Experimental Metastasis of Mouse Embryonal Carcinoma Cell Lines to Specific Locations

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ABSTRACT

Embryonal carcinoma cell lines produced tumors in highly specific and unusual sites when injected into mice. The pattern that developed when cells were injected into the left ventricle of the heart involved target organs related either to specific nerve pathways or neuronal outgrowth factors, or to pathways of primordial germ cell migration. Major sites included the ovary, testis, adrenal, iris, whiskers, and male submaxillary gland. Neither local growth responses, determined by direct injection of tumor cells into different organ parenchyma, nor initial attachments, observed upon injection of radiolabeled cells, appeared to sufficiently account for the specificity of tumor metastases occurring after arterial injection. However, tissue from un.injected target sites, but not other tissues, stimulated the in vitro migration of embryonal carcinoma cells. Conditioned medium from only target tissues had a similar effect. These results suggest that the specificity of this tumor pattern may depend on migration responses that are significant in the localization of embryonic germ and neural cells. The specific metastatic pattern observed following i.p. injection of embryonal carcinoma cells, involving only the ovary, appeared to require an additional component of high adhesivity to the target organ.

INTRODUCTION

Cancer cells metastasize to distant sites that sometimes seem quixotic (1). However, histologically similar tumors may spread spontaneously to similar or identical tissue end points (2–6), suggesting these targets are not random. While many metastatic patterns can be explained by the anatomical location of the primary tumor in relation to various drainage pathways, others cannot be understood from such considerations (2, 7). In these cases, other factors must function to determine particular localizations.

In some neoplasms, metastases occur in target organs that might be predicted from properties of the normal cell counterpart. Malignant tumors of reticular or lymphoid tissues spread in a statistically predictable pattern that was postulated to be related to the homing and migration capabilities of normal macrophages and lymphocytes (8, 9). Recent support for this idea comes from studies which show that both normal and neoplastic lymphoid cells utilize a similar cell surface receptor molecule detected by the MEL-14 monoclonal antibody for specific lymph node recognition (10–12). In addition, the "metastases" of normal cells can closely mimic the localization of the corresponding malignant cells: tissue macrophages and hepatic Kupffer cells (13, 14) or embryonic chicken pigment cells (15), not usually disseminated via the vascular system, will also specifically localize to liver and skin, respectively, after i.v. injection. Further examples of metastatic patterns related to the homing potentialities of normal cells are few, although other cell types, in particular primordial germ cells and other neural crest-derived cells, in addition to hematopoietic cells, undergo very extensive embryonic migrations.

It was previously reported that several embryonal carcinoma cell lines formed tumors specifically in the ovaries of i.p.-injected adult mice (16). LT clone 8, an EC2 line not previously selected for tumor site specificity, produced ovarian tumors exclusively in over 70% of tumor-bearing females, and nearly half of these were bilateral. Their location was postulated to be related to the localizing potential of embryonic germ cells. Teratocarcinomas have been a fruitful model system for the analysis of developmental events (17) and EC cells are known to express many molecules and genes that function in early embryonic cells, including cell surface antigens (18, reviewed in Refs. 19 and 20) and molecules involved in blastocyst cell adhesion and compaction (21), cell type-specific recognition (22) and sorting (23, 24), and embryonic migration (25), as well as proto-oncogenes (reviewed in Ref. 26) and growth factor sensitivities (27, 28), any of which might potentially account for the restricted distribution of EC tumor cells.

The present study investigates the possible mechanisms used by EC cells to specifically colonize target organs. In the course of this work, a remarkable new pattern of metastasis was observed following the intracardiac injection of EC cells that is selectively related to germ cell or neuronal patterns or nerve growth factor(s). The specificity and unique distribution of these sites, involving the ovary, testis, adrenal, iris, whisker field, and male submaxillary gland, and the results of in vitro and in vivo studies, suggest that migration factors capable of influencing the localization of embryonic germ and neural cells may be important in determining this pattern.

MATERIALS AND METHODS

Cell Lines. LT clone 8 was derived from the spontaneous LT ovarian transplantable teratocarcinoma tumor 74115 as previously described (16). 8P was established from LT clone 8 by in vitro selection of cells able to grow well on a tissue culture plastic substratum in the absence of feeder cells. Normally maintained on confluent fibroblast feeder layers, LT clone 8 cells grown from low density on plastic underwent considerable death and selection at first. However, when tested after 6 months, 8P had a growth rate similar to LT clone 8 in vitro (13.9 versus 13.3 h/generation, respectively, on a 1% gelatin substrate in standard medium plus 10−4 M mercaptoethanol; data not shown). T6-4 and T6-11 are independent multipotential teratocarcinomas derived in this laboratory by implanting the embryonic half of a 7-day egg cylinder from a CBA/T6Au embryo beneath the kidney capsule of an adult syngeneic mouse. T6-4 is female line started from in vitro culture of the primary tumor. T6-11 originated from a male embryo; EC cells developed in vitro from a tumor of the second animal transplant generation. T6-4 and T6-11 lines were preserved in liquid nitrogen within 1 to 2 months of total culture time without further animal passage. OTT6050 and F9 cells were obtained from R. Auerbach and W. Dove, respectively. All EC lines were reinitiated every 2 to 3 months from frozen stocks in order to minimize progression of tumor cell properties. NS-1 myeloma and RAG renal adenocarcinoma cell lines were obtained from the Salk Institute and the American Type Culture Collection, respectively.

Culture Methods. Cell lines were maintained in a standard medium

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2 The abbreviations used are: EC, embryonal carcinoma; DMEM, Dulbecco's modified Eagle's medium; ECS, fetal calf serum; i.e., intracardiac; DPBS, Dulbecco's phosphate-buffered saline; [2H]thymidine, [3H]I-5-ido-2'-deoxyuridine; CM, conditioned medium; NGF, nerve growth factor.
of DMEM containing 4.5 g/liter glucose and 15% FCS (GIBCO) as previously described (16). EC lines were routinely propagated on irradiated human fibroblast feeders (16) except for F9 and 8P cells. The latter were grown on Falcon tissue culture plastic dishes in standard medium supplemented with 10^-4 M mercaptoethanol. EC and RAG cell lines were dissociated following a 10-min incubation in Ca^2+-Mg^2+-free DPBS containing 2 mM EDTA and 2% chicken serum. The monolayers were then dispersed by gently pipetting in standard medium. Under the transfer regime employed, EC cells were kept in rapid proliferation and showed little or no in vitro differentiation, regardless of their potential for differentiation under more permissive conditions.

**Animal Injections.** Intraportal injections of tumor cells were at a dose of 2 x 10^6 cells/0.2 ml standard medium. Animals were killed at 1 to 6 months when tumors became obvious or were autopsied at 6 months.

Intracardiac injections were monitored by recording i.e. blood pressure using a Gilson Unigraph (Model ICT-1H) and pressure transducer (Gould Statham model P23 ID) to assure proper insertion and retention of the needle in the left ventricle. Cells filtered through 15-μm nylon cloth were resuspended at a concentration of 1 x 10^6 viable cells/ml in DPBS with 10% FCS. Cell viability was determined by trypan blue exclusion and usually exceeded 90%. The cell suspension was kept on ice until a 0.2-ml volume was removed and injected into each heart through a 25-gauge needle. Animals were anesthetized by i.p. injection of tribromoethanol. For in vivo localization studies, cells labeled with [3H]IdUrd as described below were injected into 3- to 5-month-old LT mice that were killed at intervals of 10 min to 72 h postinjection. Organs were harvested from four animals at each time point and placed in tubes containing 70% ethanol; the ethanol was changed three times over 3 days after which the specimens were counted for 10 min. Cells for labeling were plated at 5 x 10^5 cells/60-mm dish in standard medium plus 10^-4 M mercaptoethanol; no feeder cells were used. [3H]IdUrd at a concentration of 0.1 μCi/ml (5 Ci/mg; Amersham) was added in fresh medium after 24 h and the cells were collected 24 h later after being rinsed twice with DPBS. The cpm/cell was determined by counting aliquots of the cell suspension to be injected and averaged 0.04 cpm/cell. The cpm values obtained on the same day from cells, labeled organs, and unlabeled organs were used to calculate the number of cells retained in each organ.

Direct site injections were performed by inoculating mice directly in target organs with various doses of tumor cells. Ten to 15 mice were injected at each site with a given dose. Cells contained in a total 10-μl volume of DPBS with 10% FCS were delivered slowly into the organ with a Hamilton syringe and the needle was gradually withdrawn over a 1-min period. Mice were injected in most sites through a single midventral incision. Kidney and ovary were reached through a dorsal midventral incision. Ampulla cells were injected s.c. Gonectomized animals were killed 1 to 6 months after tumor cell injection.

**Adhesion Assays.** Whole ovaries and spleens were removed from adult LT or ICR mice and placed in DPBS containing 10% FCS; they were carefully dissected to remove adherent fat or mesenteries so as not to injure the surface epithelium of the organs. Four ovaries or two spleens were added to a siliconized 25-ml flask containing 4 ml DMEM supplemented with 10% FCS and 0.01 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid. Cells were labeled with 0.3 μCi/ml [3H]IdUrd as described above and collected after further incubation for 2 h in unlabeled medium. 5 x 10^6 filtered cells in 0.1 ml were added to each flask which was then rotated at 70 rpm at 37°C in a New Brunswick CO2 incubator shaker having a half-inch radius of gyration. Flasks were removed at intervals of 15–60 min and the organs were rinsed three times in 0.9% NaCl. At each time point four organs of a kind were counted separately in dry tubes for 10 min. Average surface areas of organs were determined assuming a triangular prism shape for spleens and measuring the length, base and height of each spleen; ovaries are spheroids whose major and minor axes were measured. Two control experiments to assess the amount of label released from the cells and absorbed by the organs were performed by incubating labeled cells in flasks without organs for each time period, after which the cells were removed by centrifugation. The supernatant and organs were then incubated in flasks for an equal period of time. Counts incorporated into organs averaged less than 10% of counts taken up in the presence of cells in most samples.

**Autoradiography.** LT clone 8 cells passaged twice without feeders were labeled during exponential growth with 2 μCi/ml [3H]thymidine (20 Ci/mmol; New England Nuclear) for 24 h and further incubated for 2 h in unlabeled medium. Intact organs or lobes from adult LT mice were incubated for 24 hr in vitro with 4 x 10^6 cells in 25 ml flasks on a rotary shaker as described above. They were then rinsed in DPBS, fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 7 μm. Slides were dipped in Kodak NTB2 emulsion, stored at 4°C, developed after 12–16 days, and lightly stained with hematoxylin and eosin.

**Migration Assays.** The migration assay used initially was modified from that of Obeso and Auerbach (29) and used Covasphere MX or FX latex beads (122550, lots 7E83 and 1E84; 122400, lot 6A82; Covalent Tech. Corp., Ann Arbor, MI) suspended in serum-free DMEM to directly coat the bottom of Linbro or Falcon 96-well tissue culture plastic plates. Bead monolayers were formed by adding 0.2 ml bead suspension/well and centrifuging the plate at 500 x g for 15 min at 4°C. EC cells dissociated with EDTA as described above were collected in DMEM containing 0.2% FCS, filtered through 15-μm nylon cloth, and added at 200–500 cells/well. Tissue material or CM and cells were added to the monolayer in a final concentration of 0.03–0.04% FCS. Tissue pieces were 1-mm³ or 1/5 iris from animals extensively perfused with sterile DPBS. CM was produced from identical tissue pieces incubated for 24 h at a similar concentration of 10–20 pieces/ml serum-free DMEM, or in later experiments at 20 mg wet weight/ml (except for iris, not prepared at this concentration). The medium was then centrifuged at low speed and passed through a 0.45-μm pore filter. CM protein concentrations were determined by the method of Lowry et al. (30). Background DMEM values were similarly determined and subtracted to obtain CM protein totals. In later experiments wells were precoated with fibronectin as described (see Ref. 31). More extensive details of this assay, including further modifications that became necessary and quantitative methods, are described (see Ref. 31).

**RESULTS**

EC Cells Colonizing the Ovary i.p. Selectively Adhere to Ovary and Ultimately Invade the Tissue. Differential adhesion of tumor cells to the target organ has been proposed as one possible mechanism for determining the organ specificity of metastasis (32–34; reviewed in Refs. 35 and 36). A subline of LT clone 8 cells deficient in i.p. ovary colonization was therefore selected and compared with LT clone 8 for the ability to selectively adhere to ovary. The subline, designated 8P, was obtained as described in “Materials and Methods” by simply growing the cells on a different culture substrate (plastic as opposed to fibroblast) for 6 months. When subsequently tested, 8P produced very few ovary tumors (1/41 ovary tumors/total females) when injected i.p. Few other i.p. tumors appeared in females (3/41) or males (5/41) injected similarly, as also occurred with LT clone 8 cells (16). The incidence and pattern of tumors arising in either sex after i.p. injection of LT clone 8 or 8P was unaffected by sublethal (650R) host irradiation (data not shown), suggesting that new transplantation antigens observed in some other teratocarcinoma cell lines (37) were probably not responsible for the lack of ovary tumors from 8P cells, or for the lack of other tumors from both cell lines.

LT clone 8 and 8P were tested in an in vitro adhesion assay that utilized intact organs from adult animals in order to simulate in vivo i.p. encounters. Whole ovaries and spleens were cultured for 15–60 min in suspension with [3H]IdUrd labeled
tumor cells as described in “Materials and Methods” (Fig. 1). LT clone 8 cells adhered better to the outside of whole ovaries than to intact spleens (Fig. 1A); the number of cells adhering per cm² of ovary surface area was on the average 5-fold higher than that adhering per cm² of adult spleen. In contrast, 8P cells adhered poorly to the germinal epithelium and showed little organ preference (Fig. 1B). The cell lines NS-1 (myeloma) and RAG (renal adenocarcinoma) that did not form ovarian tumors but grew i.p. nonspecifically (16) were similarly tested and showed equal adherence per cm² to the two organs (Fig. 1B).

Adherence of LT clone 8 to the outer surface was followed by specific invasion of the target organ. Autoradiography of organ sections prepared after 24 h of suspension culture of [³H]thymidine labeled cells with whole ovary and spleen or lobular portions of liver showed EC cells located beneath or in the germinal epithelial layer of the ovary (Fig. 2A), but none were found interior to the surface epithelium of the other organs (Fig. 2, B and C). These results are consistent with the idea that tumors develop in the ovary after i.p. injection of LT clone 8 cells as a consequence of two steps: the cells adhere highly to the germinal epithelium of the ovary; this preferential attachment is succeeded by and facilitates specific cell invasion into the underlying tissue.

Germ/Neuronal Pattern of Tumor Localization Apparent after i.c. Injection. EC cells were introduced into the circulation to determine if a similar ovary preference and adhesive differential existed via this route. No tumors resulted from tail vein injections of 28 LT females with $2 \times 10^6$ LT clone 8 cells. Cells were therefore injected into the left ventricle of the heart to provide a better opportunity for cells to reach peripheral organs during the first arterial pass. A very different result was obtained. The tumor pattern that became apparent after 1 to 2 months was identical for LT clone 8 and 8P cell lines, and is one that has never been seen previously (Table 1). An average of 53% of animals receiving LT clone 8 cells had adrenal tumors, 23–50% had gonadal tumors in both sexes, 37% had eye tumors, and 37% had tumors under the whiskers. Most of these tumors were confirmed by either histology or culture of EC cells in vitro from the minced organ. Lungs, spleen, and thymus from 10 animals were also cultured and all were negative. The eye tumors (Fig. 3A) were apparent first, at around 3 weeks, as a white opacity between the cornea and the iris. The iris sometimes multiplied abnormally, making the tumors appear hazy black. In two of the earliest tumor stages sectioned the bulk of the tumor lay in the anterior chamber, but lining both sides of the iris (Fig. 3B); the posterior chamber was not otherwise involved. “Whisker” tumors first appeared grossly as a lump in the general area (Fig. 3C). Tumors at 10–14 days were localized histologically approximately 4–5 mm from the tip of the nose to an area near the infraorbital fissure, through which the infraorbital artery runs together with the trigeminal...
Tumors produced by site-specific EC lines were grossly obvious in all examined sites listed in Table 2. Nevertheless, a tumor pattern very similar to that of LT clone 8 was observed following their i.e. injection (Table 2). Ovary, adrenal, eye, and "whisker" tissues were again the predominant sites, with very few tumors found in other organs examined. Two criteria were used to ascertain the presence or absence of tumors in various target organs (due to organ size, opacity, compactness, or locale of tumor origin within the organ), and (c) differences in the number of cells initiating a tumor in different organ sites. Tumors in these submaxillary glands were visible by gross inspection. Apart from male submaxillary gland (Table 2), although none of these were apparent by gross inspection. Apart from male submaxillary gland (Table 2), although none of these were noted that LT clone 8 produces tumors composed almost entirely of embryonal carcinoma, and the tumors sectioned from different sites were histologically indistinguishable from those derived from LT clone 8 cells. A, LT mouse with eye tumor; B, histological section of early eye tumor (short arrow, iris; long arrows, tumor areas on either side of the iris (× 46). Inset, higher magnification of tumor area (× 320); C, LT mouse with bulged tumor area underneath the whiskers; D, section of adrenal with multiple early tumors (arrows) located in the cortex (× 26). Inset, higher magnification of right tumor focus (× 320). E, histological section of early tumor (arrow) in rostral area. The premaxillary bone (b) is medial to the tumor, which lies among branches of the trigeminal nerve (n). The whiskers (w) project laterally. x 22.

Table 2 Tumor distribution of i.c.-injected embryonal carcinoma cells

<table>
<thead>
<tr>
<th>Site of tumors</th>
<th>Ovary/testis</th>
<th>Adrenal</th>
<th>Eye</th>
<th>Whiskers</th>
<th>Submaxillary gland</th>
<th>Liver</th>
<th>Lung</th>
<th>Brain</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Kidney</th>
<th>Other</th>
<th>None</th>
</tr>
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<tbody>
<tr>
<td>Cell line</td>
<td>No. and sex injected</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>T6-4</td>
<td>12 F</td>
<td>8 (4)*</td>
<td>11 (10)</td>
<td>3 (2)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1*</td>
<td>1</td>
</tr>
<tr>
<td>T6-11</td>
<td>9 F</td>
<td>7 (6)</td>
<td>8 (8)</td>
<td>1</td>
<td>6 (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3*</td>
<td>1</td>
<td></td>
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<tr>
<td>LT clone 8</td>
<td>10 M</td>
<td>0</td>
<td>7 (6)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>9 M</td>
<td>3 (1)</td>
<td>9 (8)</td>
<td>5 (5)</td>
<td>9 (9)</td>
<td>5</td>
<td>0</td>
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<td>6 F</td>
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<td>5 (4)</td>
<td>2</td>
<td>5 (3)</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>6050</td>
<td>5 F</td>
<td>5 (5)</td>
<td>5 (5)</td>
<td>5 (5)</td>
<td>3</td>
<td>1</td>
<td>2</td>
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<td>5</td>
<td>4</td>
<td>5</td>
<td>5 (5)</td>
<td>0</td>
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<tr>
<td>6050-F9</td>
<td>8 F</td>
<td>8 (8)</td>
<td>7 (7)</td>
<td>6 (2)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>2*</td>
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* Numbers in parentheses, animals with bilateral tumors.
† Includes one lower jaw tumor.
‡ Includes three lower jaw tumors.
§ Includes two lower jaw tumors.
¶ Includes two heart tumors.

To determine the localization pattern of other EC cells lines, two additional lines derived in this laboratory from 7-day CBA/T6 embryos (T6-4 and T6-11) were injected i.c. In contrast to LT clone 8, both T6 EC cell lines are highly multipotential and also produced many nonspecific tumors on i.p. inoculation. Nevertheless, a tumor pattern very similar to that of LT clone 8 was observed following their i.e. injection (Table 2). Ovary, adrenal, eye, and "whisker" tissues were again the predominant tumor sites, with very few tumors found in other organs examined. Two criteria were used to ascertain the presence or absence of tumors in various target organs (due to organ size, opacity, compactness, or locale of tumor origin within the organ), and (c) differences in the number of cells initiating a tumor in different organ sites. Tumors in these submaxillary glands probably arose from only one or two cells, as estimated from the tumor incidence.

For all animals included in Table 2, whole organs including liver, lung, brain, spleen, thymus, kidney, and all target site

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organs were taken at 2–6 weeks postinjection, finely minced, and cultured in vitro (on feeders if required) for up to 3 weeks to assay for the presence of EC cells. These cells are morphologically idiosyncratic and in addition aggressively overgrow the cultures. Nevertheless, to demonstrate experimentally that the in vitro assay detects metastases; that is, the EC cells obtained in in vitro are indeed malignant and capable of reforming metastases, EC cultures derived from various tumor sites were further examined. Twelve LT clone 8 cultures, two from each specific tumor site including four from individual organs not grossly positive for tumors, were tested for their ability to form tumors. 10⁵ cells injected into each testis of four to seven animals/culture resulted in tumors that were very obvious upon autopsy 3–6 weeks later in 92% of the testes. Individual failures were unrelated to the ability to grossly detect the original tumor. In addition, three other LT clone 8 specific tumor site-derived cultures were reinjected i.c. into three to 21 males or females/culture. In these cases, gross tumors formed in two to four of the specific sites, demonstrating the metastatic capability of cells derived from the in vitro test procedure. The fact that single tumor-derived cells seeded different metastatic sites also indicates that the EC cells which formed the original tumors retained the capacity to colonize other and possibly all specific sites (but see Ref. 38).

For T6-4 and T6-11, very few additional tumors were detected by in vitro culture. However, only 15% of T6-11 and 82% of T6-4 tumors that were grossly evident gave rise to morphologically detectable EC culture in cells. Presumably the multipotential stem cells of these lines frequently differentiated completely during tumor growth. It is therefore possible that small tumors from these lines were not detected, which could account for the lack of testicular and submaxillary tumors in males injected with T6-11 cells.

Little site specificity was apparent with 6050 and F9 cells; tumors formed in all or nearly all organs examined (Table 2). Both the 6050 tumor line (39) and F9 cells derived from it (40) are long-term animal transplant or in vitro cultured teratocarcinoma lines, in contrast to the four site-specific EC lines.

It is evident from Tables 1 and 2 that another, minor site of specific EC localization occurred in what is termed the “lower jaw.” This area did not include the submaxillary gland; although the exact tumor origin is uncertain, it possibly involved the base of the teeth.

Finally, the fact that 8P cells were equally as able as LT clone 8 to colonize the ovary (and other targets) from the circulation was taken to indicate that preferential adhesion of EC cells might not be responsible for their specific localizations via this route.

Initial Arrest Pattern Does Not Match i.c. Tumor Pattern.

The retention of cells in various organs was measured by injecting 2 x 10⁶ LT clone 8 cells labeled with [125I]IdUrd into the left ventricle and determining the residual radioactivity at 10 min–72 h postinjection (Fig. 4). A control experiment determined the amount of [125I]IdUrd that could be tolerated by EC cells, since 125I radiotoxicity varies considerably among cell lines (41). The treatment chosen (0.1 μCi/ml for 24 h) did not substantially affect the growth rate of LT clone 8 cells (Fig. 5) nor did radiolabel per se effect tumor incidence or location (Table 1). Preferential localization in tumor sites was not apparent, as radiolabeled LT clone 8 cells were distributed initially in organs in close proportion to the percentage of cardiac output. The values of percentage of cardiac output (42) versus percentage of inoculum retained at 10 min for ovaries and adrenals were 0.07 versus 0.07 and 0.12 versus 0.10. Heart, spleen, and kidney values, respectively, were 1.2 versus 1.6, 0.39 versus 0.84, and 3.6 versus 1.5. For organs affected by recirculation (liver and lung) the corresponding respective values were more disparate: 7.8 versus 18.0 and 2.1 versus 9.4. After 24–72 h, few if any cells could be detected in adrenals and ovaries; no more than 3% of those originally present in the organ. The i.c. tumor pattern clearly does not correlate with the initial arrest pattern of cells injected i.c. Most [125I]IdUrd-labeled cells injected i.c. are found at 10 min postinjection in lungs and liver, yet very few, if any, tumors develop in these organs (Tables 1 and 2).

Tumor Localization Pattern Does Not Correlate with the Ability to Grow Only in Specific Organs. LT clone 8 cells directly injected into the kidney formed tumors with frequencies similar in a dose-response curve to directly injected ovary or testis (Figs. 6, A and B), yet no tumors formed in kidneys from i.c.-injected cells. The growth pattern was not different when cells were injected directly into the parenchyma of organs in castrated or ovariecotomized animals (Figs. 6, C and D). It is clear that differences existed among various organs in the incidence of tumor growth from directly injected cells (Fig. 6). Ovary, testis, and kidney tissues were characterized by high dose response curves, liver was intermediate, and stomach musculature low. In another experiment, normal males each injected in multiple sites gave responses similar to the above in testis, kidney and liver, and low takes in two additional sites: spleen.
to the apparent disparity between the adrenal tumor site and induction of in vitro migration is uncertain.

To quantitate migration activity, fixed plates were viewed under a television camera and the relative light intensity of track areas covering an entire 7-mm well bottom was recorded using a Quantex QX 9200 image processor and analyzer (31). CM was diluted and a fibronectin substrate was used as it is required for EC migration at low CM doses (31). Fig. 8 shows that activity profiles of CM from two organs inducing extensive migration (ovary and submaxillary gland) are very different from those of two negative tissues (muscle and adrenal). Fig. 9 shows the phagokinetic tracks induced by different concentrations of submaxillary gland CM in some of the same wells analyzed to produce Fig. 8.

**DISCUSSION**

The most significant finding from this study is that EC cell lines display very selective and unique localizations in vivo. Adult animals develop tumors primarily in sites localized to regions of the head and neck and in the gonads and adrenals when injected i.c. Very few tumors form at other sites, even in organs that are clearly capable of supporting tumor growth and that receive by far the larger dose of injected cells. If growth support and cell number were the important factors specifying this pattern, then tumors should have been observed in at least the kidney, where both of these are high, but none were. Thus, although these factors are necessary for tumor formation, they are not sufficient to explain the observed pattern of metastasis.

The i.c. pattern holds for three independent EC cell lines tested. In addition, one of these lines (LT clone 8) produces only ovary tumors when injected i.p. This pattern difference most likely results from different modes of access to possible target organs: the cells injected i.p. have direct contact only with the ovary and do not circulate via the blood in sufficient numbers to produce tumors in the other sites. The ability to adhere strongly to the target organ may be important in ovary colonization via the peritoneal cavity, since a subline of LT clone 8 defective in ovary adhesion failed to produce tumors when injected i.p. Nevertheless, this subline (8P) did specifically metastasize to the ovary (and other target sites) when injected i.c. as well the parental line did. This result might be explained if the i.c. tumor pattern, on the other hand, depends primarily on organ-specific invasion responses after the cells arrest non-specifically. This interpretation is based on the observations that (a) the target sites correlate with the ability of the tissues themselves or CM derived from them to stimulate EC cell specifically. This interpretation is based on the observations that (a) the target sites correlate with the ability of the tissues themselves or CM derived from them to stimulate EC cell 

and ampulla s.c. space (data not shown). These results suggest that LT clone 8 tumors may grow more readily within the tissues of some sites than others once the cells are established there, but this explanation alone does not sufficiently account for the tumor distribution patterns.

**Target Tissue Stimulates Migration of EC Cells.** Using a visual assay to analyze movement, tissue pieces or CM from five of the six tumor sites tested stimulated EC migration in vitro (Figs. 7 and 9). Positive results were obtained with filtered, cell-free testis and ovary CM (Fig. 7, A and C) and submaxillary CM (Fig. 9). Migration tracks were not observed with CM from either iris or whisker follicle or nerve, but were present in a spatial gradient from tissue pieces of these organs (Fig. 7, E and G). No migration tracks occurred in the absence of added EC cells in these instances, indicating that normal tissue cells were not producing the tracks. In contrast, tissue and CM from control organs including liver, kidney, and masseter muscle, or lung or spleen CM gave no migration (Fig. 7). Migration was also not induced by either tissue or CM obtained from whole adrenal glands. Preliminary results indicate that epinephrine and norepinephrine inhibit EC migration induced by submaxillary CM (data not shown), but the relevance of this observation is uncertain.

Fig. 6. Tumor incidence in sites injected directly. LT clone 8 cells were injected immediately into the tissue of various sites as described in "Materials and Methods." Ten to 15 mice were injected at each site with a given dose. A, normal males; B, normal females; C, castrated males; D, ovariectomized females.

Fig. 8. Activity profile of migratory response of LT clone 8 to organ CM. Cells were plated on Covasphere FX bead monolayers in wells precoated with 5 µg/ml fibronectin. After 24 h wells were fixed and migration track areas were quantitated using a Quantex QX 9200 image processor and analyzer as described (see Ref. 31).
Fig. 7. LT clone 8 migration in response to tissue or medium additives. LT clone 8 cells plated on Covasphere MX bead monolayers were exposed to either tissue pieces or conditioned medium as described in the text. A, ovary CM; B, 0.04% FCS; C, testis CM; D, kidney CM; E, iris tissue; F, liver CM; G, whisker nerve tissue; H, liver tissue. Photographs were taken 30–38 h after cells were added. × 72.
frequent ectopic germ cells in the mouse embryo (45, 46), as well as of neural crest migration. Embryologically, the adrenal cortex and genital ridge share a common origin from the visceral layer of lateral plate mesoderm. Three of the sites (iris, whisker field, and lower jaw) are each innervated by one of the three main branches of the trigeminal nerve, the largest sensory nerve in the body. At least one of these trigeminal target organs is known to produce high levels of NGF, which is chemotactic for nerves as well as a necessary growth factor (47). The iris, in fact, is second only to the submaxillary gland in NGF mRNA levels (48). The extremely high levels of NGF in the tumor target submaxillary gland, on the other hand, are related not to its innervation but to its exocrine function. NGF is released into the saliva via excretory ducts that open in the mouth just posterior to the incisor teeth. Whether any of this submaxillary NGF has direct access to the blood is unknown, although appreciable to very high amounts of NGF are found in the bloodstream from the gland shortly after aggressive male behavior or pharmacological stimulation (49, 50). The rodent iris and embryonic whisker field are also known to produce other specific neuronal outgrowth factors that are distinct from NGF (51–53). There are many similarities between EC cells and both primordial germ cells and primitive neuroectoderm cells of the early embryo; murine EC cells are most analogous to inner cell mass cells that may be viewed both as close ontogenic derivatives of germ cells and as precursors to the germ and neuroectoderm lines. Neural cells are the most frequent in vivo (54, 55) and a common in vitro (56–60) differentiation of EC cells, perhaps because of related developmental programs that might also be the basis for similar sensitivities. We have investigated in more detail the EC migration stimulus derived from one specific tumor target site, the male submaxillary gland (31), and the results obtained suggest this CM factor may be a form of NGF. Definitive identification of this molecule will permit very specific questions about its involvement in the metastatic pattern of EC cells and in embryonic development to be investigated.

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