Clonal Growth of Tumors on Tissue-specific Biomatrices and Correlation with Organ Site Specificity of Metastases

Ralph Doerr, Isabel Zvilb, Diana Chuiten, James D'Olimpio, and Lola M. Reid

Departments of Surgery [R. D.], Oncology [J. D'O., D. C.], and Molecular Pharmacology [I. Z., L. M. R.], Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT

We have found that neoplastic transformation alters the ability of cells to grow on substrata of tissue extracts, "biomatrices", enriched in extracellular matrix. Tumor cells were able to survive and grow at lower densities and on more types of biomatrices than normal cells. When plated at high densities (>10^6 cells/60 mm dish), tumor cells attached with equal efficiency and grew at similar rates to and equivalent saturation densities on biomatrices derived from all tissues. However, at low (10^4-10^6/mm dish) seeding densities, the tumor cells grew only on certain types of biomatrices. For the various hepatoma and mammary carcinoma cell lines tested, the tissue specificity in clonal growth on biomatrices correlated with their organ site specificity for metastasis in vivo in immunosuppressed, athymic nude mice. Analysis of the effects of purified matrix components (adhesion proteins, collagens, glycosaminoglycans) indicated that only the glycosaminoglycans influenced density-dependent survival and growth of tumor cells with effects that differed with respect to the cell's metastatic potential. The results indicate that the ability of tumor cells to colonize specific tissues represents, in part, regulation of low density survival and growth by extracellular matrix and are suggestive that one of the matrix components responsible may be proteoglycans or their glycosaminoglycan chains.

INTRODUCTION

Metastasis, the spread of cancer cells from one organ to another not directly connected to it, is not a random process. The spread of neoplastic cells to secondary sites is a complicated, multistep process requiring detachment of tumor cells or small clumps from the primary tumor, invasion through tissue into blood or lymphatic vessels, interaction with and escape from host defense mechanisms, and infiltration and proliferation in tissues at sites distant from the origin of the tumor (1). Completion of the metastatic process requires that tumor cells grow and colonize the tissue which they have invaded. Specific types of tumors are known to metastasize to specific tissues. For example, prostatic and breast tumors spread to bone, whereas colon tumors tend to metastasize to lung or liver. Typical metastatic distribution patterns for different tumors have been described in great detail in numerous studies and reviews (1-2). Simple mechanical entrapment of circulating tumor cells in a new organ does not explain the preference of different tumors for growing as metastatic colonies in specific organs. The phenomena was described as early as 1889 by Paget (3) who proposed "the seed and soil" hypothesis. The migrating tumor cell must find a fertile "soil" in the organ to be colonized.

The factors affecting site specificity of metastasis include attachment of the tumor cells to the parenchyma of the colonized organ, and/or to the endothelial capillaries of that organ.

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2 To whom requests for reprints should be addressed, at Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

3 The abbreviations used are: ALS, antilymphocyte serum; HDM, hormonally defined medium; PBS, phosphate buffered saline; SSM, serum supplemented medium; TCP, tissue culture plastic; GAG, glycosaminoglycan.
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Fig. 1. Method of testing organ site specificity of tumor cells in vivo. Tumor cells (10^6-10^7 cells) were inoculated s.c. into athymic nude mice, some of which were immunosuppressed by one of several protocols (see "Materials and Methods"). After 3-4 weeks, the mice were sacrificed and autopsied to evaluate the invasive and metastatic potential of the tumors by microscopic examination of the host organs, by microscopic examination of sections of liver, kidney, lung, and lymphatic tissue (lymph nodes and spleen) and by screens for clones of tumor cells, identifiable by karyotype in primary cultures of mouse kidneys, lungs, liver, and lymphatic tissue. For the culture assay, the host tissues (lung, liver, lymphatic tissue, and kidneys) were individually mechanically dissociated into serum-free RPMI 1640 supplemented with antibiotics (penicillin and streptomycin) by pressing the tissue through a collector (Belico Inc., NJ) to make cell suspensions. The cell suspensions were then treated with antiserum to mouse cells (prepared as described in "Materials and Methods") and with rabbit complement to reduce the fraction of the cell suspension containing mouse cells. The remaining viable cells from each sample were then plated onto a 60-mm dish coated with type IV collagen and in RPMI 1640 supplemented with antibiotics, and with 10% fetal calf serum. After 24 h, the medium in the cultures was changed to a serum-free, HDM appropriate for the tumor cell type inoculated into the mouse. Thus, the HDM for hepatomas (12) was used when any of the hepatoma cells were inoculated into the mice, and the HDM developed by Allegra and Lippman (13) was used for mice inoculated with mammary carcinoma cells. The cultures were maintained for up to 2-3 weeks, with daily medium changes, and examined throughout for clones of epithelial cells. Any colonies were evaluated by karyotype for the presence of either human or rat cells.

Primary cultures of normal rat hepatocytes were prepared by the methods of Berry and Friend (9).

Karyotypes were done by the procedures of Comings et al. (8).

Cell Cultures

Primary cultures of the mouse tissues from animals inoculated with tumor cells were prepared by dissecting the tissues from the animals using sterile techniques, pressing the tissue through a collector with a no. 2 coarse grid, 860 μm (Belico Glass, Inc., Vineland, NJ), and suspending the cells in RPMI 1640 supplemented with 1% ALS (Whittaker M. A. Bioproducts, Walkerville, MD) plus 2% rabbit antiserum prepared against the mouse tumor cell A9, prepared as described.
Organ Site Specificity in Metastasis

RESULTS

Organ Site Specificity of Tumors in Immunosuppressed Nude Mice. The results of experiments to determine the in vivo metastatic potential of three human hepatoma cell lines are summarized in Table 1. For all the tumor cell lines, macroscopic and microscopic evaluation of the host organ tissues failed to show metastatic lesions (data not shown). Our positive results came only from the culture studies. HepG2 routinely formed tumors in nude mice only at inoculum densities of $10^7$ cells. However, even at this inoculum cell density and with immunosuppression of the nude mice, no HepG2 cells were isolated from cultures of any nude mouse tissue. Though it is impossible to state whether or not there were any metastatic HepG2 cells in any of the tissues, it is clear that this cell line has quite limited metastatic potential. PLC/PRF/5 was also shown to require high cell inoculum densities, $10^7$, for expression of full metastatic potential, due in part to host defense mechanisms that are vulnerable to ALS treatment. Thus, tumor formation required $10^7$ cells/mouse, and metastatic potential was not observed unless the mice were immunosuppressed with ALS. Metastatic lesions were found in liver, lung, and lymphatic tissue but not in kidney. SK/Hep-1, the most anaplastic of the three human hepatoma cell lines, metastasized even in nonimmunosuppressed nude mice when high cell inoculum densities ($10^7$) were used, and the occurrence of metastatic cells increased in immunosuppressed mice. Metastatic cells were isolated from all the tissues cultured: liver, lymphatics, lungs, and kidney.

The in vivo metastatic potential of two human mammary carcinoma cell lines, BT-20 and MCF-7, and of a rat cell line, MTLn3 was also assessed. As with the hepatoma cell lines, high inoculum cell densities were required and immunosuppression was helpful in observing metastatic potential, especially for the two human cell lines. In our assays, the BT-20 metastasized only in nude mice that were immunosuppressed and

previously (10) plus 1:15 dilution of rabbit complement (Cappel Laboratories, Cochranville, PA). The samples were incubated at 1h. Then the samples were centrifuged at 900 RPM and the cell pellet rinsed twice with serum-free RPMI 1640. The cell and tissue pellets were then suspended in a SSM (see below) and were plated onto 60-mm culture dishes coated with type IV collagen, each tissue being plated onto one dish. 24h later the plates were rinsed with PBS, and the cultures fed with a serum-free, HDM designed either for hepatoma cells (11-12) or for mammary carcinoma, especially (13). The cultures were then incubated at 97°C in a Wedco, water-jacketed incubator supplied with 5% CO2 and 95% air. Culture medium was changed every other day for 2 to 3 weeks. Cultures containing clones of epithelial cells were then karyotyped to ascertain if they were of human or rat origin. The sensitivity of the assay is empirically defined by the minimum seeding density needed for survival of the specific tumor cells on type IV collagen and in their respective HDM. The minimum seeding densities (on 60-mm plates) found were: HepG2 ($10^5$), PLC/PRF/5 ($10^6$), SKHepl ($10^6$), BT-20 ($10^7$), MTLn3 ($10^6$), and MCF-7 ($10^6$).

Cell Lines

Hepatoblastomas. PLC/PRF/5, a human hepatoma cell line established by Alexander et al. (14), which synthesizes HBsAg in vitro (8) was obtained from Dr. I. Millman (Fox Chase Cancer Center, Philadelphia, PA). These cells also express several liver-derived proteins (15-16). SK/Hep-1 (17) another human hepatoma cell line was a gift from Dr. Jorgen Fogh (Sloan Kettering Institute, Walker Laboratories, NY). This relatively anaplastic cell line expresses liver-specific protein but few other liver-specific functions. HepG2 (16) a minimally deviant human hepatoma cell line, was obtained from Dr. Barbara Knowles (Wistar Institute, Philadelphia, PA).

Mammary Carcinomas. The rat mammary cell line, MTLn3, was derived from a lung metastatic lesion of a mammary carcinoma and has been extensively studied and characterized by Dr. G. Nicholson and his associates (M. D. Anderson, Houston, TX) (18). They have shown that it is a highly metastatic tumor cell, especially for lung tissue in syngeneic hosts and in nude mice. The two human mammary carcinoma cell lines were obtained from the American Type Culture Collection. MCF-7 derives from a pleural effusion in a patient with metastatic mammary carcinoma (19), and BT-20 derives from a well differentiated human adenocarcinoma (20). Both have been extensively characterized in culture studies (21) and in vivo in athymic nude mice (22). MCF-7 has been shown to be dependent on estrogens for its metastatic potential.

Culture Conditions

SSM. The cells were cultured in SSM consisting of RPMI 1640 (GIBCO) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM l-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (GIBCO).

Serum. Fetal bovine serum (Hyclone Lot 1101501) was purchased from GIBCO and heat-inactivated for 30 min at 56°C. Serum lots were screened on several cell lines and on several primary cultures prior to selection of lots of serum. Serum lots were then purchased and frozen in large quantities (up to 20 liters at a time). After heat-inactivation of a liter of serum, it was aliquoted into 100-ml bottles and refrozen. The cultures were incubated for varying lengths of time, up to 3 weeks, in a Wedco water-jacketed incubator at 97°C with 5% CO2. The cultures were assayed for (a) attachment efficiency; (b) survival; (c) growth rate and saturation density; (d) clonal growth efficiency; and (e) DNA content. The assays were done as described previously (11-12).

Evaluation of Survival and Growth on Substrata of Biomatrix

Suspensions of cells from stock cell cultures or freshly isolated rat liver cells from liver perfusion (see above) were plated at densities from $10^5$ to $10^6$ cells per 60-mm culture dishes and on the following substrata: tissue culture plastic dishes, and biomatrix derived from liver, lung, kidneys, and lymphatics (spleen + lymph nodes). Experiments were performed using triplicate plates per cell line per time point. The cultures were incubated for varying lengths of time, up to 3 weeks, in a Wedco water-jacketed incubator at 37°C with 10% CO2. The cultures were assayed for (a) attachment efficiency; (b) survival; (c) growth rate and saturation density; (d) clonal growth efficiency; and (e) DNA content. The assays were done as described previously (11-12).

Sources of Glycosaminoglycans. Reference standards for glycosaminoglycans (dermatan sulfate, chondroitin sulfate, heparan sulfate, heparin, and hyaluronate) were provided by Dr. Martin Mathews and Dr. J. A. l'itini-li of the University of Chicago (contract NO1-AM-5-2205). For preliminary studies, glycosaminoglycans were obtained from Sigma Chemical Company including chondroitin-4-sulfate from whale cartilage; chondroitin 6-sulfate from shark cartilage; derman sulfate from porcine skin; heparins from porcine intestinal mucosa or from bovine lung; and hyaluronic acid from human umbilical cord.

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metastatic lesions were isolated from lung and kidney tissues. Similarly, MCF-7 metastasized in estrogenized nude mice but also required immunosuppression. Tumor cells were found in the lungs, kidney, and lymphatic tissue. The rat mammary carcinoma, MTLn3, metastasized in both immunosuppressed and untreated nude mice, but as with SK/Hep-1, spread with greater frequency in the immunosuppressed nude mice. Metastatic lesions were found in lungs, lymphatics, and kidneys.

Growth Parameters of Normal Cells versus Tumor Cells at High Seeding Densities. We tested the ability of normal hepatocytes and of tumor cells of different metastatic potential, to attach and grow on biomatrix prepared from different organs. Representative data from the normal hepatocytes and from three of the cell lines are shown in Table 2. The other cell lines yielded similar results (data not shown). Primary cultures of normal liver cells seeded in SSM and on all substrata, the tumor cell lines did not show any evidence of growth. After 2–3 weeks, survival of the primary liver cultures occurred only at high densities (10^7 or greater) and only on biomatrix derived from liver.

By contrast to the normal liver cells, the tumor cell lines attached readily and proliferated on all substrata. The proliferation rates of the minimally deviant tumor cell lines (HepG2 and BT-20) were reduced twofold or more (BT-20 data not shown) when the cells were plated on biomatrix substrata as compared with their rates on tissue culture plastic. Similarly, shown) when the cells were plated on biomatrix substrata and BT-20 were reduced twofold or more (BT-20 data not shown) when the cells were plated on biomatrix substrata as compared with their rates on tissue culture plastic. Similarly, the saturation densities reached by all the cell lines did not vary in a pattern that correlated with metastatic potential.

The experiments shown in Table 2 were done in SSM. However, all the experiments were also done in the appropriate HDM for the cell type. The results in HDM and at high cell densities also did not show any pattern that correlated with organ site specificity (data not shown).

In conclusion, there were distinctions in the proliferation rates between cells growing on TCP or matrix substrata, but there was no differential effect on the various tumor cell lines

### Table 1 Organ site specificity of metastases in hepatomas and mammary carcinomas in immunosuppressed nude mice

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>No. of animal(s) with metastatic colonies in</th>
<th>Summary of tissues colonized</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>Liver Lung Kidney Lymphatics</td>
<td></td>
</tr>
<tr>
<td>0/9</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>4/9</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>8/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>10/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>0/12</td>
<td>Liver, lung, lymphatics</td>
</tr>
<tr>
<td>8/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>10/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>SK/Hep-1</td>
<td>10/18</td>
<td>Liver, lung, lymphatics, kidney</td>
</tr>
<tr>
<td>10/18</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>10/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>Mammary carcinomas</td>
<td>2/18 10^6-5x10^6</td>
<td>None</td>
</tr>
<tr>
<td>BT-20</td>
<td>2/18 10^6-5x10^6</td>
<td>None</td>
</tr>
<tr>
<td>7/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>8/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>6/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>4/15 10^6</td>
<td>None</td>
</tr>
<tr>
<td>18/21</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>9/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>9/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>MTLn3</td>
<td>6/7 10^6</td>
<td>None</td>
</tr>
<tr>
<td>10/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>9/10</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>10/10</td>
<td>0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2 Tumor cell growth parameters on biomatrix which did not correlate with metastatic potential

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>TCP</th>
<th>Liver</th>
<th>Lung</th>
<th>Lymphatics</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat hepatocytes</td>
<td>46 ± 6</td>
<td>44 ± 6</td>
<td>49 ± 3</td>
<td>40 ± 7</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>66 ± 18</td>
<td>80 ± 15</td>
<td>78 ± 8</td>
<td>72 ± 12</td>
<td>77 ± 11</td>
</tr>
<tr>
<td>SK/Hep-1</td>
<td>81 ± 3</td>
<td>86 ± 4</td>
<td>93 ± 6</td>
<td>89 ± 9</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>MTLn3</td>
<td>90 ± 4</td>
<td>92 ± 5</td>
<td>100 ± 2</td>
<td>92 ± 8</td>
<td>90 ± 5</td>
</tr>
</tbody>
</table>

* Form of immunosuppression. See "Methods" for full explanation.
* With or without immunosuppression.
* In prior studies by Liotta and associates (22), it has been shown that the metastatic potential of MCF-7 in nude mice requires estrogen (E) treatment. Therefore, the mice were treated with 20 mg pellets of 17β-estradiol on the contralateral side to the injected tumor cells.

# References

For a detailed bibliography, please consult the original research article. This table and discussion are based on specific experiments and findings that highlight the differences in cell behavior under various conditions. Understanding these differences is crucial for advancements in cancer research and treatment strategies.
of the matrices from the various organs on the attachment, proliferation rates, or saturation densities.

**Low Density Growth Efficiency of Normal Hepatocytes versus Tumor Cell Lines.** The biomatrices from different organs were assessed for their influence on growth and survival of hepatocytes, hepatoma cells, and mammary carcinomas cell lines seeded at low densities. In Fig. 2A is shown a positive and negative clonal growth efficiency assay of the human hepatoma cell line, SK/Hep-1. The screen of clonal growth efficiency on various types of biomatrices for the partially differentiated human hepatoma cell line PLC/PRF/S, is shown in Fig. 2B. In Table 3 are given the quantitative data from studies of clonal growth efficiency of normal versus neoplastic cells when plated onto various substrata. Although they attached to the matrices, the normal hepatocytes did not grow clonally on any of the substrata tested. By contrast, the neoplastic cells did grow clonally on TCP and on some, but not all types of biomatrix. HepG2, showed a low clonal growth efficiency, requiring a seeding density of at least \(10^5\)–\(10^6\) cells/dish for any colonies to form. On liver biomatrix, the HepG2 cells attached with greater than 90% attachment efficiency. However, the attached cells survived but did not form colonies throughout the 2–3-week time course of the assay. The PLC/PRF/S and SKHep1 cells formed colonies at 100 cells/dish. They both grew well clonally on biomatrix prepared from liver, lung, and lymphatic tissue. Both could form colonies at higher seeding densities (500–1000 cells/60-mm dish) on kidney biomatrix, although PLC/PRF/S did show a slight ability to form colonies on kidney biomatrix at a density of \(10^5\) cells/60-mm dish.

The poorly metastatic mammary carcinoma cell lines also showed a strongly density-dependent clonal growth that was influenced by the types of matrix used as substratum. Thus, BT-20, the low metastatic cell line did not form colonies on any biomatrix type unless at least \(5 \times 10^5\) cells/dish were seeded. However, at \(5 \times 10^5\)–\(10^5\) cells/dish, BT-20 could grow only on matrix substrata derived from lung and kidney. It was not able to grow on the other matrix substrata except at densities above \(10^6\) cells/dish. A sharp change in BT-20’s ability to survive and grow occurred at densities of \(10^5\) cells/dish and higher: the cells were able to attach and grow on all types of biomatrix. However, even at \(10^5\) cells/dish, its ability to grow clonally was somewhat restricted on biomatrix from lymphatic tissue.

At the other extreme, the carcinoma cell lines MCF-7 and MTLn3, formed colonies on most types of biomatrix at seeding densities of 100 cells per plate. However, even these poorly differentiated, highly metastatic cell lines showed some differential clonal growth efficiency depending on the type of biomatrix. MCF-7 and MTLn3 grew well on biomatrix derived from lung, kidney and lymphatic tissue but required higher seeding densities for survival and growth on biomatrix from liver. An exception to the correlation between the in vivo data and the culture results was evident in MTLn3’s high clonal growth efficiency on kidney biomatrix and its relatively low metastatic potential to kidney in vivo.

In conclusion, the minimally deviant hepatoma and mammary carcinoma cells required high seeding densities in order to overcome the inhibitory effects of biomatrix on their survival and growth. Almost all the data was consistent and indicated that the hepatomas and mammary carcinoma cell lines had a selective advantage and formed more colonies when seeded at low densities on matrices from those organs to which the cells metastasized in vivo. This pattern is evident even in the case of the highly metastatic mammary carcinomas MCF-7 and MTLn3: both required higher seeding densities for growth on liver biomatrix and showed no evidence of metastasis to the liver in vivo.

**Effect of Individual Matrix Components on Survival of Highly versus Poorly Metastatic Cells.** Our next question was which one of the matrix components was affecting the growth and survival of cells seeded at low densities. The different matrix components were tested separately for their effect on the survival and proliferation of normal cells (hepatocytes) and of the tumor cells. Representative data is given in Table 4.

Under all conditions, normal hepatocytes required high seed-
ing densities for survival. However, the density at which they could survive when on TCP was approximately 10 times higher than the density at which they could survive on collagens (type I and type IV). They did not proliferate in the SSM on any of the substrata tested (data not shown).

All of the tumor cells were helped in their attachment and survival on laminin, fibronectin, collagen type I and collagen type IV when plated at low seeding densities. However, none of these matrix components distinguished between the density-dependent growth of highly metastatic versus poorly metastatic cells (Table 4). The only extracellular matrix molecules found to inhibit the survival (and of growth; no data shown) of poorly metastatic cells at low densities, but not of highly metastatic cells at low densities, was the glycosaminoglycan, heparin (Table 4).

Table 3 Influence of biomatrices on density-dependent growth of highly versus poorly metastatic tumor cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Metastatic potential (organ sites)</th>
<th>Seeding density</th>
<th>TCP</th>
<th>Average number of colonies/60-mm plate* on Biomatrix derived from</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Hepatoma cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HepG2</td>
<td>Low (none)</td>
<td>$10^2$-3</td>
<td>6.8 ± 4</td>
<td>Liver: 0, Lung: 3 ± 5, Kidney: 1 ± 2, Lymph: 1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^4$</td>
<td>3.7 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>29 ± 7</td>
<td>7.5 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^4$</td>
<td>Too many to count</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>$10^6$</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^7$</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^8$</td>
<td>Too many to count</td>
</tr>
<tr>
<td>B. Mammary carcinomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT-20</td>
<td>Low (lungs, kidney)</td>
<td>$10^2$-3</td>
<td>0</td>
<td>Liver: 0, Lung: 0, Kidney: 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5 	imes 10^3$</td>
<td>0</td>
<td>Too many to count</td>
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<tr>
<td></td>
<td></td>
<td>$10^4$</td>
<td>2 ± 1</td>
<td>Too many to count</td>
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<td></td>
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<td></td>
<td>43 ± 3</td>
<td>21 ± 4</td>
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<td>$10^7$</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^8$</td>
<td>Too many to count</td>
</tr>
</tbody>
</table>

* Number of clones/number of cells seeded × 100.

**Lymph. = lymphatic tissue (lymph nodes and spleen).

The cells were seeded on 60-mm plates at the densities indicated, and the number of colonies formed was determined after 14 days. The results are an average of seven experiments.

The results were the same with chondroitin 4-sulfate and with chondroitin 6-sulfate.

DISCUSSION

Two mechanisms relevant to organ-site specific metastasis have been identified and characterized in numerous studies:

(a) Adhesion mechanisms acknowledging a recognition system between tumor cells and the parenchymal cells or endothelial cells of the colonized tissues (27–33).

(b) Tissue-specific stimulatory or inhibitory growth factors affecting the growth of metastatic tumor cells (34–35).

In addition, we have identified a third mechanism relevant to colonization of tissues by metastatic tumor cells: differential clonal growth properties of tumor cells in chemically distinct forms of extracellular matrix found in different tissues.

Adhesion Mechanisms Necessary but Not Sufficient to Explain Organ Site-specific Metastatic Properties of a Tumor. Recognition between metastatic cells and the colonized organ have been documented in a variety of studies: specific heterotypic aggregation between normal cells and organ specific metastatic variants (27–30); attachment of tumor cells to cryostat sections of normal tissues (31); blocking the spread of tumor cells to a specific tissue by use of monoclonal antibodies against an antigen in that tissue (36) or by injecting animals with peptides representing the binding site for adhesion proteins (37); the identification of distinct cell surface antigens on capillary endothelial cells from different tissues (38); and recognition and adhesion of metastatic tumor cells to capillary endothelial cells of different organs (32).

However, the studies of Kahn and Shin (39) indicate that tumor cells can be found in many tissues in which they ultimately do not grow. Also, some of the attachment assays of tumor cells on endothelial cells show the distinct patterns of attachment only if the assay is a few hours in length but not after 24 h (40). In our own studies, the various neoplastic cell lines, plated at high seeding densities (above $10^3$ cells/60-mm plate), showed no tissue specificity for attaching to types of biomatrix even though they usually attached better to matrix than to plastic dishes (Table 2).

Thus, although differential adhesion is clearly relevant to metastatic properties, the data indicate that attachment to specific tissues is a necessary but not sufficient mechanism to
explain organ site specificity. Tumor cells might attach to many tissues but survive and grow only in certain ones. Blocking the attachment process, as with antibodies against appropriate antigens, will, of course, block metastases in those tissues.

Tissue-specific Soluble Growth Factors May also be Contributory to Organ-site Specificity. The effect of the new environment to either stimulate or inhibit the proliferation of the metastasizing tumor cells also can derive from soluble factors produced by the tissue. Conditioned medium obtained from slices of organs in vitro has been shown to stimulate or inhibit tumor cell proliferation depending on which organs are used and which tumor cells: conditioned medium from lung stimulated the growth of lung-colonizing B16 melanoma cells; ovary-derived factors stimulated ovary-colonizing cells; while liver and kidney tissue factors inhibited the growth of B16 melanoma. The patterns of tissue factors stimulation or inhibition in culture correlated with the ability of the tumor cells to colonize those organs in vivo (34). Similarly, lung-conditioned medium was found to stimulate, while liver-derived factors inhibited the growth of murine mammary tumors, supporting the hypothesis that tissue factors may play a role in the lung metastasis frequently seen in naturally occurring murine mammary tumors (35). The relevant factors from these conditioned media have not yet been purified. Therefore, although it is clear that the factors exist, it is difficult, at this time, to assess the contribution of these soluble regulatory signals to metastatic potential.

Relevance of Extracellular Matrix to Metastatic Properties. The extracellular matrix is known to be a potent regulator of the growth and differentiation of cells (11-12, 34, 41). Furthermore, the matrix chemistry is known to be distinct in each tissue (41). Therefore, extracellular matrix is another candidate for regulating organ site specificity of metastases. Tumor cells are known to be altered in their matrix synthesis and turnover in general and must be able to degrade matrices in order to be invasive (42-44). However, tumor cells can be transiently "normalized" by plating them on an appropriate matrix (45) or by inducing the malignant cells to produce a matrix that shifts them from a proliferative to a differentiative pathway (46).

To test the hypothesis that extracellular matrix contributes to selective growth of tumor cells in some tissues and not others, we have screened a panel of tumor cells for their current metastatic potential in immunosuppressed hosts and correlated that with their growth properties on "biomatrices." Our studies assessing the current metastatic potential of the tumor cell lines confirmed many prior studies indicating that several host defense mechanisms are available in athymic nude mice to control the metastatic potential of even those cells that form tumors in the mice (6, 8, 10). Immunosuppression of the mice with ALS, silica or anti-interferon can minimize these host defense mechanisms, and, thereby, permit fuller expression of tumorigenic and metastatic potential for many types of tumor cells (6, 8, 10). Using this assay, distinctive patterns of organ site specificity were ascertained for the various cell lines tested, and highly metastatic and poorly metastatic cell lines were identified. Our data on MCF-7 was somewhat different from that of prior reports (22). We did not find metastatic lesions in estrogenized nude mice injected with MCF-7. In our study both estrogen treatment and immunosuppression were required to observe metastases. This may reflect either (a) that the subline of MCF-7 that we are using is antigenically distinct from the one used by Liotta and associates and solicits more host defense responses or (b) that it is merely a statistical artifact that would be resolved by doing the experiments in more animals. Clearly, there are still limitations to the assay, in that unidentified variables affecting tumorigenicity of any tumor cell may exist when it is screened in any host other than a syngeneic host. Still, tumorigenicity in immunosuppressed nude mice represents the best assay available, especially for human tumor cells even though the data must be interpreted with caution.

Although labor intensive and time consuming, culturing the host tissues under conditions to select for any tumor cells has certain advantages over the usual assays for metastasis in which metastasis is diagnosed by either histological studies of several sections of tissue (complete serial sections of all the tissues are rarely done) or simple macroscopic identification of metastatic lesions in particular tissues. One can screen for microscopic lesions often not seen either macroscopically or in random histological sections. The sensitivity of the assay is inherently dependent on the clonal growth efficiency of the tumor cells under the selective conditions. In our studies, this meant a sensitivity adequate to detect 10^2-10^3 cells in the tissues cultured. In the culture assays, the tumor cells showed distinctive patterns of growth on biomatrices derived from different tissues. At high seeding densities (above 10^6 cells/60-mm dish), their growth properties were affected by matrix but usually did not reflect any pattern indicative of their metastatic properties. All of the tumor cells grew more slowly on matrix substrata than on tissue culture plastic, but both minimally deviant and highly metastatic tumor cells were equally affected. However, more informative were the patterns of growth at low seeding densities (100-1000 cells/60-mm plates). Tumor cells formed clones of growing cells on some but not all types of biomatrix, and their pattern of proliferation on different biomatrix types reflected their in vivo pattern of metastasis (Table 3). This phenomenon may be analogous to the metastatic process in vivo in the early stages of dissemination. With small tumor loads, small numbers of tumor cells could spread throughout the body, colonizing only those tissues in which they can adapt to the tissue-specific forms of extracellular matrix. Increasing tumor load in a host should prove analogous to our findings at high seeding densities in cultures: increasing numbers of tumor cells are able to overwhelm the growth-inhibiting matrix factor(s), resulting in the growth of the tumor in more and more tissues.

The Matrix Component(s) Discriminating between Poorly and Highly Metastatic Tumor Cells. The extracellular matrix of all tissues has been incompletely chemically defined (for reviews see References 47-48). Of the known components, all forms of extracellular matrix contain adhesion proteins (e.g., laminin, fibronectin), collagens (e.g., fibrillar and network collagens), and proteoglycans and their glycosaminoglycan chains. Of these, only the GAG chains of the proteoglycans are known to have extensive chemical heterogeneity that is cell-type specific (48). Also, the proteoglycans and their GAGs have dramatic affects on cell motility and growth (49-53), and the chemistry of the GAGs is modified in a density-dependent fashion (49). With respect to epithelial cells, the sulfation patterns and iduronic acid content of the heparan sulfate chains change with cell density. At low density, the cells produce proteoglycans with heparan sulfate chains, whereas the same cells at confluence or in quiescence produce proteoglycans with GAG chains that are similar to heparins (49). Certain heparins and heparan sulfates have been shown to inhibit the proliferation of some cells as indicated in the recent studies of Karnovsky et al. (51) and Rosenberg and associates (50, 52-53). Reilly and Rosenberg showed that this inhibition of growth in smooth muscle cells by the active species of heparan sulfate and heparin is due to a specific decrease in the number of epidermal growth factor...
receptors without effects on their insulin or platelet-derived growth factor receptors (52–53). The physiological (as opposed to pharmacological) relevance of heparin inhibition of smooth muscle cell growth was further stressed when endothelial capillary cells were shown to secrete heparin-like molecules (51). However, heparins (or heparin sulfates) cannot be categorized as general inhibitors for cell growth, since endothelial cells are dependent on the presence of specific types of heparins complexed with fibroblast growth factors (54–55). Therefore, the specificity of the response, whether growth or inhibition of growth, can be dependent on the cell type, on the type or source of the glycosaminoglycan, and on the presence or absence of relevant soluble signals (e.g., the growth factors).

In our studies, the adhesion proteins, (laminin and fibronectin), and the collagens (type I and type IV) improved low density growth of all cells and did not discriminate between low and high metastatic tumor cells. Thus, they did not prove relevant to the density-dependent inhibition of growth shown by biomatrices but rather are matrix factors that are positive in helping cells attach, survive, and grow. These findings are not surprising given the ubiquitous distribution and relative lack of chemical heterogeneity of these molecules in the different tissues. The role of both laminin and fibronectin and their receptors on the surface of metastatic cell variants has been shown by others to be important to the invasive properties of metastatic cells (56–58) and to the adhesion properties needed by metastatic tumor cells (59).

By contrast, the one species of heparin tested but not the other GAGs tested, were found to differentially inhibit poorly metastatic but not highly metastatic tumor cells when the cells were plated at clonal densities. The ability of the highly metastatic tumors to grow at clonal densities in the presence of this heparin may reflect their ability to secrete heparinases or other glycosidases that might eliminate such growth regulators associated with the matrix (60). Thus, this class of matrix molecules fulfills three of the criteria necessary for a matrix factor involved in organ-site specificity of metastasis: (a) they are tissue-specific in their chemistry; (b) they are density dependent in their effects; and (c) they discriminate appropriately in their effects on poorly versus highly metastatic tumor cells. Therefore, our working hypothesis is that heparins and related molecules are one of the classes of matrix molecules involved in dictating differential growth patterns of tumor cells in different tissues.

In summary, the ability of tumor cells to colonize a particular tissue requires an ability to grow at low densities in the presence of specific forms of extracellular matrix, and in particular, in the presence of glycosaminoglycan chains that are part of the proteoglycan components in the matrix. Thus, there are matrix components such as adhesion proteins and collagens which facilitate cell attachment, survival and growth, and other matrix components, the glycosaminoglycans, that can inhibit cell growth. A tumor cell must either “tolerate,” be stimulated in its growth by, or be able to degrade such growth regulators in the extracellular matrix of the tissue to which it migrates if it is to form a metastatic colony. Furthermore, the repertoire of matrix-degrading enzymes secreted by a specific tumor cell could vary depending on the environment in which the tumor is located. Thus, the enzymes secreted by the tumor cell during escape from its original site could be different than those required for colonization of a distant tissue.

How Does the GAG or Proteoglycan Regulate Density-dependent Growth of Tumor Cells? Tumor cells have long been known to have an enhanced ability, when compared to their normal counterparts, to grow at low densities. At least one known mechanism facilitating the ability of the tumor cells to survive autonomously from neighboring cells is the secretion of autocrine growth factors (61). Many studies have shown that the conditioned medium from tumor cells can improve the low density survival and growth of their normal counterparts (62–67). A few such density-dependent growth factors have been purified and characterized. They include transforming growth factor-α, transforming growth factor-β, insulin-like growth factor-I, and insulin-like growth factor-II (68–70).

An interpretation for our findings is that autocrine factors are secreted by the tumor cells and are regulatable by proteoglycans (or their glycosaminoglycan chains). Studies ongoing at present, indicate that indeed heparins can regulate autocrine growth factor synthesis in minimally deviant tumor cells but not in highly metastatic tumor cells.

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Ralph Doerr, Isabel Zvibel, Diana Chiuten, et al.


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