Loss of the Metastatic Phenotype in Murine Carcinoma Cells Expressing an Antisense RNA to the Insulin-like Growth Factor Receptor

Li Long, Raphael Rubin, Renato Baserga, and Pnina Brodt

Abstract

The ability of malignant cells to form metastases in secondary sites remains a major obstacle to the curative treatment of cancer. Previously, we identified type 1 insulin-like growth factor (IGF-1) as a paracrine mitogen for highly metastatic murine carcinoma, H-59 cells. Here the role of IGF-1 and its receptor (IGF-1R) in metastasis was further investigated using H-59 cells transfected with a plasmid vector expressing IGF-1R cDNA in the antisense orientation. The transfecteds had a markedly reduced expression of IGF-1R and lost the ability to respond to IGF-1 in vitro. When injected in vivo, either directly into the microvasculature of the liver or lung (experimental metastasis) or s.c. to allow the growth of primary local tumors (spontaneous metastasis), these cells did not give rise to any metastases under conditions which allowed wild-type or control transfectants to form multiple hepatic and pulmonary metastases. The results demonstrate that the IGF-1R can play a critical role in the regulation of carcinoma metastasis.

Introduction

Despite major advances in our understanding of the molecular mechanisms underlying malignant transformation, progress in the clinical management of cancer remains limited. This is due in large part to the limited success of conventional therapy in the treatment of secondary metastatic lesions (1). Cancer metastasis is a complex multistep process involving detachment of tumor cells from the primary site, invasion of and migration through blood or lymph vessels, extravasation into distant organs, and cell proliferation in the target organ in response to autocrine and/or locally released growth factors (2, 3). Numerous studies have shown that the acquisition of a malignant phenotype is associated with altered responses to various growth factors due to changes in the expression of growth factor receptors and/or the altered regulation of growth factor-induced signal transduction mechanisms (4).

Evidence is rapidly accumulating that IGF-1 (5) and IGF-1R play a crucial role in malignant transformation (5). Recent studies have shown that antisense expression plasmids to IGF-1 (6) or the receptor (7) and antibodies to the IGF-1R (8) could inhibit tumorigenicity. In vitro, monolayer cultures of the tumor were maintained in RPMI containing 5% FCS as detailed elsewhere (2).

Materials and Methods

Cell Lines. Tumor H-59 was established from a hepatic metastases of the parent line 3LL (9). The tumor was maintained in vivo by s.c. implantation of liver metastases derived from tumor-bearing mice into new recipient animals. In vitro, monolayer cultures of the tumor were maintained in RPMI containing 5% FCS as detailed elsewhere (2).

Construction of IGF-1R Plasmids. An XbaI-Xhol fragment corresponding to 1–309 base pairs of the IGF-1 receptor cDNA (13) was ligated into the XbaI-BamHI site of the CVN vector (14) in the sense or antisense orientations. We sequenced, both under the control of the SV40 early promoter gene. The vector also contains the dihydropyrimidinase reductase (DHFR) and neomycin resistance (NeoR) coding sequences, both under the control of an SV40 promoter.

Transfections. The cells were transfected by electroporation (15) and then cultured in RPMI 1640 containing 10% FCS, which was supplemented from day 2 onward with 100 μg/ml G-418 (GIBCO-BRL, Burlington, Ontario, Canada). Resistant clones were isolated 12–14 days later.

Tumor Cell Proliferation Assay. H-59 cells and transfectants were cultured in SF-RPMI for 24 h and then dispersed and seeded into 96-well plates (Falcon, Lincoln Park, NJ) at a density of 2 × 10^5 cells/well and incubated for 54 h with medium containing 5% FCS, IGF-1, or hepatocyte-conditioned medium prepared as we described previously (11). The cells were pulsed with 0.1 μCi/ml of ^3H]thymidine (Du Pont Canada, Mississauga, Ontario, Canada) for 18 h, and thymidine incorporation was monitored as detailed elsewhere (11).

Northern Blot Analysis. Cellular RNA was extracted from H-59 and transfected cells by the phenol-chloroform method of Chomczynski and Sacchi (16) as we described in detail elsewhere (11). Blots were probed with a radiolabeled 0.7-kilobase EcoRI restriction fragment of the human IGF-1 receptor cDNA (American Type Culture Collection, Rockville, MD), analyzed by laser densitometry, and normalized relative to the 18S rRNA.

Ligand Binding Assay. To quantitate the binding of IGF-1 to the tumor cells, the method of Phillips et al. (17) was used as we described in detail previously (11), except that cells were grown in RPMI containing 5% FCS prior to assay.

Metastasis Assays. Tumor cells were inoculated by the intrasplenic route to generate experimental liver metastases (18) or by tail vein injections to generate lung metastases. Liver metastases were enumerated immediately after the removal of organs. Lungs were fixed in Bouin’s fixative, and nodules were enumerated as reported previously (19). For spontaneous metastasis assays, the tumor cells were injected s.c. into the lateroabdominal region. The mean tumor diameter for individual tumors was calculated from measurements in two planes at right angles. Animals were sacrificed, and metastases were enumerated when the mean tumor diameter for the group reached 1.4 cm. In some animals, tumor immunogenicity was assessed by simultaneous injection of 10^5 hepatic metastasis in this model correlated with increased responsiveness to hepatic IGF-1, which was in turn due to increased expression of the IGF-1R (11). To study the role of IGF-1R in H-59 growth and metastasis in vivo, the tumor cells were transfected with a vector in which IGF-1R cDNA is constitutively expressed in the antisense or (as control) sense orientations relative to the SV40 promoter. We show here that the antisense transfectants lost the ability to respond to IGF-1 and hepatocyte-conditioned medium in vitro and failed to give rise to metastases in vivo.
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wild-type and transfected cells at symmetrical contralateral sites in the latero-abdominal region.

Results

To study the role of IGF-1R in H-59 growth and metastasis in vivo, the tumor cells were transfected with a vector in which IGF-1R cDNA is constitutively expressed in the antisense or (as control) sense orientations relative to the SV40 promoter. Several neomycin-resistant clones were obtained, and two were selected for further study. We found that the expression of IGF-1R mRNA in the antisense-transfected clone SA-9 was reduced by 75% relative to parental H-59 cells or the sense-transfected clone SS-2 (Fig. 1). This was reflected in reductions of 65 and 75%, respectively, in the number of IGF-1 binding sites on antisense-transfected SA-9 cells (22,137 sites/cell) relative to sense transfected (64,490 sites/cell) or parental H-59 cells (87,297 sites/cell), as assessed by a radiolabeled ligand binding assay.

In vitro, cell growth in serum-supplemented (5%) medium was not affected, as shown in Fig. 2. This was also confirmed in a second assay where 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (20) was used to monitor cell growth daily for 5 days (results not shown). However, the antisense-transfected cells lost their ability to respond to IGF-1 or hepatocyte-conditioned medium, which are highly mitogenic for parental H-59 and sense-transfected cells (Fig. 2; Ref. 11).

To test the effect of IGF-1R suppression on the ability of the tumor cells to colonize secondary organs, they were inoculated into syngeneic mice by three different routes. Following inoculation by the intrasplenic route, as means of delivering the cells into the hepatic circulation via the portal vein (18), all the animals which were injected with 3 × 10⁷ H-59 or sense-transfected cells developed multiple hepatic nodules by day 14, ranging from 81—280 and from 31—226 nodules per liver, respectively. In contrast, none of the animals inoculated with IGF-1R antisense-transfected cells developed any nodules for up to 30 days following inoculation (Table 1).

In a second experimental metastasis assay, animals were inoculated i.v. via the tail vein with 3 × 10⁷ tumor cells. Previously, we have shown that H-59 cells preferentially colonize the lungs when inoculated by this route (9). In the present experiment, animals injected with parental or sense-transfected cells developed multiple lung colonies within 21 days of the injection, ranging from 100—317 and 81—357 colonies per lung, respectively. However, none of the animals

![Fig. 1. Northern blot analysis of IGF-1R mRNA in tumor H-59 and transfected cells. Total RNA (50 μg/lane) derived from parental H-59, sense-transfected SS-2, antisense-transfected SA-9 (A, Lanes 1, 2, and 3, respectively), as well as from s.c. tumors of SS-2 and SA-9 cells (B, Lanes 1 and 2, respectively) was analyzed using a 0.7-kilobase IGF-1R cDNA as hybridization probe. The 28S and 18S ribosomal RNAs were used as size markers. The results of hybridization of the same filter with an 18S oligonucleotide probe are shown at the bottom. Bands were scanned using the LKB Bromma Ultrascan XL enhanced laser densitometer, and the difference in the expression of mRNA transcripts was calculated relative to the 18S standards. Results of this analysis are shown in the bar graph.

![Fig. 2. Loss of response to IGF-1 and hepatocyte-conditioned medium (HCM) in IGF-1R antisense transfected cells. A, serum-starved H-59 (○), SS-2 (△), and SA-9 (□) cells were seeded in 96-well plates at a concentration of 2 × 10⁶ cells/well and incubated with different concentrations of IGF-1 at 37°C for 72 h. [³H]Thymidine was added during the last 18 h of incubation. B, tumor H-59 and the transfected cells were incubated in the absence (△) or presence (□) of 5% FCS or with HCM (○), and thymidine uptake was measured 72 h later. Results are of a representative experiment done in triplicates. For cells cultured in serum-containing medium, the relative increases in [³H]thymidine incorporation were 12.0-, 12.6-, and 10.1-fold, and for cells cultured with HCM, they were 4.1-, 4.5-, and 1.2-fold for H-59, SS-2, and SA-9 cells, respectively. Bars, SD.](cancerres.aacrjournals.org)
Tumor cells were injected intrasplenically (i.s.), and the animals were splenectomized and sacrificed 28 days later when the mean diameter of H-59 tumors was 1.4 cm.

Animals in all groups developed local tumors, although in animals inoculated with antisense-transfected cells had detectable lung nodules (Table 1). We injected H-59 and antisense-transfected cells simultaneously at symmetrical, contralateral sites of the lateroabdominal region. In both experiments, parental H-59 cells formed local tumors, which were indistinguishable in time of appearance and rate of growth from tumors in the corresponding control groups, suggesting that the antisense-transfected cells did not induce an effective immune response in these animals (Table 1).

### Discussion

Our results show that H-59 cells expressing IGF-1R antisense RNA lost their ability to metastasize spontaneously to the liver or lung from primary s.c. tumors and could not colonize these organs, even when inoculated directly into their microvasculature. These results are in agreement with several recent studies which implicated the IGF-1R in the control of tumor growth (5, 7) and further extend these findings by showing that IGF-1R can play a crucial role in the regulation of tumor cell potential to disseminate and form metastases in secondary organs.

Severe mechanisms may be invoked in interpreting our findings. Previously we reported that H-59 cells do not produce detectable levels of IGF-1 but express high levels of IGF-1R (Ref. 11; Fig. 1). The cells require, therefore, an exogenous source of IGF-1 for activation of the IGF-1R signal transduction pathway. This dependence on paracrine mechanisms renders the liver and lung favorable target organs for metastasizing H-59 cells because high levels of IGF-1 are produced in both organs (21). In cells expressing IGF-1R antisense RNA, the growth advantage in these sites is apparently lost. This may lead to tumor cell death due to apoptosis, a process of cell death which can normally be inhibited by IGF-1R (22), or it may increase tumor cell vulnerability to natural host resistance mechanisms, including the tumoricidal effects of resident Kupffer cells in the liver and alveolar macrophages in the lung (19), with a resulting rapid clearance of the cells from the local circulation. It remains to be determined, however, whether the failure of IGF-1R-suppressed cells to metastasize is yet another manifestation of the critical role this receptor and its ligand play in growth control or whether other tumor cell properties essential for metastases formation, such as invasiveness, are also affected.

### Table 1 Loss of metastatic potential in H-59 cells expressing IGF-1R antisense RNA

<table>
<thead>
<tr>
<th>Cell Route of inoculation</th>
<th>Tumor cell inoculum</th>
<th>Tumor incidence</th>
<th>Metastases (incidence)</th>
<th>Nodules/organ (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-59</td>
<td>10/10</td>
<td>169 (81-280)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS-2</td>
<td>10/10</td>
<td>115 (31-226)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS-2</td>
<td>0/10</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-59</td>
<td>3x10^6</td>
<td>8/8</td>
<td>178 (100-217)</td>
<td></td>
</tr>
<tr>
<td>SS-2</td>
<td>3x10^6</td>
<td>8/8</td>
<td>168 (81-357)</td>
<td></td>
</tr>
<tr>
<td>SA-9</td>
<td>0/8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-59</td>
<td>2x10^3</td>
<td>5/5</td>
<td>24 (8-69)</td>
<td>9 (4-17)</td>
</tr>
<tr>
<td>SS-2</td>
<td>2x10^3</td>
<td>5/5</td>
<td>8 (4-13)</td>
<td>12 (6-27)</td>
</tr>
<tr>
<td>SA-9</td>
<td>0/5</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-59r</td>
<td>2x10^3 + 10^3</td>
<td>5/5</td>
<td>18 (11-25)</td>
<td>4 (2-6)</td>
</tr>
<tr>
<td>H-59s</td>
<td>0/5</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-59s</td>
<td>5/5</td>
<td>10 (5-15)</td>
<td>4 (3-5)</td>
<td></td>
</tr>
<tr>
<td>H-59s</td>
<td>5/5</td>
<td>10 (5-15)</td>
<td>4 (3-5)</td>
<td></td>
</tr>
</tbody>
</table>

- a Mice were anesthetized by an i.p. injection of 28.6 mg/kg sodium pentobarbital. Tumor cells were injected intrasplenically (i.s.), and the animals were splenectomized 1 min later, as described previously (25). Mice were sacrificed, and livers were examined 14 days later.
- b Animals were sacrificed 21 days following tumor inoculation.
- c Animals were sacrificed when the mean tumor diameters for each group reached 1.4 cm.
- d Animals were sacrificed 28 days after tumor cell inoculation when the mean diameter of the local tumors was 1.4 cm.
- e The mice which were initially injected with 2x10^6 SA-9 cells and mice which failed to develop local tumors were rechallenged with a s.c. injection of 10^6 H-59 cells 35 days later. The animals were sacrificed 28 days following the second injection when the mean tumor diameter was 1.4 cm.

Similar findings were recently reported by Resnicoff et al. (7) using the C6 rat glioblastoma. In these and other studies with the glioblastoma model, the reduced tumorigenicity of cells transfected with IGF-1 or IGF-1R antisense cDNA was attributed to increased immunogenicity of the transfected cells (6). To test whether the host immune response also played a role in the failure of SA-9 cells to develop local tumors when injected with the lower dose of 2x10^3 cells, we rechallenged the tumor-free mice with a second inoculum of 10^3 wild-type H-59 cells 35 days after the first injection. Additionally, inoculated with antisense-transfected cells had detectable lung nodules (Table 1).

To test the effect of IGF-1R suppression on spontaneous metastasis from a primary site, animals were injected s.c. with 10^6 tumor cells. Animals in all groups developed local tumors, although in animals injected with IGF-1R antisense-transfected cells, tumor appearance was delayed (Fig. 3). The animals were sacrificed when the mean diameter of their local tumors reached approximately 1.4 cm (24 days later). The cells require, therefore, an exogenous source of IGF-1 for activation of the IGF-1R signal transduction pathway. This dependence on paracrine mechanisms renders the liver and lung favorable target organs for metastasizing H-59 cells because high levels of IGF-1 are produced in both organs (21). In cells expressing IGF-1R antisense RNA, the growth advantage in these sites is apparently lost. This may lead to tumor cell death due to apoptosis, a process of cell death which can normally be inhibited by IGF-1R (22), or it may increase tumor cell vulnerability to natural host resistance mechanisms, including the tumoricidal effects of resident Kupffer cells in the liver and alveolar macrophages in the lung (19), with a resulting rapid clearance of the cells from the local circulation. It remains to be determined, however, whether the failure of IGF-1R-suppressed cells to metastasize is yet another manifestation of the critical role this receptor and its ligand play in growth control or whether other tumor cell properties essential for metastases formation, such as invasiveness, are also affected.

![Fig. 3. Growth of H-59 and transfected cells in vivo. One million H-59 (○), SS-2 (■), and SA-9 (□) cells were injected s.c. into C57BL/6 mice. The mean tumor diameter for individual tumors was calculated from measurements in two planes at right angles. To determine mean tumor diameter (bars, SD) for the group, the sum of the individual measurements was divided by the number of tumor-bearing (10 of 10) mice.](image-url)
following results argue in support of the latter: (a) the growth of antisense transfected cells in vitro in serum-supplemented medium was unchanged relative to controls; and (b) the antisense transfecants which grew s.c. and gave rise to primary tumors failed to metastasize to the liver or lung (Table 1; Fig. 3).

The failure of IGF-1R antisense-transfected cells to give rise to local tumors following the s.c. injection of 2 × 10^6 but not 10^6 cells suggests that IGF-1R is also a limiting factor for tumor cell survival and growth in the subcutis and that, in this microenvironment, the requirement for IGF-1R can be overcome under conditions of high cell density, as has also been suggested previously (2). It is possible that, at high cell densities, cell growth in the subcutis could proceed through autocrine growth mechanisms or other paracrine factors independent of IGF-1 (2, 3). Alternatively, since the antisense-transfected cells expressed residual low levels of IGF-1R (Fig 1), it is also possible that, under conditions of high cell density, this was sufficient to rescue a small subpopulation of the cells expressing the required threshold level of IGF-1R; these cells eventually giving rise to the s.c. tumors. This is consistent with our observation that, in animals injected with 10^6 antisense-transfected cells, the latent period preceding the appearance of tumors was considerably prolonged as compared to controls. It is also in accordance with the reported role of IGF-1R in the control of cell survival through inhibition of apoptosis (22).

In view of these findings, the possibility cannot be ruled out that the complete failure of IGF-1R antisense-transfected cells to colonize the liver or lung is related to the conditions inherent in the metastatic process, i.e., the arrest in the microvascular bed of small numbers of single cells and/or small tumor cell emboli (23). This implies that micrometastases are likely to be highly susceptible to targeted disruption of growth processes during these early stages of dissemination.

New approaches for cancer therapy are being developed that aim to intervene at various steps in growth factor receptor-signaling pathways. One of the most promising targets of more specific therapy is the growth factor receptor itself, the activity of which can be suppressed by monoclonal antibodies inducing receptor down regulation (8), by receptor kinase inhibitors (24), or by the use of an antisense strategy (6, 7). Our data suggest that, for tumors which are dependent on IGF-1 for proliferation, the IGF-1R could provide a specific target for effective antimetastatic therapy.

References

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