Characterization of Two Metastatic Subpopulations Originating from a Single Human Colon Carcinoma

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ABSTRACT

Two separate cell lines originating from distinct metastatic deposits in a patient with a primary colon carcinoma have been established both in vivo and in vitro. One metastasis, OM-1, was found in the omentum, and the other, HOT-3, was located on deposits in a patient with a primary colon carcinoma. These cell lines should prove useful in studies that investigate the biology and therapy of human solid tumors. Furthermore, since both metastases were from the same individual, these materials will be helpful in investigating the functional heterogeneity found within a single human carcinoma.

INTRODUCTION

Intratumor heterogeneity has now been demonstrated in many neoplasms, including human carcinomas. Indeed, the evidence is overwhelming that many solid tumors contain subpopulations of neoplastic cells that differ from one another in a multiplicity of phenotypic characteristics (for reviews, see Refs. 4–7, 9, 15, 17). Reports from several workers that certain cells within a cancer are specialized for colonization of distant sites have documented the existence of functional heterogeneity in tumors (2, 11, 18, 21). The preexistence within individual neoplasms of cells with increased invasive and metastatic potential compared to the great majority of cells in the primary tumor has obvious and important clinical implications. The demonstration by several laboratories that cells from metastases also differ from cells in primary tumors in their responses to antineoplastic agents further complicates the task of the clinician (16, 23, 26). Moreover, differences among metastases originating from the same primary tumor have also been documented (1, 3, 25, 26). These studies indicate that cell lines established from human tumor metastases should be investigated to provide data relevant to the design of clinical protocols effective against micrometastatic disease already developing in patients prior to surgical removal of their primary tumors.

This report describes the establishment and characterization of cell lines established from biopsy material that was obtained from distinct metastases found simultaneously in a patient with a primary colon carcinoma. These cell lines should prove useful in studies that investigate the biology and therapy of human solid tumors. Furthermore, since both metastases were from the same individual, these materials will be helpful in investigating the functional heterogeneity found within a single human carcinoma.

MATERIALS AND METHODS

Case History. The patient from whom these 2 cell lines were established was a 45-year-old white female. She presented with a 6-month history of anorexia, a 10- to 15-pound weight loss, and low back pain. Her general physical examination was negative except for mild hypertension and evidence of recent weight loss. Laboratory evaluation revealed a CEA4 of 540 ng/ml. Sigmoidoscopy showed a lesion at 35 cm; a biopsy of which was positive for well-differentiated adenocarcinoma. At the time of exploratory laparotomy, biopsies, each of which was handled separately, were taken from 2 distinct metastases, one involving the left ovary and the other involving the omentum. These clinical specimens, designated HOT-3 and OM-1, respectively, were used to establish xenograft tumors in nude mice. The primary colon tumor was not removed, since the patient did not have clinical symptoms of obstruction, and a formal debulking procedure was not deemed feasible. She was treated with several antineoplastic agents, including 5-fluorouracil and Adriamycin, but failed to respond.

Establishment of Cell Lines. Metastatic tissue from the ovary or omentum was transplanted s.c. into the flank regions of 6-week-old nude (nu/nu) mice that were bred and maintained in the Roger Williams Cancer Center Animal Care Facility. Portions of each biopsy sample were also explanted into 35-mm tissue culture dishes (Falcon 3001; Falcon Plastics, Oxnard, Calif.) or were treated with 2.5% trypsin solution to prepare single-cell suspensions that were plated in 35-mm plastic tissue culture dishes. RPMI-1640, supplemented with 20% fetal calf serum and antibiotics [penicillin, 100 units/ml; streptomycin, 100 μg/ml; Fungizone, 2.5 μg/ml (all from Grand Island Biological Co., Grand Island, N. Y.); and gentamycin, 20 μg/ml (Scherer Corp., Kenilworth, N. J.)] was used as the culture medium, as reported previously from our laboratory (8, 14).

Permanently growing cell lines were not established from these cultures. However, the tumor fragments implanted in nude mice did produce s.c. tumors, which could be readily passaged in athymic hosts. Tumor tissue from each of these heterotransplants after one passage in vivo

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4 The abbreviations used are: CEA, carcinoembryonic antigen; RPMI-1640, Roswell Park Memorial Institute medium 1640; CSAP, colon-specific antigen; CMA, colon-specific antigen; NCA, nonspecific cross-reacting antigen.

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was minced, enzymatically dissociated, and plated as described below and in a previous report from our laboratory (10).

Xenograft tissue was washed in 0.9% NaCl solution containing penicillin (100 units/ml), streptomycin (100 µg/ml), and Fungizone (2.5 µg/ml) (all from Grand Island Biological Co.). This material was then minced, and small pieces of tumor tissue were removed and placed into a 75-ml Wheaton graduated trypsinizing flask (Wheaton Scientific, Millville, N. J.) containing 20 ml of enzyme solution for enzymatic dispersal of tumor cells. The enzyme solution used was a mixture of the following components in Hanks’ balanced salt solution, pH 7.2, trypsin (1 mg/ml; Difco Laboratories, Detroit, Mich.), Dispase neutral protease Grade II (0.5 mg/ml), DNase Grade II (10 µg/ml), and collagenase (1 mg/ml) (all from Boehringer Mannheim, Indianapolis, Ind.). This enzyme solution was sterilized by filtration through a 0.2-µm Nalgene filter (Sybron Corp., Rochester, N. Y.). After a 1-hr exposure to the enzyme solution, single-cell suspensions of OM-1 or HOT-3 were transferred to tubes containing fetal calf serum, and cells were centrifuged and resuspended in serum-free RPMI-1640 supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), Fungizone (2.5 µg/ml), and gentamycin (20 µg/ml), and buffered with 10 µM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10 µM tris(hydroxymethyl)methylglycine, and 0.075% sodium bicarbonate solution. Aliquots of the resuspensions were then added to flasks containing RPMI-1640 with antibiotics and serum supplements.

The elimination of any murine fibroblasts originating from the stromal component of the xenograft tumor tissue was accomplished in both cultures by brief exposures to 0.06% trypsin:0.02% EDTA. This procedure was repeated for 5 consecutive days, after which time no further fibroblast growth was observed. Both cell lines are presently maintained in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics as described above. This procedure was successful in establishing the HOT-3 and OM-1 cell lines.

**Karyotypic Analysis.** Karyotypic analyses of the OM-1 and HOT-3 cell lines were performed by the Rhode Island Hospital Cytogenetics Laboratory, under the supervision of Dr. Tariseta Mendoza. Cytogenetic studies were performed on exponentially growing cultures using standard Giemsa trypsin G-banding techniques. Randomly selected metaphase spreads were counted to determine modal chromosome number. Photomicrographs (<3000) were prepared and examined to identify chromosome markers.

**Histopathologic Characterization of OM-1 and HOT-3.** The 2 metastases and the primary colon carcinoma obtained from the patient were examined by the Clinical Pathology Laboratory of the Roger Williams General Hospital. Xenograft tumor tissue from heterografts which had been serially transplanted in vivo, and from those which had been produced by the inoculation of cultured cells, were also examined histologically. Xenograft tumor tissue was fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 to 6 µm, stained with hematoxylin and eosin, and examined by light microscopy. In addition, tissue sections were examined for mucus content by staining with Alcian blue (pH 2.5, with and without hyaluronidase) and with periodic acid-Schiff (with and without diastase).

**In Vitro and In Vivo Growth Characteristics.** The methods used for determining doubling times, saturation density, and cloning efficiency in agar have been reported previously (8, 10). Briefly, to determine doubling times, replicate 35-mm culture dishes (Falcon 3001) received an inoculum of 1.5 x 10⁶ cells. Cells from 3 of the dishes were harvested separately each day and counted with a hemocytometer. Plating efficiencies were calculated by dividing the number of cells counted at 16 hr after inoculation by the number initially plated. Saturation densities were determined from data obtained from the experiments done to determine cell doubling times. The number of cells present at confluency was used to determine the maximum cell number per sq cm. Cloning efficiency in agar was determined by resuspending cells in 0.5% agar (Difco Laboratories, Detroit, Mich.) in complete growth medium. One ml of this suspension was layered onto a 2-ml base of 1.0% agar in medium in a 35-mm dish (Falcon 3001). Duplicate dishes received either 1 x 10⁶ or 3 x 10⁶ cells.

Cloning efficiencies were determined by counting, under a light microscope, all colonies larger than 50 µm on Day 14. The in vivo doubling times of HOT-3 and OM-1 cells were obtained by injecting HOT-3 (5 x 10⁶ cells) or OM-1 (7.5 x 10⁶ cells) into 5 mice and measuring tumor dimensions twice weekly with a caliper. Tumor weights were estimated from:

\[ \text{mg of tumor} = L \times W^2/2 \]

Dimensions were given in mm. Estimated xenograft tumor weight was plotted versus days post inoculation, and the doubling time was calculated from the graph.

**Immunochemical Characterization of OM-1 and HOT-3.** Xenograft tumors of each line were studied for the expression of several colon carcinoma-related immunochemical markers.

Tumors were excised from host mice when they measured approximately 1 x 1 x 1.5 cm, and were stored at −20°C until use. The xenograft tumors were minced into 1- to 2-mm pieces, mixed with 5 volumes of cold 0.1 M NaHCO₃ containing 0.5 µM NaCl, and homogenized on ice for two 2-min cycles in a Sorvall Omnimixer. The 2 cycles were separated by a 2-min cooling period. The homogenate was centrifuged at 48,000 x g in a Sorvall RC2B centrifuge for 45 min. The resulting supernatant was collected and recentrifuged under the same conditions to obtain a second supernatant which was stored at −20°C.

CSAp was measured as described previously (22). Briefly, the assay used 125I-radiolabeled, affinity-purified goat anti-CSAp immunoglobulin (10 µCl/µg), to which was added either the supernatant prepared from the tumor tissue or a standard CSAp sample. After incubation at 37°C for 3 hr, insolubilized CSAp (colon tumor homogenate bound to polyvinylidene fluoride powder, Kynar grade 301F; Perwatt Corp., King of Prussia, Pa.) was added, and the suspension was incubated at 37°C for 90 min and centrifuged. The pellet was washed once, and then the radioactivity in the pellet was determined in a γ-scintillation counter. The dilution of a standard CSAp preparation that resulted in approximately 92% maximum binding (90 to 94%) was selected as one CSAp unit.

Radioimmunoassay for CMA (12, 13) was performed as follows. Rabbit anti-CMA (100 µl) was added to either the sample prepared from xenograft tumor tissue or to a standard CMA sample (100 µl) and incubated at 37°C for 30 min. 125I-radiolabeled CMA (100 µl), containing approximately 10,000 cpm, specific activity, 7 µCl/µg was added, and the mixture was incubated for an additional 30 min at 37°C. All dilutions were prepared in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride and 1% bovine serum albumin. One ml of zirconyl phosphate gel (prepared in 0.01 M ammonium acetate buffer, pH 5.2) was added, and the mixture was vortexed. The suspension was centrifuged at 2000 x g for 10 min, and the supernatant was decanted. The gel was washed once with 3 ml of 0.01 M ammonium acetate buffer, pH 5.2; the wash fluid was decanted after centrifugation at 2000 x g for 10 min; and the radioactivity in the gel was determined using a β-scintillation counter. B/B₀ was plotted against antigen concentration. B was 125I bound to the gel in the presence of antigen, and B₀ was the amount bound in its absence.

Measurement of NCA was performed by adding goat antibody, specific for NCA, to 5.0 ml of 0.01 M ammonium acetate, pH 6.25, containing 1.0% normal rabbit serum and antigen. Standard inhibition curves were generated using 0.8 to 16 ng of purified NCA obtained from liver metastases of colon adenocarcinomas (19). After incubation for 30 min at 45°C, approximately 1.0 ng of radiiodinated NCA (30 µCi/µg) was added to the tubes followed by incubation at 45°C for another 30 min. A solid-phase donkey anti-goat IgG was used to separate free and bound labeled antigen (20).

CEA assays were performed on 3-day-old spent media (without fetal calf serum) from actively growing cultures and on complete medium which had not been exposed to cultured cells. All assays were performed by radioimmunoassay using the Roche kit and procedure manual (Hoffman-LaRoche Inc., Nutley, N. J.) in the Roger Williams General Hospital Toxicology Laboratory under the direction of Dr. I. Diamond. The cells...
The xenograft tumors showed more extracellular mucin and from each other (Figs. 4 to 6). While very similar to the patient's biopsy specimens, they did show some differences from them. It developed as a result of the inoculation of cultured cells. Acid and neutral mucins were present and about an 80% higher saturation density than OM-1 cultures. Saturation densities, with HOT-3 cells showing better attachment times of OM-1 and HOT-3 cells were 42 hr and 61 hr, respectively. Creased nuclearcytoplasmic ratio and a higher mitotic rate. been established in vivo and with xenograft tumors which de

HOT-3 xenograft tumors were indistinguishable from each other (Figs. 4 to 6). While very similar to the patient's biopsy specimens, they did show some differences from them. The xenograft tumors showed more extracellular mucin and larger cystic spaces, although the amount of intracytoplasmic mucin was identical. The xenograft tumors were also more cellular, and the cells composing them showed a slightly increased nuclear:cytoplasmic ratio and a higher mitotic rate.

**Growth Properties**. Cultured OM-1 cells differed from cultured HOT-3 cells in several growth parameters (Table 1). The doubling times of OM-1 and HOT-3 cells were 42 hr and 61 hr, respectively. The 2 lines also had different plating efficiencies and saturation densities, with HOT-3 cells showing better attachment and about an 80% higher saturation density than OM-1 cultures. The cloning efficiencies of the 2 lines in agar were similar.

The doubling times of xenograft tumors from the 2 lines also differed; HOT-3 heterotransplants had a tumor volume-doubling time of 9 days, whereas OM-1 tumors propagated in nude mice doubled every 15 days. The latency periods for tumor development were 13 and 14 days for HOT-3 and OM-1, respectively. Thus, the 2 cell lines differed significantly for several growth parameters that were examined.

**RESULTS**

**Histology and Morphology**. Histologically, biopsy materials from the patient's primary colonic neoplasm and from her 2 metastatic sites were very similar (Figs. 1 to 3). All 3 specimens showed well-differentiated adenocarcinoma. The tumors were composed of large and small cystic structures arranged in a fibrous stroma and lined by tall columnar cells with large oval nuclei typical of well-differentiated adenocarcinoma of the colon. The cysts contained necrotic cellular, proteinaceous, and mucinous material. The cells lining the cysts formed distinct glandular and tubular structures. Solid, noncystic, glandular areas of tumor cells were also present. Acid and neutral mucins were present both within the tumor cells and in the cystic spaces. These histological specimens were compared to those prepared from either OM-1 or HOT-3 xenograft tumors which had originally been established in vivo and with xenograft tumors which developed as a result of the inoculation of cultured cells.

OM-1 and HOT-3 xenograft tumors were histologically identical (Figs. 4 to 6). While very similar to the patient's biopsy specimens, they did show some differences from them. The xenograft tumors showed more extracellular mucin and larger cystic spaces, although the amount of intracytoplasmic mucin was identical. The xenograft tumors were also more cellular, and the cells composing them showed a slightly increased nuclear:cytoplasmic ratio and a higher mitotic rate.

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**Antigens**. OM-1 and HOT-3 xenograft tumors and cultured cells were analyzed for expression of several immunohistochemical markers (Table 2). Both OM-1 and HOT-3 tumors contained essentially equivalent amounts of CEA (40 and 42 µg per g of tissue for OM-1 and HOT-3, respectively) and of NCA (20.8 and 21.2 µg per g of tissue for OM-1 and HOT-3, respectively). In contrast, the 2 lines differed significantly in the expression of each of 2 other marker antigens. CSAP was undetectable in OM-1 xenograft tumor tissue, whereas HOT-3 xenograft tumors contained 1200 units per g of tissue. CMA levels were also clearly different between the 2 lines. OM-1 nude mouse tumors contained 2500 µg CMA per g of tissue, whereas HOT-3 tumors contained 300 µg CMA per g of tissue.

Interestingly, culture media from the 2 cell lines differed significantly in their amounts of CEA. OM-1 cultured cells produced

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (hr)</th>
<th>Saturation density (x 10⁶ cells/sq cm)</th>
<th>Plating efficiency (%/1.5 x 10⁵ cells)</th>
<th>Tumor volume-doubling time (days)</th>
<th>Latency period (days)</th>
<th>Cloning efficiency in agar (% of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM-1</td>
<td>42</td>
<td>2.3</td>
<td>48</td>
<td>15</td>
<td>14</td>
<td>1.0</td>
</tr>
<tr>
<td>HOT-3</td>
<td>61</td>
<td>4.2</td>
<td>65</td>
<td>9</td>
<td>13</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*3 x 10⁵ cells inoculated.

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**Table 2**

<table>
<thead>
<tr>
<th>Tumor-associated antigens detected from the OM-1 and HOT-3 cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA production in vitro (ng/10⁵ cells)</td>
</tr>
<tr>
<td>---------------------------------------</td>
</tr>
<tr>
<td>OM-1</td>
</tr>
<tr>
<td>HOT-3</td>
</tr>
</tbody>
</table>

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518 ng CEA per $10^6$ cells, whereas HOT-3 culture media contained only 92 ng CEA per $10^6$ cells.

**DISCUSSION**

Several studies have documented that only some subpopulations within primary tumors possess the ability to invade and metastasize (11, 18, 21). Investigators have also reported heterogeneity between the primary tumor and its metastases or among the metastases themselves. Sugarbaker and Cohen (25) used cells from a primary methylcholanthrene-induced sarcoma and its metastases to document differences among some of these cell lines in their antigenicities, tumor growth rates, and latency periods. Heterogeneity, both between the primary and its metastases and among metastases, has also been found within human tumors. Stich and Steel (24) reported that a metastasis may consist of cells with an amount of DNA differing from that present in the stemline of the primary tumor. Using flow cytometric DNA analysis of biopsies of human small cell carcinoma of the lung, Vindelev et al. (27) documented differences between discrete metastatic foci found within the same patient. Abeloff et al. (1) documented differences in markers such as histaminase and DOPA decarboxylase among primary tumors and their metastases. A study of marker differences that is directly clinically relevant was reported by Brennan et al. (3). These authors determined the level of estrogen receptor present in primary and metastatic breast cancer in individual patients. They concluded that clinically significant differences in estrogen receptor content exist not infrequently between primary breast cancers and their metastases as well as among different metastases originating from the same primary tumor. The authors suggested that these findings could account both for the lack of responsiveness of some of the metastases from estrogen receptor-positive primary tumors and for the "mixed" responses often observed when patients receive hormonal modes of therapy. In addition, there are differences between primary and metastatic tumors in their responses to cytotoxic agents. Siroway (23) exposed cells from both primary ovarian cancers and their metastases to antineoplastic drugs and then used tritiated thymidine to label suspensions of these cells. He reported that differential labeling was found both between the primary tumor and its metastases and among the metastases.

The significant differences in the doubling times of HOT-3 and OM-1 cells both in vitro and in vivo indicate that each has distinctive growth parameters. Although the growth kinetics of these 2 metastases in the patient are unknown, one might speculate that they were also different. We are presently examining the chemotherapeutic sensitivity of each cell line to several drugs; if differences are observed, this would suggest that one metastasis, while in the patient, might be more responsive to treatment than the other.

Culture media from these 2 cell lines differed significantly in their CEA content. This difference suggests that treatment effective against the omental metastasis might result in a marked decrease in CEA levels in the patient, even though the metastasis to the ovary might continue to proliferate, since the omental metastasis appears to produce more of this marker. However, levels of circulating CEA in nude mice hosting OM-1 and HOT-3 xenografts were not determined. It should also be noted that xenograft tissue from both metastases had equivalent amounts of CEA. Furthermore, localization studies for immunodetection of occult disease would fail to detect the OM-1 metastatic deposit if an anti-CSA p reagent antiserum were used. A similar problem might be encountered with localization studies designed to identify disseminated disease using CMA as a marker. An anti-CMA reagent antiserum would not be as useful in identifying the presence of the ovarian metastasis as it would be in localizing the omental tumor. This points out the necessity of using multiple markers for clinical protocols. Our data on differences in markers elaborated or expressed by these 2 metastases underscore questions already raised about the reliability of tumor markers in evaluating disease response or in predicting relapse. Certainly, information from diagnostic markers in patients with heterogeneous solid tumors must be interpreted with caution (4, 9).

On the other hand, similarities were also found between the OM-1 and HOT-3 cell lines. This encourages a continued search for properties common to metastases from one patient, and hopefully, to secondary neoplasms in general. Properties of metastases could be evaluated to determine whether any common denominator(s) could be found among: (a) metastases in one patient; (b) metastases from patients suffering from one type of solid tumor, i.e., colon carcinoma; and (c) metastases from patients with any one of a variety of solid tumors. Such commonalities could then be assessed to see whether they could be exploited in a therapeutic protocol. Approaches that address this issue include the use of activated macrophages and differentiation-inducing chemicals (5, 15). Such an approach has as its goal the rational design of treatment strategies effective both for patients with macroscopic metastases and for those who will receive adjuvant therapy.

In summary, we have shown that 2 metastases from a patient with primary carcinoma of the colon have both similarities and differences with respect to several phenotypic characteristics. We cannot discount the possibility that selection pressures may have been exerted on these 2 cell lines during tissue culture or nude mouse passage; i.e., some subpopulations of cells may have failed to grow. The differences found in this study could not be predicted based on the (almost identical) histopathological descriptions of the 2 metastases. These findings are expected from a consideration of, and are at least in part a consequence of, intraneoplastic diversity. This model system of cultured lines and xenograft tumors should aid investigations of functional tumor heterogeneity and of the therapeutic consequences of such intraneoplastic diversity.

**REFERENCES**

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Fig. 1. Biopsy of patient's colon tumor showing well-differentiated adenocarcinoma with glandular structures infiltrating connective tissue. Tumor cells are arranged in simple rows with basally oriented hyperchromatic and irregularly shaped nuclei. H & E, × 63 (original magnification).

Fig. 2. Section of patient's ovarian metastasis showing a similar pattern with glandular structures more closely approximated. H & E, × 63 (original magnification).

Fig. 3. Patient's omental metastasis with same pattern seen in other sites. H & E, × 63 (original magnification).

Fig. 4. Nude mouse xenograft tumor produced by injecting cultured cells from the ovarian metastasis, HOT-3. Well-formed glandular pattern is maintained, although the tumor appears more cellular due to relative lack of stromal connective tissue and the "piling up" of cells within the glandular structures. H & E, × 63 (original magnification).
Fig. 5. Xenograft tumor established from the ovarian metastasis, HOT-3, showing more "piling up" of cells in a large cystic structure (left). Nuclear-cytoplasmic ratios appear greater than those seen in the patient's tumors. H & E, x 63 (original magnification).

Fig. 6. Xenograft tumor produced by inoculating cells cultured from the omental metastasis, OM-1, showing an appearance identical to the other xenografts. H & E, x 63 (original magnification).
Fig. 7. Karyotype and banding of OM-1 cells.

Fig. 8. Karyotype and banding of HOT-3 cells.
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