**In Vivo** Selection of Highly Metastatic Cells from Surgical Specimens of Different Primary Human Colon Carcinomas Implanted into Nude Mice

Kiyoshi Morikawa, Shirley M. Walker, J. Milburn Jessup, and Isaiah J. Fidler

Departments of Cell Biology [K. M., S. M. W., J. J. F.] and Surgery [J. M. J.], The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, 1515 Holcombe Boulevard, Houston, Texas 77030

ABSTRACT

The purpose of these studies was to select and isolate cells with increased liver-metastasizing potential from heterogeneous primary human colon carcinomas (HCCs). Cells derived from a primary HCC classified as Dukes' stage B2, were directly established in culture or were injected into the subcutis, cecum, or spleen of nude mice. Progressively growing tumors were excised, dissociated, and established in culture. Subsequent to implantation into the cecum or spleen of nude mice, cells from all four lines produced only a few liver tumor foci. HCC cells from the few liver metastases were expanded in culture and then injected into the spleen of nude mice to provide a source for further cycles of selection. With each successive *in vivo* selection cycle, the metastatic ability of the isolated propagated cells increased. Four cycles of selection yielded cell lines with a very high metastatic efficiency in nude mice. In parallel studies using another primary HCC classified as Dukes' stage D, we isolated cell lines that were highly metastatic in nude mice. Successive selection cycles for growth in the liver increased the metastatic properties of the HCC cells, albeit to a lesser extent than it did those of the Dukes' B2 stage HCC.

The ability of the HCC cells to produce liver metastases was not due to simple trapping in the liver. *In vivo* distribution studies using [3H]iododeoxyuridine-labeled tumor cells revealed that, shortly after injection into the spleen, a comparable number of cells with either low or high metastatic properties arrested in the liver. The differences between the low- and high-degree metastatic cells became apparent by 24 h after injection and, by 72 h, only highly metastatic cells survived in the liver. These results demonstrate that hepatic metastasis by HCC cells is a selective process and that the nude mouse model can be useful for isolating highly metastatic HCC cells and for studying the relevant host organ factors that regulate the pathogenesis of metastasis.

INTRODUCTION

Most of the data describing the metastatic heterogeneity of neoplasms have been obtained from analysis of rodent neoplasms (1). Little comparable data are available detailing the heterogeneous nature of human neoplasms. In part, this has been due to the lack of available *in vivo* models for such studies. The initial success of heterotransplantation of human neoplasms into the athymic nude mouse has encouraged investigation of human cancer metastasis (2, 3). However, the usefulness of the nude mouse to study metastatic behavior of human tumors has been limited because human tumors transplanted into nude mice, especially at a s.c. site, rarely produce metastasis in recipient mice (3).

Recent studies from our laboratory (4–6) and others (7, 8) have shown that the implantation of HCC cells into the spleen or cecum in nude mice allows for both the growth and metastasis of HCC to the regional lymph nodes and the liver. These studies using well-established cell lines (4) or cell lines recently established from HCCs of different metastatic behavior in patients (5, 6) revealed that the implantation of HCC cells into the spleens of nude mice allows differentiation between HCCs of low- and high-grade malignancy. These studies did not determine whether cells populating individual HCCs are heterogeneous for metastatic properties or whether the nude mouse could be used to isolate highly metastatic subpopulations of HCC.

We report studies with two different surgical specimens of HCC. We show that highly metastatic cells can indeed be selected from low-metastatic HCC and that the ability of the metastatic cells to grow in the liver environment is a major factor contributing to their metastatic potential.

MATERIALS AND METHODS

Mice. Male athymic BALB/c mice were obtained from the Animal Production Area, National Cancer Institute–Frederick Cancer Research Facility (Frederick, MD). Mice were used in the studies when 8 wk old and were maintained in a laminar flow cabinet under specific pathogen-free conditions.

Establishment of Cell Lines. Tumor specimens from two primary HCCs (KM12, Dukes' B2; and KM20, Dukes' D) were enzymatically dissociated with collagenase type I (200 units/ml) and DNase (270 units/ml) (Sigma Chemical Co., St. Louis, MO) as detailed elsewhere (5, 6). After enzymatic dissociation, the cell suspension was filtered through a 4-layer sterile gauze and washed 3 times in serum-free medium. The procedure yielded a suspension of single tumor cells or very small clumps of cells (<5) with a viability >80% (trypan blue exclusion). One aliquot of the cell suspensions was directly established in culture (KM12c and KM20c). From the other aliquot, 2 x 10⁴ viable single cells were injected into the subcutis, cecum, and spleen of nude mice. When tumors exceeded 20 mm in diameter or when the mice became moribund, the mice were killed. HCC tumors were excised, enzymatically dissociated, and established as individual cell lines in culture (subcutis tumor, KM12sc; spleen tumor, KM12sp; and cecal tumor, KM12cc). All cell lines were shown to be of human origin by both karyotypic and isoenzyme analyses (Authentic Kit; Corning Medical, Corning, NY). No contamination with mouse cells was detected.

Selection of Cell Lines with Enhanced Metastatic Ability. Cell lines with enhanced metastatic ability were selected in a manner analogous to that originally described for the B16 melanoma (9). HCC foci in the livers of nude mice ( injected into the spleen with HCC cells prepared from the surgical specimens) were harvested, established in culture, and designated as lines KM12L1a, KM12L1b, KM12L1c, KM20L1a, and KM20L1b. Cells from *in vivo* passages 4–5 were then injected into spleens of nude mice. Liver tumor lesions were harvested and established in culture as individual cell lines. This procedure was repeated 4 (Fig. 1) or 2 (Fig. 2) times. With each successive cycle of selection, the ability of HCC cells to produce metastasis in nude mice was determined.

Tissue Culture. All tumor cell lines were maintained in Eagle's minimum essential medium (M. A. Bioproduct, Walkersville, MD) supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids, l-glutamine, and 2-fold vitamin solution (Gibco, Grand Island, NY). All of the cell lines were examined and found free of mycoplasma, retrovirus type 3, mouse pneumonia virus, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus,
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extremeliva virus, and lactate dehydrogenase virus (Microbiological Associate, Bethesda, MD).

Preparation of Tumor Cell Suspension for in Vivo Injections. Tumor cells were harvested from subconfluent monolayer cultures by a 1-min treatment with 0.25% trypsin and 0.02% EDTA. The cells were washed in supplemented medium and then resuspended in HBSS for injection. Only suspensions of single cells with greater than 90% viability were used for the in vivo injections.

Liver Metastasis Assay: Intrasplenic Injection of HCC Cells. The ability of HCC cells implanted into the spleen to produce liver tumor nodules was determined by techniques described in detail previously (5). Briefly, mice were anesthetized with methoxyflurane and the left flank was prepared for sterile surgery. A small abdominal incision was made in the left flank and the spleen was isolated and exteriorized. Viable tumor cells (1 × 10^6/0.05 ml HBSS) were injected into the spleen by a 27-gauge needle. The spleen was returned to the abdominal cavity and the wound was closed in one layer with metal wound clips. All of the mice survived this procedure. Several weeks later, moribund mice were killed and autopsied. Liver tumor burden was evaluated based on findings in terms of the size and number of lesions as follows: 0, tumor free; I, histological evidence of tumor growth; II, <10 tumor foci of <1-2 mm in diameter; III, 10-100 tumor foci of <3-5 mm in diameter; and IV, >100 tumor foci of >5 mm in diameter.

Spontaneous Metastasis Assay: Intracecal Injection of HCC Cells. Mice were anesthetized with methoxyflurane, and the abdomen was prepared for sterile surgery. A small midline abdominal incision was made, and the cecum was exteriorized and supported on a sterile gauze. Viable tumor cells (1 × 10^6/0.03 ml HBSS) were injected into the wall of the cecum from the serosal side using a 30-gauge needle. A visible bullock formation between the submucosal and subserosal tissues and a lack of extracecal leakage of fluid were the two criteria needed for a successful injection. The cecum was returned to the abdominal cavity and the wound was closed in one layer with metal wound clips. All of the mice survived this procedure. Several weeks later, when the mice became moribund, they were killed and autopsied.

In Vitro Labeling of Cells with [125I]IdUdR. Tumor cells were seeded into 150-cm² tissue culture flasks at 4 × 10^5 cells/flask in supplemented medium and 24 h later, 0.3 μCi of [125I]IdUdR/ml medium was added (New England Nuclear, Boston, MA; 2000 mCi/μmol). One day later, the monolayer cultures were rinsed twice with excess HBSS to remove nonbound radioiodine. The cultures were overlaid with 0.25% trypsin-0.02% EDTA solution for 1 min. The cells were dislodged and suspended in supplemented medium, then washed and resuspended in HBSS at a final concentration of 5 × 10^5 cells/0.05 ml, the inouclus volume per mouse.

Distribution and Fate of [125I]IdUdR-labeled Cells after Intrasplenic Injection. The in vivo distribution and fate of HCC cells with high- or low-metastatic potentials were studied following methods described previously (10). Radiolabeled HCC cells from parental cell lines KM12c and KM20c and from selected lines KM12L4a and KM20L2a were injected into the spleens of nude mice at the dose of 5 × 10^5 cells per mouse. Groups of 3 mice were killed at various intervals thereafter. Spleen, liver, lung, and kidneys collected from each mouse were placed in test tubes containing 70% ethanol. The ethanol was replaced daily for 3 days to remove all soluble [125I]IdUdR (10). Blood samples (0.2 ml/mouse) were placed directly into test tubes for radioactive monitoring. The organs were monitored for radioactivity in a gamma counter (TM Analytic, Elk Grove Village, IL). Triplicate tubes containing the inoculum dose were retained, and the radioactivity was monitored at the same time as the sample organs. The mean counts in organs from each group of mice (n = 3) were expressed as the percentage of input counts, which is equivalent to viable cells (10). All measurements were corrected for radioactive decay.

RESULTS

Isolation and Selection of Highly Metastatic Lines in Nude Mice from Two Primary HCCs with Different Clinical Stages. Single cell suspensions obtained from a surgical specimen of primary Dukes’ B-stage HCC were directly adapted to growth
in culture and designated as line KM12c. The freshly isolated cells were also injected into several organs of different nude mice. Tumors growing at the injection sites were recovered, enzymatically dissociated, and established in culture, and cell lines were designated as KM12c, KM12cc, and KM12sp. Aliquots of the cells harvested from the surgical specimens were injected into the spleens of several nude mice. A few weeks later, some of the mice developed 1–5 small visible liver tumor colonies. Each HCC liver nodule was harvested under sterile conditions and dissociated, and the cells were placed into culture to provide a source of HCC cells for a further cycle of selection. The cell lines derived from the first (KM12L1a, KM12L1b, KM12L1c) and the second (KM12L2a, KM12L2b, KM12L2c) selection-isolation cycles produced liver metastases in 23 of 35 nude mice and 15 of 20 nude mice, respectively. All 20 nude mice injected with cells derived from the third (KM12L3a, KM12L3b) and the fourth (KM12L4a, KM12L4b) selection-isolation cycles had extensive liver metastases (Fig. 3). Thus, with each successive cycle, the number and volume of metastases in the affected livers increased. This was most pronounced with the cells isolated at the end of the fourth selection cycle. The cell lines KM12L4a and KM12L4b produced grade IV hepatic tumor foci in 9 of 11 mice. The severity of the disease shortened the life span of the mice (Table 1).

The same phenomenon was observed in a freshly isolated cell line derived from a primary Dukes’ D-stage HCC. Cells isolated from the surgical specimen of KM20 tumor were more metastatic than were cells isolated from the KM12 tumor (incidences of liver metastasis, 8 of 11 versus 3 of 25, respectively; Tables 1 and 2). Moreover, the metastatic ability of the KM20c cells equaled that of cells isolated after the first selection cycle from the KM12 tumor: KM12L1a, MK12L1b, and KM12L1c. KM20L2a or KM20L2b cells isolated after two selection cycles from the KM20 HCC produced extensive hepatic metastases (grade IV) in 7 of 10 mice. This too was reflected in a shortened life span of the recipient mice (Table 2).

Spontaneous Metastatic Capabilities of Selected HCC Cell Lines after Implantation into the Cecum. Differences in spontaneous metastatic capability between cells of the primary nonselected lines and cells of the successive selected lines were also found for tumors growing in the wall of the cecum. None selected KM12-derived cell lines (KM12c, KM12sp, KM12cc, and KM12sc) produced visible mesenteric lymph node metastases in 10 of 20 injected mice and hepatic metastases in only 1 mouse (Fig. 4). Cells isolated from hepatic tumor foci (following implantation into the spleen) exhibited increased metastasis to regional lymph nodes. For example, cell lines from the second selection cycle (KM12L2b, KM12L2c) produced metastasis to lymph nodes in all of the injected mice and hepatic metastases in 2 of 10 mice (Table 3).

HCC cells isolated from Dukes’ D-stage HCC were more metastatic in nude mice after implantation into the spleen and cecum than were cells of the nonselected KM12 (Dukes’ B-stage) lines. The incidence of lymph node metastasis produced by KM20 cells implanted into the cecum (8 of 10 mice) equaled that found for the selected KM12 sublines KM12L1a, KM12L1b, and KM12L1c (13 of 15 mice).

Distribution and Fate of [125I]IdUd-labeled HCC Cells Subsequent to Their Injection into the Spleen. The organ distribution and fate of [125I]IdUdR-labeled HCC KM12c and KM20c cells and their selected sublines KM12L4a and KM20L2a were determined from 10 min to 72 h after intrasplenic injection (Table 4). By 10 min after intrasplenic injection, more than 60% of the HCC cells had reached the liver, with less than 20% of the cells remaining in the spleen. At 10 min, 1 h, and 4 h after the injection, no significant differences in the number of cells arrested in the liver were discernible among the four cell lines. By 24 h, however, significant differences in the number of cells surviving in the liver were found among the lines. In the liver, levels of radioactivity associated with viable tumor cells were low for KM12c (1.43%), and high for KM20c, KM20L2a, and KM12L4a (6.20, 10.6, and 13.9%). Thus, the differences in production of hepatic metastases observed among the cell lines could not be due to the inability of HCC cells to reach the liver. Rather, these distribution data suggest that the production of hepatic metastasis is associated with the capacity of HCC cells to survive and proliferate in the liver. The distribution of radiolabeled cells to the kidneys, lung, heart, and blood was unremarkable and the negative data were omitted from Table 4.

**DISCUSSION**

We investigated the metastatic potential and heterogeneity of two human HCC specimens obtained at surgery. One tumor
Table 1 Selection of tumor cell lines with increased metastatic potential from a surgical specimen of a Dukes' B2 HCC

<table>
<thead>
<tr>
<th>Metastatic selection cycle</th>
<th>Tumor cell line</th>
<th>In vitro doubling time (h)</th>
<th>No. of mice with spleen tumor/total mice</th>
<th>Mean survival days ± SD</th>
<th>No. of mice with liver tumor/total mice</th>
<th>Grade of metastasis*</th>
<th>Other visceral metastasis</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>KM12c</td>
<td>24.0</td>
<td>10/10</td>
<td>79 ± 15</td>
<td>2/10</td>
<td>0/10</td>
<td>8/10</td>
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<td></td>
<td>KM12sp</td>
<td>28.8</td>
<td>5/5</td>
<td>&gt;90</td>
<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
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<td>KM12ce</td>
<td>26.4</td>
<td>4/5</td>
<td>80</td>
<td>1/5</td>
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<td>&gt;85</td>
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<td>62 ± 21</td>
<td>11/15</td>
<td>4/15</td>
<td>0/15</td>
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<td>KM12L1b</td>
<td>29.3</td>
<td>8/10</td>
<td>100 ± 9</td>
<td>6/10</td>
<td>4/10</td>
<td>1/10 (Lung)</td>
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<td></td>
<td>KM12L1c</td>
<td>38.2</td>
<td>9/10</td>
<td>79 ± 19</td>
<td>6/10</td>
<td>10/10</td>
<td>0/10</td>
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<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>23/35</td>
<td>12/35</td>
<td>1/35 (Lung)</td>
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<tr>
<td>Second</td>
<td>KM12L2a</td>
<td>26.4</td>
<td>6/6</td>
<td>42 ± 12</td>
<td>5/6</td>
<td>1/6</td>
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<td></td>
<td>KM23L2b</td>
<td>28.7</td>
<td>5/5</td>
<td>41 ± 13</td>
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<td>0/5</td>
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<tr>
<td></td>
<td>KM12L2c</td>
<td>30.2</td>
<td>9/9</td>
<td>49 ± 17</td>
<td>5/9</td>
<td>4/9</td>
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<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>15/20</td>
<td>6/20</td>
<td>2/20 (Lung)</td>
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<td>Third</td>
<td>KM12L3a</td>
<td>32.4</td>
<td>4/4</td>
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<td>KM12L3b</td>
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<td>36 ± 3</td>
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<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>9/9</td>
<td>0/9</td>
<td>2/9 (Lung)</td>
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<td>Fourth</td>
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<td>28.4</td>
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<td>34 ± 8</td>
<td>6/6</td>
<td>0/6</td>
<td>4/6</td>
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<tr>
<td></td>
<td>KM12L4b</td>
<td>26.9</td>
<td>5/5</td>
<td>31 ± 5</td>
<td>5/5</td>
<td>0/5</td>
<td>5/5</td>
</tr>
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</table>

* Liver metastasis was graded from 0 to IV. See "Materials and Methods.

Table 2 Selection of tumor cell lines with increased metastatic potential from a surgical specimen of a Dukes' D HCC

<table>
<thead>
<tr>
<th>Metastatic selection cycle</th>
<th>Tumor cell line</th>
<th>In vitro doubling time (h)</th>
<th>No. of mice with spleen tumor/total mice</th>
<th>Mean survival days ± SD</th>
<th>No. of mice with liver tumor/total mice</th>
<th>Grade of metastasis*</th>
<th>Other visceral metastasis</th>
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<td>0</td>
<td>KM20c</td>
<td>23.6</td>
<td>11/11</td>
<td>98 ± 2</td>
<td>8/11</td>
<td>3/11</td>
<td>0/11</td>
</tr>
<tr>
<td>First</td>
<td>KM20L1a</td>
<td>26.9</td>
<td>9/10</td>
<td>106 ± 13</td>
<td>5/10</td>
<td>2/10</td>
<td>1/10 (Lung)</td>
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<tr>
<td></td>
<td>KM20L1b</td>
<td>25.8</td>
<td>14/15</td>
<td>93 ± 19</td>
<td>14/15</td>
<td>9/15</td>
<td>1/15 (Lung)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>19/25</td>
<td>11/25</td>
<td>7/25 (Lung)</td>
</tr>
<tr>
<td>Second</td>
<td>KM20L2a</td>
<td>27.2</td>
<td>6/6</td>
<td>46 ± 6</td>
<td>6/6</td>
<td>1/6</td>
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<td>KM20L2b</td>
<td>22.4</td>
<td>5/5</td>
<td>61 ± 12</td>
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<td>5/5</td>
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<td></td>
<td></td>
<td>11/11</td>
<td>1/11</td>
<td>3/11 (Lung)</td>
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</table>

* Liver metastasis was graded from 0 to IV. See "Materials and Methods.

was classified as a Dukes' B2 stage (local invasion, no evidence for metastasis) and the other as a Dukes' D stage (clinical evidence of visceral metastasis). The primary HCCs were enzymatically dissociated and cells were established in culture or implanted into various organs of nude mice. Although the HCCs produced tumors at all organ sites, hepatic metastasis was found only in the mice with HCC cells implanted into their spleen. Cells from the Dukes' B2-stage tumor produced only few liver tumor nodules, whereas cells from the Dukes' D-stage tumor produced many hepatic tumor nodules. These data confirmed our earlier observations that the athymic nude mouse can be used to differentiate between low- and high-metastatic-potential HCC (5, 6). The few liver metastases in nude mice whose spleens were injected with HCC cells (isolated from the Duke's B2-stage surgical specimen) were harvested and dissociated, and individual cell lines were established in culture. These cells were then implanted into the spleens of new nude mice and subsequent liver metastases were harvested to provide a source for new in vitro growing cell lines. With each successive selection cycle (spleen-liver culture), the metastatic ability of the HCC cells increased. This was not due to mere ability of HCC cells to grow in nude mouse organs because the growth of HCC in the subcutis, spleen, and cecum of nude mice did not enrich the tumors for cells with increased metastatic properties. Regardless of the site of growth, all HCC cells we isolated were shown to be of human origin subsequent to karyotypic and isoenzyme analyses.

The metastatic potential of the HCC cells in nude mice was determined by two different assays. The first involved the implantation of cells into the spleen and the production of liver tumor foci (experimental metastasis). The second assay measured the ability of HCC cells to produce lymph node and liver metastases subsequent to implantation into the wall of the cecum (spontaneous metastasis). In general, there was agree-
In general, Dukes' B tumors are likely to be an earlier manifestation of HCC than Dukes' D tumors. Clinical observations of various neoplasms have suggested that tumors tend to evolve with the passage of time. Neoplasms that are first diagnosed as noninvasive-nonmetastatic can progress to become metastatic (review, Ref. 1). In the case of HCCs, it is entirely possible that an early Dukes' B neoplasm can progress to become a Dukes' D neoplasm. If such were the case, Dukes' B tumors should contain only few metastatic cells, whereas Dukes' D tumors should contain a large number of metastatic cells. Our present data support this assumption.

In both the experimental and spontaneous metastasis assays, cells isolated from the surgical specimens of Dukes' D-stage tumor (KM20) were more metastatic than were cells isolated from the Dukes' B-stage tumor (KM12). These data suggest that more HCC cells in Dukes' D cancers possess metastatic properties than do HCC cells in Dukes' B cancers. The majority of HCC cells in a Dukes' B tumor can invade the colon wall but may lack the ability to spread via the lymphatic or hematogenous route. This may explain, in part, the findings of a retrospective study analyzing 391 cases of HCC that found the mean diameter of Dukes B2-stage tumor to be significantly greater than that of Dukes' D-stage cancers (13). Stated differently, if metastasis were a random process, a greater tumor burden should result in a higher incidence of metastasis. After a single-step selection from the low-metastatic KM12 cells, the metastatic potential of the selected KM12L1a, KM12L1b, and KM12L1c cell lines increased significantly. In fact, these cells produced an incidence of metastasis similar to that of cells isolated from the KM20 tumor. At surgery, the KM20 tumor (Dukes' D) further progressed toward metastasis than had KM12 tumor (Dukes' B). Indeed, after two cycles of selection, the lines selected from KM20 tumor (KM20L2a and KM20L2b) produced metastasis in all mice tested. In the KM12 system, three cycles of selection were required to reach this level. For this reason, we speculate that the selective pressure(s) in the nude mouse bioassay may have simulated the selective process that is likely to occur in a patient with a progressive Dukes' D-stage HCC.

There are several mechanisms to explain the selection of HCC with higher metastatic capacity in nude mice: (a) selection for cells with high proliferative activity (1); (b) selection of cells with high invasive characteristics (14, 15); (c) selection of cells with high adhesive characteristics (16); and (d) selection of cells able to survive local host-defense mechanisms (17, 18). Because no significant differences in growth rate in vitro were discernible among the cell lines, the differences in production of liver metastases observed among the cell lines could not be due only to proliferation rate of the cells. The metastatic growth in the liver was also not influenced by the ability of HCC cells to reach the liver vasculature. The proportion of [\textsuperscript{125}I]dUdrlabeled HCC cells that arrested in the liver by 10 min, 1 h, and 4 h after intrasplenic injection was similar for the low- and high-metastatic cell lines. These data agree with those from our previous studies, showing that the pattern of initial tumor cell arrest in an organ capillary bed does not necessarily predict subsequent formation of metastasis (1, 6, 19–21). The differences in the survival rates of HCC cells from high- and low-metastatic cell lines were apparent by 24 h after implantation. Highly metastatic cells survived in liver to produce metastases, whereas low-metastatic cells did not. Collectively, these data demonstrate that unique tumor cell characteristics may be responsible for tumor cell invasion, dissemination, arrest, survival, and growth. All of these phenotypes are affected by the microenvironment of the tumor cells.
The prognosis in a patient with colorectal cancer deteriorates once metastasis occurs (12). Currently, there is no effective therapy for metastases of HCC. For this reason, rational approaches to the treatment of HCC must be based on an understanding of the pathogenesis of its metastasis and on characterization of subpopulations of tumor cells most likely to metastasize. The isolation of HCC cells with enhanced metastatic ability should be a useful model for studying tumor and host interaction that control metastasis formation by these cells.

REFERENCES

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