Differential Gene Expression in Metastasizing Cells Shed from Kidney Tumors

Maximilian Bockhorn, Sylvie Roberge, Cristina Sousa, Rakesh K. Jain, and Lance L. Munn

Edwin L. Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

ABSTRACT

We developed a novel orthotopic mouse tumor model of renal cell carcinoma to collect and characterize cells spontaneously shed from SN12C (renal cell carcinoma) and SN12L1 (high metastatic variant of SN12C) tumors grown in kidneys of severe combined immunodeficient mice. Viability of the shed cell population was greater for SN12L1 tumors (25%) compared with SN12C tumors (11%, P < 0.05). Gene array analysis of 23 genes involved in metastasis showed that CD44, o3 integrin, and caveolin were down-regulated in the shed tumor cells compared with their primary counterparts, and blocking o3 integrin or CD44 function inhibited attachment and migration of both cell lines. These results suggest that cohesion of the cells within the primary tumor mediated by CD44 and o3 integrins hinders metastasis and that shedding is a passive process not necessarily mediated by cell migration in these tumors. Furthermore, resistance to apoptosis may enhance metastasis in the higher metastatic tumor.

INTRODUCTION

Seminal observations of Butler and Gullino (1) and of Liotta et al. (2) have shown that although millions of cells are shed from a tumor each day, these cells produce relatively few clinically detectable metastases. These findings and others suggest that once in the vasculature, metastatic cells encounter barriers that render metastasis a relatively inefficient process. Our recent studies of cell shedding in an ectopic tumor model of a human colon carcinoma grown in the ovarian pedicle have concluded that low viability and clonogenicity of shed cells might be responsible for metastatic inefficiency (3).

The genetic changes required for the intravasation step in metastasis are also a subject of debate (4). Recently, Bernard and Weinberg (5) proposed that metastatic propensity is acquired very early during tumorigenesis, and Ramaswamy et al. (6) found expression “signatures” in primary tumors that were predictive of successful metastasis. But these studies were not designed to detect modulation of expression in the few cells actively involved in metastasis.

We have developed and implemented a novel orthotopic kidney tumor model that allowed us to characterize the cells shed into the blood stream from a renal carcinoma and compare them with cells from the primary tumor. Here, we (a) quantify viability and apoptosis in cells shed from primary tumors with low and high metastatic potential, (b) identify genes differentially expressed between the shed cells and the respective primary tumors and (c) assess the biological significance of the differentially expressed genes.

MATERIALS AND METHODS

Cell Lines and Tumor Model. The human renal cell carcinoma cell line SN12C and its higher metastatic counterpart SN12L1 were kind gifts of Dr. Isiah J. Fidler (7; The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston). Tumor cells were maintained in Eagle’s MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum. Cells from sub-confluent monolayers were harvested by trypsinization and resuspended in MEM to a final concentration of 2 × 10^6 cells/ml.

Male severe combined immunodeficient mice, 6–10 weeks of age, bred in our gnotobiotic animal facility, were used in accordance with an approved protocol by the Massachusetts General Hospital. Mice were anesthetized using a ketamine (100 mg/kg body weight; Parke-Davis, Morris Plains, NJ) and xylazine (10 mg/kg body weight; Miles, Shawnee Mission, KS) mixture administered by i.m. injection. For tumor implantation, the hair on the left flank was first shaved. A small left lateral laparotomy in the kidney area was performed, and the kidney was carefully exteriorized. A suspension of 1 × 10^6 SN12C or SN12L1 cells in 0.1 ml of MEM was slowly injected under the capsule of the kidney. The retroperitoneal wall was sutured with 5–0 prolene (Ethicon, Somerville, NJ) and the skin closed with wound clips. Tumors were allowed to grow for 10–14 days and monitored every 5 days. Before injection, >95% of the cells were viable, assessed by trypan blue exclusion. Ninety to one hundred percent of all mice developed renal cell carcinoma.

Comparison of Metastatic Potential of SN12C and SN12L1. To verify the metastatic potential of the two cell lines originally characterized by Naito et al. (7), 1 × 10^6 SN12C or SN12L1 cells in 0.1 ml of MEM were injected slowly under the capsule of the kidney. Eight and seven mice were used for SN12C and SN12L1 cells, respectively. When the primary tumor overtook the kidney (visibly covering >80% of the organ), the animal was sacrificed and autopsied. Because the less aggressive SN12C tumors grew more slowly than the SN12L1 tumors, the time of tumor growth varied. Three of the SN12C mice were autopsied at 4 weeks and four at 6 weeks. All of the SN12C mice were analyzed at 8 weeks after implantation. Using this protocol, we likely underestimated the difference in metastatic ability, because the SN12C tumors were allowed to grow much longer. Metastases in the spleen, liver, contralateral kidney, and the mesentery were examined macroscopically, and lung specimens were examined histologically.

Tumor Perfusion and Shed Cell Collection. A midline laparotomy was performed to expose the entire abdominal cavity with the tumor growing in the left kidney. The animals were kept on a heating pad adjusted to 37°C. The left renal vein was separated from the artery and ligated. A PE-10 tube (0.28-mm inside diameter; 0.61-mm outside diameter; Becton Dickinson, Franklin Lakes, NJ) inserted into the carotid artery was connected via a three-way stopcock to a pressure transducer (Gould, Inc., Valley View, OH) through heparin-filled PE-10 tubing. Pressure measurements were processed digitally and recorded continuously using MacLab (AD Instruments, New South Wales, Australia). A PE-50 tube (0.58-mm inside diameter; 0.965-mm outside diameter) was introduced into the left jugular vein and connected to a reservoir of Oxyglobin (Biopure, Cambridge, MA). A peristaltic pump (Ismatec 7618-3; Cole-Parmer, Chicago, IL) was used to perfuse the system, and the flow rate was adjusted to maintain the blood pressure of the mice between 60 and 100 mm Hg. Oxygenation of the perfusate solution was achieved by a gas exchanger in which the perfusate was led through 16 feet of silastic tubing (Becton Dickinson) while equilibrating with warm humidified 95% O2 and 5% CO2.

During the experiments, the perfusate was kept in a heated bath and led via the pump through the gas exchanger and then into the jugular vein and through the circulation of the mice. To collect shed cancer cells, the renal vein of the left kidney, in which the tumor was growing, was cannulated with a PE-10 tube (0.28-mm inside diameter; 0.61-mm outside diameter). The perfusate leaving the kidney was collected in a 15-ml tube containing MEM (Fig. 1) and immediately processed to isolate the shed cell RNA. This procedure allowed us to perfuse the anesthetized mice for a period of about 2 h.

Quantification of Shed Cell Viability and Apoptosis. Because the SN12 tumor lines are of human origin, we were able to identify tumor cells using an anti-human leukocyte antigen-class II antigen antibody (One Lambda, Inc., Canoga Park, CA) and flow cytometry. The shed cells of the SN12C tumor were compared with the higher metastatic SN12L1 cells. Blood collected from

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Requests for reprints: Lance L. Munn, Department of Radiation Oncology, Massachusetts General Hospital, 100 Blossom Street, COX-736, Boston, MA 02114. Phone: (617) 726-4085; Fax: (617) 726-1962; E-mail: lance@steele.mgh.harvard.edu.
tumor-free mice was used as a negative control. To differentiate between viable, apoptotic, and dead cells, we also labeled the cells with Annexin (CALTAG, Burlingame, CA) and 7-amino actinomycin D (Calbiochem-Novabiochem Corp., San Diego, CA). Annexin is largely found on the cytosolic face of plasma membranes, and 7-amino actinomycin D is an intracellular DNA marker. Both antibodies are useful in distinguishing early apoptotic cells, which have lost membrane integrity, from apoptotic and live cells. Flow cytometry and sorting were performed with a FACScan Vantage SE (Becton-Dickinson). To compare the shed tumor cells with the tumor cells of the primary tumor, kidney tumors were minced and incubated for 1 h at 37 °C in a Trypsin-EDTA (0.25%)/collagenase (1 mg/ml) solution. The tissue suspension was filtered through a 70-μm nylon cell strainer to produce a suspension of single cells. The cells were then labeled with human leukocyte antigen-class II antigen antibody, Annexin, and 7-amino actinomycin D using the same procedure applied to the shed cells to sort only the viable tumor cell population using the FACScan.

Gene Expression Analysis. Viable sorted cells were stored in the mRNA-later RNA Stabilization Reagent (Ambion, Inc., Austin, TX) and pooled after obtaining sufficient viable tumor cells for RNA isolation (10–15 mice for each tumor type). RNA was extracted using the RNAeasy kit according to the manufacturer’s instructions (Qiagen, Germantown, MD).

Because of the low number of viable shed tumor cells (~3–5 × 10^3/tumor) and the corresponding small amount of extracted RNA, we amplified the total RNA with the RiboAmp RNA Amplification kit according to the manufacturer’s instructions (Arcturus, Mountain View, CA). In brief, a first strand synthesis reaction incorporates cDNA, a T7 promoter; a second-strand synthesis reaction using exogenous primers yields double-stranded cDNA. After purification, in vitro transcription using T7 RNA polymerase yields antisense RNA, which is again purified. To maintain consistency in the comparison, we also performed this amplification procedure on the RNA from the primary tumors.

For the analysis of the differential expression of multiple genes involved in human metastasis, we used the GEArray human metastasis-specific expression arrays (hGEA9005040; SuperArray Inc., Bethesda, MD). For every tumor, 5 μg of collected total RNA were used in the cDNA probe synthesis with DCTP (6000 Ci/mmol; NEN Life Science Products, Inc., Boston, MA). Purification, hybridization, and washings were performed according to the manufacturer’s specification (SuperArray Inc.). Each GEArray membrane contained 96 or 23 coordinates containing specific cDNA fragments of genes involved in human metastasis, spotted in quadruplet as well as the negative control pUC18 DNA and loading controls (β-actin and glyceraldehyde 3-phosphate dehydrogenase). The spots were quantified using ScanAlayze 2.441 as well as GEArray software (SuperArray Inc.). The relative abundance of a particular transcript was estimated by comparing its signal intensity to the signal derived from β-actin after subtraction of pUC18 DNA intensity.

Real-Time PCR. cDNA of our four samples was subjected to PCR with Taqman PCR Core Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. The incorporation of the specific Taqman probe into the PCR products was monitored in real time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA), resulting in the calculation of a cycle threshold (ct) value that defines the PCR cycle number at which an exponential growth of PCR product begins. To account for possible concentration errors, we also created a Taqman probe for β-actin as a reference control. The oligonucleotide primers for CD44, cavelin, integrin, and β-actin are listed in Table 1.

Migration Assay. Cell migration was assessed using Falcon HTS FluoroBlok 24-well inserts (Becton-Dickinson) with 8-μm pores. Inserts were coated with a combination of laminin (5 μg/ml; Chemicon International Inc., Temecula, CA) and collagen IV (5 μg/ml; Sigma Chemical Co., St. Louis, MO) for 1 h and then washed once in PBS. Cells (6 × 10^3) suspended in 300 μl of MEM (Life Technologies, Inc.) with 10% fetal bovine serum (Sigma) were placed in each insert, and 900 μl of the same medium were added to each outer well. To examine the effect of blocking antibodies on cell migration, cells were pretreated for 30 min at 37 °C before being placed in the inserts with antihuman α3 integrin antibody (clone P1B5; Chemicon International Inc., Temecula, CA) or antihuman CD44 (clone DF1485; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a final dilution of 1:250 and 1:50, respectively. Cell aggregates were disrupted during the incubation by repeated pipetting through a 1000-μl pipette tip.

After 6 h, the cells were incubated with calcine-AM (2 μM; Molecular Probes, Eugene, OR) for 15 min at 37 °C to allow epifluorescence visualization of the cells that had migrated through the pores to the bottom of the filter. Four random pictures from the bottom of each insert were acquired, and the area covered by the migrating cells was quantified using NIH Image 1.62.

Statistical Analyses. For comparison of the percentage of viable, apoptotic, and nonviable tumor cells shed into the circulation and cell migration, Student’s t test was used to determine statistical significance (at P < 0.05). Metastasis of the SN12C versus SN12L1 tumors was tested using a χ^2 test. In the gene array analysis, >4-fold changes in expression were considered significant.

RESULTS

SN12L1 Is More Metastatic than SN12C. We first verified that the two cell lines have maintained their disparate metastatic potentials (7). Table 2 shows the distribution of metastases in mice receiving injection of SN12C and SN12L1 cells. In general, the SN12C mice had fewer metastases (P < 0.01, χ^2 test). In mice with SN12C tumors, the metastases in the lungs were micrometastases only visible via microscopic examination. SN12L1-bearing mice had macroscopic lung metastases that involved a large portion of the lobes. For the mesentery, mice with SN12L1 tumors had invasive secondary colonies throughout the abdomen, but only one SN12C-bearing mouse had (a few small) abdominal metastases. Also interesting was the fact that the SN12L1 tumors spread to the opposite kidney in two animals.

High and Low Metastatic Tumors Shed Cells at the Same Rate. The perfused SN12C and SN12L1 tumors shed an average of 249 ± 69 × 10^3 (mean ± SD) and 275 ± 67 × 10^3 tumor cells/h/g, respectively (not significantly different, P > 0.05). This corresponds to 6.0 and 6.6 million cells/day/g. All cultured primary tumor cells stained positive for human leukocyte antigen-class II antigen when taken out of culture.

Shed Cells Are Mostly Apoptotic or Nonviable. Although there was little apoptosis in the primary tumors, few of the shed cells were viable. Apoptosis in intact SN12L1 and SN12C1 primary tumors was assessed histologically using the ApopTag Peroxidase In Situ Apo-

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after blocking CD44 and assessed the migration of the SN12C and SN12L1 cells before and after blocking CD44 only affected migration of the SN12L1 cells ($P < 0.05$).

**DISCUSSION**

By collecting cells shed into the isolated vasculature of the kidney, we have demonstrated that relatively few viable cancer cells are shed into the bloodstream by these tumors. This result is in agreement with our previous studies of cell shedding using isolated perfusion techniques (3) and suggests that shedding is an inefficient, passive process.

The lower levels of apoptosis and higher viability in the cells shed

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' -&gt; 3')</th>
<th>Reverse (5' -&gt; 3')</th>
<th>Taqman-probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>CTGGGAGGACAGAAGGCCA</td>
<td>CAAAACCCCATTTCCTAGAC</td>
<td>TGGACTTCAGAGGAGGCCACGA</td>
</tr>
<tr>
<td>Caveolin</td>
<td>CTACAGCCCAACACAAAGGC</td>
<td>GGTTTGCGCTGTGTCACCT</td>
<td>ATGGCAAGAGGCAAGCTGAAGCTA</td>
</tr>
<tr>
<td>α3 integrin</td>
<td>GACTACCGCTCAGCGCCA</td>
<td>GGTTGAGATGCCTCCAGTGAG</td>
<td>TGGGCTTGGCCTCTGCAG</td>
</tr>
<tr>
<td>β-actin</td>
<td>ACCGGCGTCAAGCGTTCA</td>
<td>TCTCTTAAATGTCCAGCCAGT</td>
<td>CACCACCGGCGAGCGGGA</td>
</tr>
</tbody>
</table>

**Table 2 Secondary colony formation in mice with primary kidney tumors: SN12C (eight animals), SN12L1 (seven animals). Δ% is the percent increase of SN12L1 over SN12C.**

<table>
<thead>
<tr>
<th></th>
<th>SN12C</th>
<th>SN12L1</th>
<th>Δ%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>6/8</td>
<td>3/7</td>
<td>33</td>
</tr>
<tr>
<td>Liver</td>
<td>1/8</td>
<td>3/7</td>
<td>243</td>
</tr>
<tr>
<td>Mesentery</td>
<td>1/8</td>
<td>7/7</td>
<td>700</td>
</tr>
<tr>
<td>Kidney (opposite)</td>
<td>0/8</td>
<td>2/7</td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>4/8</td>
<td>4/7</td>
<td>14</td>
</tr>
</tbody>
</table>

Fig. 2. Percentage of viable, apoptotic, and nonviable tumor cells shed into the circulation. There is a statistically significant difference in the number of viable shed tumor cells between the low and high metastatic tumors ($P < 0.05$; Student’s $t$ test).
from the SN12L1 tumors may contribute to the higher metastatic potential of these tumors. In support of this theory, Glinsky et al. (8) reported that higher metastatic cell lines are less prone to apoptosis attributable to a loss of apoptosis execution mechanisms.

Previous work has shown that modulation of gene expression during metastasis can be a dynamic, orchestrated process, with critical genes being up-regulated at each stage (9, 10). In the present study, we found that the adhesion-related molecules CD44 and α3 integrin were modulated in the shed cell populations. CD44 is a glycoprotein with a variety of functions including homotypic cell adhesion (11). Down-regulation of CD44 may disrupt cell contact with neighboring cells, making escape from the primary tumor easier, but additional studies are needed to establish the prognostic value and metastatic involvement of CD44.

α3 integrin is a receptor that recognizes a variety of extracellular matrix proteins (12). Several studies showed an increased expression of α3 integrin associated with higher metastatic ability (13, 14). Using immunohistochemistry, Miyamoto (15) showed that reduced α3-integrin expression in endometrial cancers correlates with histological grade and metastasis. Adachi et al. (16) obtained similar results in lung adenocarcinoma. We also observed a loss of expression in the high metastatic shed tumor cells. A simple explanation could be that α3 integrin helps maintain cell-matrix adhesion, and loss of this molecule increases the probability of cell shedding.

There was down-regulation of caveolin in our shed cell populations. Caveolin is the major structural protein of caveolae, specialized plasma membrane invaginations that regulate multiple signal transduction pathways (17, 18). Bender et al. (19) showed that caveolin mRNA levels were reduced in human colon carcinoma cell lines and that re-expression significantly reduced the tumorigenicity of these cell lines. Fiucci et al. (20) concluded that the high expression level of caveolin-1 in human breast cancer cells exerts a negative modulatory effect on anchorage-independent growth by inhibiting cell proliferation and that caveolin-1 expression also inhibits matrix invasion and blocks laminin-dependent activation of extracellular signal-regulated kinase 1/2. Our results suggest that loss of caveolin expression in the high metastatic shed tumor cells might facilitate the escape of these cells from the primary tumor.

Interestingly, we did not find a difference in gene expression between the low and high metastatic primary tumors, although the SN12L1 tumors are known to be more metastatic. This conflicts with the hypothesis of Bernards and Weinberg (5), which proposes that genes involved in the metastatic cascade already exist at the very early stages of tumorigenesis. It is also at odds with the recent report of Ramaswamy et al. (6) who found genetic signatures in primary tumors that correlated with metastatic ability. Contrary to widely accepted paradigm, they claim that the cells in primary tumors are relatively stable, and any cell in a primary tumor with correct genetic signature is capable of metastasizing. But the existence of a background expression profile signature predictive for metastasis does not preclude the possibility of transient changes in expression of genes in a subpopulation of cells. We propose that local microenvironmental pressures within the tumor can affect gene expression in a few cells (which would not change the overall “signature” in a detectable manner), and these are the cells that initiate metastasis. Our results suggest that modulation of genes such as CD44, α3 integrin, and caveolin are associated with this step. These changes in gene expression of a few cells were not the focus of the Ramaswamy et al. study, which considered only snapshots of expression in the primary and secondary colonies.

In the functional studies, we found that the cells with higher metastatic potential migrated faster than the low-metastatic cells. We also found that blocking α3 integrin or CD44-inhibited migration, although loss of these genes in the in vivo experiments apparently contributed to cell shedding. This, together with the low viability of the shed cell population, suggests that active migration/extravasation is not important in these tumors, but that passive shedding, enhanced by loss of cell-cell and cell-matrix adhesion, dominates the process.

In summary, our results suggest that cell shedding is one of the rate-limiting steps of metastasis and that the few viable tumor cells must have a high metastatic potential. The fact that the SN12L1 tumors shed more viable cells in the circulation might contribute to the higher metastatic potential of these cells. Furthermore, down-regulation of CD44, α3 integrin, and caveolin appears to give shed cells a metastatic advantage.
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