrate it paralleled their meta-

Fig. 2. Liver metastases from experimental cecal tumors. A, macroscopic liver metastases (arrowheads). × 2; B, tumor cells similar to those of the primary tumor invading the liver parenchyma from a portal tributary. PV, portal vein. Hematoxylin-eosin, × 120; C, intraparenchymal focus of tumor cells. Hematoxylin-eosin, × 400.
Fig. 3. Electron micrograph demonstrating tumor cells (T) in relation to hepatic sinusoidal endothelium (E) and hepatic parenchymal cells (H). S, sinusoid; rbc, red blood cell; c, collagen. Arrow, apparent contact between tumor cell and endothelium. × 9600.
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Table 1 Metastatic ability of tumor cell lines

The liver-metastasizing ability of cancer cell lines was evaluated after injection and formation of experimental cecal primary tumors. Animals were sacrificed 4 weeks after cecal injections of 5 \( \times 10^6 \) tumor cells into the cecal wall. Population doubling times were determined from the logarithmic portion of the growth curve. Each time point of the growth curve represented the mean of three separate cultures. Macroscopic metastases were those visible to the naked eye. Microscopic metastases were determined by examination of serial tissue sections as described in the text. The number of hepatic tumor foci per animal was determined by examining 5 \( \mu \text{m} \) microscopic sections after the entire liver was sectioned at 0.2-cm intervals. Animals given injections of parental cell line SIB were studied initially and as simultaneous controls with cycles 4 and 5. The low incidence of liver metastasis for the parental cell line noted initially was reproduced in subsequent experiments (0 of 10 and 2 of 11 animals given injections).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Metastatic cycle</th>
<th>Doubling time (h)</th>
<th>No. of hepatic metastasis-bearing mice</th>
<th>No. of hepatic tumor foci/animal (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIB</td>
<td>Parent</td>
<td>10.6</td>
<td>2/21</td>
<td>1-3</td>
</tr>
<tr>
<td>SIB LiM-1</td>
<td>1</td>
<td>10.1</td>
<td>0/7</td>
<td>4</td>
</tr>
<tr>
<td>SIB LiM-2</td>
<td>2</td>
<td>10.6</td>
<td>4/6</td>
<td>3-6</td>
</tr>
<tr>
<td>SIB LiM-3</td>
<td>3</td>
<td>11.5</td>
<td>6/8</td>
<td>5-10</td>
</tr>
<tr>
<td>SIB LiM-4</td>
<td>4</td>
<td>12.3</td>
<td>8/9</td>
<td>5-15</td>
</tr>
<tr>
<td>SIB LiM-5</td>
<td>5</td>
<td>10.6</td>
<td>10/10</td>
<td>50-100</td>
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In addition, the cells were found to produce collagen IV-degrading enzyme levels proportional to their metastatic activity. These studies suggest that the increased metastatic activity observed in the selected cells could be related, in part, to their ability to pass the basement membrane barriers which limit the spread of cells from the cecum and from the hepatic sinusoids to the liver parenchyma.

Clinical experience and experimental studies (36, 52, 53) strongly suggest that many human and animal tumors metastasize to preferential sites. Although this might be in part based on their site of entry into the circulation (52), other factors must be involved (1, 52). Such factors may include tissue-specific chemoattractants (29, 54-59) and preferential adhesion of metastatic tumor cells to target organ parenchymal cells or endothelium. In our study liver extract stimulated the movement of highly liver-metastasizing tumor cells to a significantly greater degree than did bovine serum albumin or extracts of lung or brain (Fig. 6). This same liver extract enhanced the invasion of tumor cells through the reconstituted basement membrane. These results are similar to those reported by Hujanen and Terranova (29) who demonstrated preferential migration of melanoma, sarcoma, and breast carcinoma cells to extracts from the organs for which they show metastatic predilection. The nature of such attractants remains to be determined, but these workers found the factors in brain and liver to be of different molecular weights. According to these results, the metastasizing tumor cells can disseminate among different tissues based on the presence of local factors stimulating their migration.

Preferential organ attachment of metastatic tumor cells has been studied in vitro by a number of investigators (60-66). These studies have examined adhesion of tumor cells to frozen tissue sections (61), organ culture slices (60), or cultured cell monolayers (63-66). Using highly purified monolayer cultures of target cells, we have demonstrated specific in vitro adherence of radionabeled metastatic colon cancer cells to hepatic sinusoidal endothelial cells but not hepatocytes (Table 3). Rat target cells were used since highly purified murine cells cannot be easily prepared by our method in sufficient numbers from the mouse to allow such studies. Our results are not surprising, since blood-borne metastatic cells must first adhere to endothelial walls and invade through basement membranes before en-

Fig. 4. Regional lymph node metastasis showing tumor cells (T) similar to those of the primary tumor in a mesenheric lymph node. Hematoxylin-eosin, × 250.
ANIMAL MODEL FOR COLON CANCER METASTASIS

Table 2  Type IV collagen-degrading activity of tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type IV collagen-degrading activity (cpm released/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51B</td>
<td>191 ± 8.5</td>
</tr>
<tr>
<td>51B LIM-4</td>
<td>475 ± 106</td>
</tr>
<tr>
<td>51B LIM-5</td>
<td>1704 ± 23</td>
</tr>
</tbody>
</table>

* P < 0.05.  
* * P < 0.005 compared to 51B.

Table 3  Percentage of tumor cell adhesion to liver-derived cells

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Endothelial cells</th>
<th>Hepatocytes</th>
<th>Plastic</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>47.7 ± 4.3 (8)</td>
<td>8.0 ± 3.9 (8)</td>
<td>11.2 ± 1.5 (8)</td>
</tr>
<tr>
<td>30</td>
<td>64.7 ± 9.5 (5)</td>
<td>17.1 ± 11.6 (5)</td>
<td>29.8 ± 6.2 (5)</td>
</tr>
</tbody>
</table>

* Mean ± SD.  
* * P < 0.001.  
* Numbers in parentheses, number of experiments.  
* * P < 0.005 compared to either hepatocytes or plastic.

countering organ parenchymal cells. Nicolson et al. (60) found that B16 melanoma cells often bound to exposed blood vessels, and therefore attached to endothelial rather than parenchymal cells in organ culture. Others, however, have shown that hepatocyte surface molecules may be involved in the adhesion of some types of carcinoma cells to rat hepatocyte cultures (64) and that the mechanism of adhesion of different tumor cells to hepatic organ target cells may be different (66). We have not yet tested whether the adhesion of our colon cancer cells to hepatic sinusoidal endothelial cells is specific for this endothelium in particular or endothelial cells in general. Experiments by Alby and Auerbach (65) have demonstrated that capillary endothelial cells derived from different organs are not alike and that differences expressed at the cell surface of these cells can be distinguished by tumor cells.

The model which we have described should be useful for studying many factors which might affect the invasive activity and metastasis of colon cancer cells. Similarly, the highly liver-metastasizing cells selected may be used in this model to assess therapeutic regimens that could lessen the spread of this disease.

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REFERENCES

ANIMAL MODEL FOR COLON CANCER METASTASIS


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