Genetic Factors and Suppression of Metastatic Ability of Prostatic Cancer

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ABSTRACT

Progression of prostatic cancer from nonmetastatic to high metastatic ability may involve the loss of a metastasis suppressor gene. To test this possibility, nonmetastatic and highly metastatic Dunning rat prostatic cancer cells were fused. Hybrid clones were isolated which expressed androgen and were not killed by hypoxanthine-guanine phosphoribosyltransferase-lacking clone (i.e., AT2.1 Neo/HPRT–). To do this, 5 × 106 AT2.1 Neo cells in 10 ml of standard media containing 500 mg/ml of G418 were seeded into a T-75 flask and 24 h later the medium was replaced with standard media containing 500 μg/ml of G418 and 260 μg/ml of ethyl methanesulfonate. After 36 h, the ethyl methanesulfonate medium was exchanged with standard media containing 500 μg/ml of G418 and 10 μg/ml of neomycin. After 1 week, the TG concentration was lowered to 1.7 μg/ml; 2 weeks later, the flask was trypsinized and all of the cells were added to one T-75 flask containing 10 μg/ml of TG. The cells were passaged every week at a 1/10 split for 3 weeks and then cloned by limited-dilution cloning. A HPRT– clone (i.e., AT2.1 Neo/HPRT–) was isolated, expanded, and then tested for its ability to be killed by hypoxanthine-aminopterin-thymidine selection. No AT2.1 Neo/HPRT– cells grew in standard media containing 500 μg/ml of G418, and AT2.1 Neo/HPRT– cells were maintained in the standard media containing 500 μg/ml of G418 and 10 μg/ml of TG.

AT2.1 Neo/HPRT– cells were hybridized with AT3.1 cells according to the polyethylene glycol method of Davidson et al. (12) For control cell hybridization, a highly metastatic AT3.1 cell was fused with a AT3.1 cell itself. AT3.1 cells were transfected with either the pY3 plasmid that encodes the neomycin resistance gene as described previously (11) and then a neomycin-resistant AT3.1 clone (i.e., AT2.1 Neo) was mutagenized with ethyl methanesulfonate treatment to produce a hypoxanthine-guanine phosphoribosyltransferase-lacking clone (i.e., AT2.1 Neo/HPRT–). To do this, 5 × 106 AT2.1 Neo cells in 10 ml of standard media containing 500 μg/ml of G418 were seeded into a T-75 flask and 24 h later the medium was replaced with standard media containing 500 μg/ml of G418 and 260 μg/ml of ethyl methanesulfonate. After 36 h, the ethyl methanesulfonate medium was exchanged with standard media containing 500 μg/ml of G418 and 10 μg/ml of TG. After 1 week, the TG concentration was lowered to 1.7 μg/ml; 2 weeks later, the flask was trypsinized and all of the cells were added to one T-75 flask containing 10 μg/ml of TG. The cells were passaged every week at a 1/10 split for 3 weeks and then cloned by limited dilution cloning. A HPRT– clone (i.e., AT2.1 Neo/HPRT–) was isolated, expanded, and then tested for its ability to be killed by hypoxanthine-aminopterin-thymidine selection. No AT2.1 Neo/HPRT– cells grew in standard media containing 500 μg/ml of G418, and AT2.1 Neo/HPRT– cells were maintained in the standard media containing 500 μg/ml of G418 and 10 μg/ml of TG.

INTRODUCTION

Studies of experimental and human cancers have demonstrated that transformation of a normal cell to a fully malignant cancer cell requires a series of genetic changes (1–3). This transformation involves gain in function of certain genes coupled with losses or inactivations of other genes (4, 5). These studies have led to the realization that carcinogenesis is a competing process between genes that determine the induction versus the suppression of malignancy. An unresolved question, however, is whether the acquisition of metastatic ability by already tumorigenic cancer cells also involves both positive and negative genetic changes. In the present studies, the mechanism of acquisition of metastatic ability in prostatic cancer was studied utilizing the Dunning rat prostatic cancer system. The original Dunning R-3327 tumor is a androgen-responsive, slow growing, well differentiated, nonmetastatic prostatic adenocarcinoma which spontaneously developed in a Copenhagen inbred male rat (6). From the original Dunning tumor, a large variety of additional sublines (i.e., ≥20 sublines) have developed during serial passage (7). These lines exhibit a wide range of tumor phenotypes with regard to androgen sensitivity, growth rate, histological and biochemical differentiation, and metastatic ability. If the progression from nonmetastatic to high metastatic ability involves only gains of certain functions (i.e., expression of oncogenes), then fusing a nonmetastatic with a highly metastatic prostatic cancer cell should produce a highly metastatic hybrid cell. In contrast, if loss of a certain function (i.e., loss or inactivation of suppressor gene(s)) is also involved, then such hybrids should be nonmetastatic since chromosomes from the nonmetastatic parental cell should supply the lost suppressor function. Therefore, such somatic cell fusion studies can be used to determine if gains in functions alone are involved in the acquisition of high metastatic ability or whether losses in functions are also required. To test this possibility, two Dunning rat prostatic cancers, the nonmetastatic AT2.1 and the highly metastatic AT3.1 sublines, were fused and the behaviors of the hybrid cells were analyzed.

MATERIALS AND METHODS

Animals. All animals used in these studies were adult male inbred Copenhagen rats (Cop/NHsBr) obtained from Harlan Sprague-Dawley. Tumor cells (1 × 106) were injected s.c. into Cop rats and the tumor volume-doubling time was determined as described previously (8).

Cells, Growth Medium, and Cell Hybridization. The cells used for these studies were nonmetastatic, androgen-independent, and anaplastic rat prostatic cancer cells (AT2.1 cells) and highly metastatic, androgen-independent, and anaplastic rat prostatic cancer cells (AT3.1 cells). The characteristics of AT2.1 and AT3.1 cells have been described in detail previously (9). Both cell lines have been maintained in RPMI 1640 containing 10% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 unit/ml), and dexamethasone (250 nm) (standard media) at 37°C with 5% CO.

For cell fusion, AT2.1 cells were transfected with the pZipNeoSV(X) plasmid (10) that encodes the neomycin resistance gene as described previously (11) and then a neomycin-resistant AT2.1 clone (i.e., AT2.1 Neo) was mutagenized with ethyl methanesulfonate treatment to produce a hypoxanthine-guanine phosphoribosyltransferase-lacking clone (i.e., AT2.1 Neo/HPRT–). To do this, 5 × 106 AT2.1 Neo cells in 10 ml of standard media containing 500 μg/ml of G418 were seeded into a T-75 flask and 24 h later the medium was replaced with standard media containing 500 μg/ml of G418 and 260 μg/ml of ethyl methanesulfonate. After 36 h, the ethyl methanesulfonate medium was exchanged with standard media containing 500 μg/ml of G418 and 10 μg/ml of neomycin. After 1 week, the TG concentration was lowered to 1.7 μg/ml; 2 weeks later, the flask was trypsinized and all of the cells were added to one T-75 flask containing 10 μg/ml TG. The cells were passaged every week at a 1/10 split for 3 weeks and then cloned by limited dilution cloning. A HPRT– clone (i.e., AT2.1 Neo/HPRT–) was isolated, expanded, and then tested for its ability to be killed by hypoxanthine-aminopterin-thymidine selection. No AT2.1 Neo/HPRT– cells grew in standard media containing 500 μg/ml of G418, and AT2.1 Neo/HPRT– cells were maintained in the standard media containing 500 μg/ml of G418 and 10 μg/ml of TG.

AT2.1 Neo/HPRT– cells were hybridized with AT3.1 cells according to the polyethylene glycol method of Davidson et al. (12) For control cell hybridization, a highly metastatic AT3.1 cell was fused with an AT3.1 cell itself. AT3.1 cells were transfected with either the pY3 plasmid that encodes the hygroycin B resistance gene (13) or the pZipNeoSV(X) plasmid (10) as described previously (11) to select hybrid cells. Hygromycin B-resistant AT3.1 clones and G418-resistant AT3.1 clones were selected in the standard media containing 500 μg/ml of hygromycin B and in the standard media containing 500 μg/ml of G418, respectively. An AT3.1 Hygro cell clone and an AT3.1 Neo clone were used as described above and then hybrid cells were selected in the standard media containing both 500 μg/ml hygromycin B and 500 μg/ml of G418.

Spontaneous Metastatic Assay. To test metastatic ability, animals were inoculated with 1 × 106 cells s.c. in the leg. The animals were allowed to go undisturbed until tumors reached 8–10 cm3 and the
RESULTS

When injected s.c. into the leg of Copenhagen rats, AT2.1 Neo/HPRT~ cells produced no distant metastases in 10 animals given injections, whereas AT3.1 cells produced distant metastases in all 10 animals given injections (Table 1). The modal chromosomal numbers for the AT2.1 Neo/HPRT~ cells and AT3.1 cells were 69 and 61, respectively, with each line possessing several characteristic structurally rearrangements (Table 2; Fig. 1, A and B). AT2.1 Neo/HPRT~ cells were fused with AT3.1 cells. AT2.1 Neo/HPRT~ x AT3.1 hybrid clones which conserved all (e.g., hybrid 1) or nearly all (e.g., hybrid 2) of the normal and traceable aberrant chromosomes from their parental AT2.1 Neo/HPRT~ and AT3.1 cells were identified (Table 2; Fig. 1C). When such hybrids were injected s.c. in the leg of rats, primary tumors developed in all animals; however, no distant metastases were observed in any animals (Table 1). Since hybrid 1 conserved all of the normal as well as marker chromosomes of both parents without any additional changes (Table 2; Fig. 1C), it was selected for further studies.

When the nonmetastatic hybrid 1 cells were injected s.c. in the flank of rats and primary tumors were passaged in vivo, occasional animals developed distant metastases. In detail, four Cop rats were inoculated with 1 × 10⁶ AT2.1 Neo/HPRT~ x AT3.1 hybrid 1 cells (in vitro passage 1) s.c. in the flank. After 42 days, each nonmetastatic primary tumor was transplanted s.c. in the flank of 3–4 Cop rats (second passage). Five of 14 animals bearing the second passage hybrid 1 tumor developed axillary lymph node and/or lung metastases. Two of nine second passage tumors which produced no metastases were transplanted in the flank of eight Cop rats (third passage). Distant metastases were observed in seven of eight animals bearing third passage hybrid 1 tumor. The average number of lung metastases per metastatic animal bearing the third passage hybrid 1 tumor was 51 ± 31 (SE). Five individual lung metastases and three individual axillary lymph node metastases from animals bearing these second and third in vivo passage hybrid 1 tumors were established in culture and analyzed cytogenetically (Table 2). These metastatic revertant cells showed 1–6 chromosomal changes (i.e., <5% changes in total chromosomes). All of these changes were simple chromosomal losses. A loss of a single copy of a normal chromosome 2 was consistently observed in all eight metastatic revertants. One of eight metastatic revertants (i.e., Ly1) had only one change which is a loss of a chromosome 2. A loss of Xp+ (i.e., structurally abnormal chromosome “o” (Table 2; Fig. 1, B and C)) was observed in seven of the eight metastatic revertants. When injected into animals to retest metastatic ability, all eight of these metastatic revertant lines showed high metastatic ability (Table 1).

To test whether the suppression of metastatic ability observed in the AT2.1 Neo/HPRT~ x AT3.1 hybrids was a specific effect as opposed to a nonspecific effect of cell hybridization itself, highly metastatic AT3.1 cells were fused to themselves. To do this, AT3.1 cells were transfected with either the hygromycin B resistance gene or the neomycin resistance gene and appropriate resistant clones were isolated and characterized cytogenetically. An AT3.1 hygromycin B-resistant clone (i.e., AT3.1 Hygro) and a neomycin-resistant clone (i.e., AT3.1 Neo) were

Table 1 In vivo characteristics of AT2.1 Neo/HPRT~ and AT3.1 parental cells, AT2.1 Neo/HPRT~ x AT3.1 hybrid 1 and 2 cells, and highly metastatic revertants of AT2.1 Neo/HPRT~ x AT3.1 hybrid 1 cells

<table>
<thead>
<tr>
<th>Metastatic ability</th>
<th>% of group with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>Tumor-doubling time (day)</td>
</tr>
<tr>
<td>AT2.1 Neo/HPRT~ (n = 10)*</td>
<td>2.5 ± 0.1*</td>
</tr>
<tr>
<td>AT3.1 (n = 10)</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>AT2.1 Neo/HPRT~ x AT3.1 Hybrid 1' (n = 13)</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Hybrid 2' (n = 7)</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Highly metastatic revertants of AT2.1 Neo/HPRT~ x AT3.1 hybrid 1 Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>Ly1 (n = 4)</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Ly2 (n = 4)</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Ly3 (n = 4)</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Ly4 (n = 4)</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Ly5 (n = 4)</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Lung metastasis</td>
<td></td>
</tr>
<tr>
<td>Lu1 (n = 4)</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Lu2 (n = 4)</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Lu3 (n = 4)</td>
<td>4.0 ± 0.4</td>
</tr>
</tbody>
</table>

* Number of animals given injections of cells.

* Mean ± SE.

* In vitro passage 1 cells injected in vivo.
Fig. 1. Representative karyotypes of AT2.1 Neo/HPRT" (A), AT3.1 (B), and AT2.1 Neo/HPRT" × AT3.1 hybrid 1 (C). Giemsa-banded karyotypes were constructed as described in “Materials and Methods.” Large arrowheads with capitals, traceable structurally rearranged chromosomes of AT2.1 Neo/HPRT": A, t(1;?)p11:]; B, del(1)(q42); C, t(3;7)(p11:7); D, del(15)(p14); E, 7p+; F, del(15)(p14); G, del(x)(q35); H, der(Y). Small arrowheads with small letters, traceable structurally rearranged chromosomes of AT3.1: a, rob(1:4); b, rob (2:6); c, rob(2:11); d, rob(2q-5); e, t(3:7)(p11:7); f, rob(X:4); g, 6p+; h, rob(7:8); i, 9q+; k, (?::3q11--3p42::7q11--10qter); l, t(11:?)(p11:7); m, t(11:2)(p11:7); n, del(15)(p14); o, Xp+. M1, untraceable marker chromosome but distinguishable from the other small markers. As shown in C, AT2.1 Neo/HPRT" × AT3.1 hybrid 1 conserved all of these traceable and untraceable marker chromosomes. Bottom line, small marker chromosomes the origins of which are unknown.
GENETIC FACTORS AND SUPPRESSION OF METASTASES

Table 3. Characteristics of AT3.1 Hygro and AT3.1 Neo parental clones and AT3.1 Hygro × AT3.1 Neo hybrid clones

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Modal chromosomal no.</th>
<th>Tumor-doubling time (day)</th>
<th>Metastatic ability* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3.1 Hygro</td>
<td>57</td>
<td>2.2 ± 0.2</td>
<td>100 (8/8)</td>
</tr>
<tr>
<td>AT3.1 Neo</td>
<td>60</td>
<td>1.7 ± 0.1</td>
<td>100 (8/8)</td>
</tr>
<tr>
<td>AT3.1 Hygro × AT3.1 Neo hybrid</td>
<td>116</td>
<td>3.1 ± 0.4</td>
<td>100 (8/8)</td>
</tr>
<tr>
<td>Clone 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Percentage of animals with axillary lymph node and/or lung metastases.
† Mean ± SE.
‡ Numbers in parentheses, number of animals with metastases/number of animals inoculated with cells.

identified which retained the major numerical and structural chromosomal aberrations of the parental AT3.1 cells. When inoculated into animals, both AT3.1 Hygro and AT3.1 Neo clones conserved their high metastatic ability (Table 3). These AT3.1 Hygro and AT3.1 Neo cells were fused and hybrid clones were isolated which conserved all of the normal and traceable aberrant chromosomes from both parents. When such hybrid clones were inoculated into animals, they maintained high metastatic ability, although tumor volume-doubling time did increase slightly in both hybrid clones (Table 3). This demonstrates that cell hybridization itself does not suppress the high metastatic ability of the AT3.1 parental cells.

DISCUSSION

The present studies demonstrate that high metastatic ability is suppressed in hybrid cells produced by fusing nonmetastatic and highly metastatic rat prostatic cancer cells when the hybrid cells retain all chromosomes from their parental cells. This suggests that acquisition of high metastatic ability by rat prostatic cancer cells involves loss of metastasis suppressor gene(s) function. These studies also suggest that for rat prostatic cancer such a metastasis suppressor gene(s) is located on chromosome 2. A loss of Xp+ was also observed in seven of the eight metastatic revertants. However, this loss does not seem to be related to the reacquisition of metastatic ability, because Xp+ is one of the structurally abnormal chromosome characteristics for the highly metastatic AT3.1 cells. If Xp+ carried a metastasis suppressor gene(s), then the parental AT3.1 cells should not be highly metastatic. In contrast, if Xp+ carried a positive function(s) which is important for the metastatic phenotype of the AT3.1 cells, then the metastatic revertants would be expected to retain this Xp+ chromosome. Thus, the loss of the Xp+ marker in the revertants appears to be a random event.

Indirect evidence for the existence of a metastasis suppressor gene(s) has been demonstrated by several somatic cell fusion studies (17–20). In these studies, metastatic potential was suppressed when rat metastatic mammary carcinoma cells were fused with various nonmetastatic cells (17), when mouse metastatic melanoma cells were fused with normal cells (18, 19), and when mouse metastatic lung carcinoma cells were fused with tumorigenic but nonmetastatic mouse L-cells (20). However, no specific chromosomes possessing metastasis suppressor genes have been identified from these earlier studies. The present studies demonstrate that acquisition of high metastatic ability by rat prostatic cancer somatic cell hybrids is associated with the loss of a gene(s) on chromosome 2.

Direct demonstrations of the suppression of tumorigenicity
or the induction of cellular senescence by means of microcell-mediated chromosome transfer have been reported (21–26). No human chromosome completely corresponds to rat chromosome 2. However, human chromosomes 1, 4, 5, 6, 10, and 15 have portions which correspond to rat chromosome 2 based on comparative gene mapping (27). Introduction of these human chromosomes via microcell-mediated chromosome transfer into highly metastatic rat prostatic cancer cells is currently under way.

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REFERENCES

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