Suppression of Tumorigenicity in Human Teratocarcinoma Cell Line PA-1 by Introduction of Chromosome 4

I. Jean McGowan-Jordan, Marsha D. Speevak, David Blakey, and Mario Chevrette

Department of Biochemistry, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, K1H 8M5 Canada [I. J. M-J., M. D. S., M. C.], and Environmental Health Centre, Tunney's Pasture, Ottawa, Ontario, K1A 0L2 Canada [D. B.]

Abstract

Teratocarcinomas are tumors that develop spontaneously in the gonads and usually contain a rapidly dividing, undifferentiated stem cell population. Immature ovarian teratocarcinomas are highly malignant with only 30-60% of patients surviving for 2 years after diagnosis. We have used microcell fusion to introduce individually tagged normal human chromosomes into the PA-1 human teratocarcinoma cell line. Introduction of human chromosome 4 caused a change of cell morphology in culture and suppressed PA-1 tumorigenicity in nude mice, whereas addition of portions of either chromosome 7 or 12 had no effect on the cell phenotype. The PA-1 cell line regained its tumorigenicity when the tagged chromosome 4 was lost under negative selection. We conclude that there is a putative tumor suppressor gene on human chromosome 4 whose expression interferes with the tumorigenicity of PA-1 cells.

Introduction

The human ovarian teratocarcinoma cell line PA-1 was isolated from the ascites fluid of a 12-year-old girl suffering from recurrent malignant teratocarcinoma (1). Malignant ovarian teratocarcinomas differ from their benign counterparts in that they are a rarer form of tumor and usually occur in prepubertal females. They consist of many immature elements and can arise by several mechanisms including failure of meiosis I or II or fusion of two ova (2). The PA-1 cell line is believed to have developed from stem cells prior to the first meiotic division. Recently, other ovarian teratocarcinomas of this origin have been identified (3). High passage PA-1 cells are tumorigenic in nude mice upon s.c. injection, form embryoid bodies under nonadherent culture conditions, and form colonies in soft agar. Cytogenetically, PA-1 has a stable diploid female karyotype with a single, balanced translocation between chromosomes 15 and 20.

Results from previous experiments have suggested that tumor suppressor genes have been inactivated in PA-1. Early passage PA-1 cells are not tumorigenic in nude mice, whereas late passage cells are tumorigenic and carry an activated N-ras oncogene (4). Whole cell fusions between early and late passage cells resulted in suppression of the ras-transformed phenotype, suggesting that an active dominant tumor suppressor gene was present in the early passage cells (5). As these whole cell hybrids were cultured, the pattern of chromosome loss and reversion to tumorigenicity was monitored. The pattern suggested the existence of tumor suppressor genes on chromosomes 1, 4, and 11, since tumorigenic cells were often found to have lost one or more of these chromosomes. Two of these chromosomes, 1 and 11, harbor known tumor suppressor genes, K-revl/rapla (6) and WT-1 (7), respectively. However, no tumor suppressor gene has yet been mapped to chromosome 4.

In order to determine the chromosomal location of a putative tumor suppressor gene, we have used microcell fusion to introduce tagged normal human chromosomes into the human teratocarcinoma cell line PA-1. While the independent transfer of several chromosomes was insufficient to suppress PA-1 tumorigenicity, introduction of human chromosome 4 resulted in a suppressed phenotype. Furthermore, loss of the tagged chromosome resulted in the reappearance of the tumorigenic phenotype.

Materials and Methods

Cell Lines and Culture Conditions. PA-1 (obtained at passage 332 from the American Type Culture Collection, Rockville, MD) and all resulting cell lines were maintained as monolayer cultures at 37°C under 5% CO2 in minimal essential media (GIBCO) supplemented with 10% FBS (Gibco). Electroporation (960 µFD, 200V) was used to introduce pSV2neo (8) into PA-1. A G418-resistant clone (200 µg/ml; Gibco), PA1N5, with growth properties similar to those of PA-1 was chosen for further experimentation. Human fibroblasts were established from foreskins and grown in Dulbecco's modified Eagle medium/Ham's F-12 (1:1 medium; PDL) supplemented with 10% FBS. Cells (2.5 x 10⁶) were electroporated with the IgCMV/HyTK plasmid, which confers resistance to hygromycin B and sensitivity to ganciclovir (9). B78 mouse melanoma cells (a gift from M. Thayer, Oregon Health Sciences University, Portland, OR) were grown in high glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS. B78MC microcell hybrids were cultured as B78 cells but in the presence of hygromycin B (400 µg/ml). Segregants of PAB1O8 were isolated after 3 weeks in the presence of ganciclovir (100 µM; Syntex, Inc., Palo Alto, CA).

Microcell-mediated Chromosome Transfer. All microcell fusions were performed as described by Fournier (10). The B78MC mouse/human microcell hybrids used to transfer the TNHC into PA1N5 will be described elsewhere. Microcell fusion between PA1N5 and B78MC microcell hybrids were cultured as B78 cells but in the presence of hygromycin B (400 µg/ml). Segregants of PAB1O8 were isolated after 3 weeks in the presence of ganciclovir (100 µM; Syntex, Inc., Palo Alto, CA).

Tumorigenicity in Nude Mice. Cells were trypsinized and counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Following resuspension in sterile saline, 10⁴ cells were injected s.c. into nu/nu athymic mice (Charles River). Tumor formation was monitored weekly for 6 months or until tumors arose.

Hygromycin B Resistance. To determine the hygromycin B resistance of cell lines, 2.5 x 10⁵ cells were plated in triplicate in complete media with and without hygromycin B (400 µg/ml) in six-well plates. After 6 days, cells were trypsinized and counted using a Coulter counter.

Cytogenetic Analysis. Metaphase chromosome spreads were prepared and G-banded according to standard procedures.

FISH. FISH, as described by Pinkel et al. (11), was used to confirm that Hsa 4 was introduced into PA1N5 to create the PAB1O8 cell line. The DNA probe for Hsa 4, provided by Dr. J. Gray (University of California, San...
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Results

Introduction of TNHC. To facilitate selection of hybrid teratocarcinoma cells containing single TNHC, the parental cell line, PA-i, was first transfected with pSV2neo. Several G148-resistant clones (PA1N5) were tested for growth in soft agar and the ability to form tumors upon s.c. injection in nude mice (not shown). Clone PA1N5 had the same karyotype and exhibited growth properties similar to the parental PA-1 cells and was chosen for further experiments.

The dual selectable marker tGCMV/HyTK (9) was introduced into normal human skin fibroblasts to ensure the subsequent retention of the transferred chromosome in the tumorigenic cell line. In the first round of microcell fusions, human chromosomes were transferred into an immortal cell line. TNHC from skin fibroblasts were transferred, via microcell fusion (10), into a mouse melanoma cell line, B78, thus generating a mouse-human microcell hybrid panel (B78MC). Microcell fusions between B78MC hybrids containing TNHC and G418-resistant PA1N5 were performed. PAB hybrids containing a TNHC are resistant to both G418 and hygromycin B but are sensitive to ganciclovir due to the herpes thymidine kinase gene encoded by tGCMV/HyTK (9).

Microcell hybrids generated from each fusion were analyzed to confirm the introduction of the TNHC (Table 1). Karyotype analysis (data not shown) revealed that all microcell hybrids carry a supernumerary chromosome; PAB63 contains an extra centromeric portion of Hsa 7 [der(7)], while an intact Hsa 4 was transferred into PAB108. The cytogenetic identification of the introduced chromosome in PAB108 was confirmed by FISH using an Hsa 4-specific chromosome “painting” probe (11), which revealed the presence of a third copy of chromosome 4 (Fig. 1A).

We used ganciclovir to segregate the TNHC from PAB108. These segregants (PAB108-S) are sensitive to hygromycin B (Table 1) and were shown by Alu-PCR FISH (12) to contain only two copies of Hsa 4 (Fig. 1B).

Tumorigenicity of Microcell Hybrids. The tumorigenicity of several hybrids was tested (Table 1) by monitoring tumor growth after s.c. injection of cells into nude mice. The parental cell line PA1N5 formed tumors by 21 days after injection. Microcell hybrid PAB63, containing an extra portion of Hsa 7, also formed tumors by 21 days post injection (Table 1). In contrast, numerous injections of three independent clones of PAB108 (PAB108–1, PAB108–3, and PAB108–4) failed to induce tumors in nude mice, whereas two PAB108-S clones (PAB108-S25 and PAB108-S33) which had segregated the tagged Hsa 4 rapidly formed tumors (Table 1). These results indicate that there is a tumor suppressor gene on Hsa 4 which is inactivated in PA-1 cells. It is of interest to note that the putative tumor suppressor gene on Hsa 4 shows some specificity in its action since the mouse melanoma cell line B78MC108 (from which Hsa 4 was transferred) was tumorigenic when injected into nude mice (data not shown).

Tumor-derived Cell Lines. In two instances, injection of PAB108 resulted in tumor formation (Table 1). Karyotype analysis and Alu-PCR FISH were performed and showed that tumor-derived cell lines had either lost a copy of Hsa 4 (PAB108-TD1; data not shown) or had a rearranged Hsa 4 (PAB108-TD2; Fig. 2). The G-banding pattern seemed to indicate that the rearranged Hsa 4 present in PAB108-TD2 contained an extra portion of the long arm (Fig. 2). Moreover, FISH analysis seemed to indicate that the additional material in the short arm was derived from chromosome 4 material (data not shown). The PAB108-TD1 cell line was sensitive to hygromycin B (Table 1), indicating that the TNHC had been lost and was responsible for the tumor suppression seen in PAB108. The TNHC was likely lost from the cells due to the nonselective environ-

<table>
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<th>Hybrid</th>
<th>TNHC</th>
<th>No. of independent clones injected</th>
<th>No. of tumors/No. of injections</th>
<th>Growth in hygromycin (400 μg/ml)</th>
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<tr>
<td>PA1N5</td>
<td>None</td>
<td>4/9</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>PAB63</td>
<td>der(7)</td>
<td>2/4</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>PAB108</td>
<td>4</td>
<td>3/12</td>
<td></td>
<td>+</td>
</tr>
<tr>
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<td>None</td>
<td>2/6</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>PAB108-TD1</td>
<td>*</td>
<td>+/–</td>
<td></td>
<td></td>
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<tr>
<td>PAB108-TD2</td>
<td>der(4)</td>
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* Tumors were scored 21 days after subcutaneous injection of 10^6 cells; * Alu-PCR FISH (data not shown) shows that the majority of cells (22 of 35 observed) had lost the third Hsa 4.

Francisco, CA) was biotinylated and used according to standard procedure. A Nikon Optiphot microscope fitted with a B filter was used for viewing.

Fig. 1. FISH of microcell hybrids. A, PAB108 metaphase spread with chromosome 4-specific “painting” probe. B, PAB108-S metaphase spread with Alu-108 probe which hybridizes to chromosome 4. At least 30 spreads of each cell line were examined. All PAB108 spreads had three hybridizing chromosomes, while all PAB108-S spreads had only two hybridizing chromosomes.
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Fig. 2. Karyotype analysis of Hsa 4 in PAB108-TD2. Three G-banded copies of Hsa 4 from PAB108-TD2 showing two normal copies and one rearranged copy. Arrow, the rearranged copy which was present in over 80% of the analyzed spreads.

Fig. 3. Morphology of subconfluent cells in culture. A, PA1N5; B, PAB63; C, PAB108; D, PAB108-S; E, PAB108-TD1; F, PAB108-TD2. All photographs were taken at × 100.

Morphology. Not only did the introduction of Hsa 4 into PA1N5 cause a suppression of tumorigenicity, it also resulted in a change in cell morphology. Both PA-1 and PA1N5 have an epithelial morphology when grown in tissue culture flasks (Fig. 3A and data not shown). Addition of either der(7) or der(12) to PA1N5 had no effect on its morphology (Fig. 3B and data not shown). However, all three PAB108 clones obtained, each containing a TNHC 4, exhibit a flattened appearance in culture (Fig. 3C). Loss of this chromosome from PAB108 resulted in a cell line, PAB108-S, with a morphology similar to that of PA1N5 (Fig. 3D). The tumor-derived cell lines, PAB108-TD1 which lacks the tagged Hsa 4, and PAB108-TD2 which has a rearranged tagged Hsa 4, also reverted to an epithelial morphology (Fig. 3, E and F). Although we cannot rule out the possibility that another gene on Hsa 4 could be responsible for these morphological changes, the fact that they are always associated with the tumorigenic phenotype indicates that they are directly linked to the expression of the tumor suppressor gene.
**Discussion**

We have shown that introduction of a normal copy of Hsa 4 into PA1N5 resulted in a change in cell morphology and was sufficient to suppress its tumorigenicity, whereas the addition of den(7) had no effect. Loss of the tagged Hsa 4 reverted both the tumorigenic phenotype and the morphology of PA1B08 to that of PA1N5, providing strong evidence that a tumor suppressor locus is present on this chromosome. As in colorectal cancer, the tumorigenicity of PA-1 appears to result from a combination of mutations of oncogenes and inactivation of tumor suppressor genes. Late passage PA-1 is known to have an activated N-ras oncogene (4) as well as one mutated p53 allele (13). Addition of a normal Hsa 4 to PA1N5 suppressed the effect of these two genetic changes, suggesting that a tumor suppressor gene located on Hsa 4 was inactivated in PA-1. Thus, at least three events contribute to the tumorigenic phenotype of this teratocarcinoma cell line.

Changes in cell morphology, as we have observed when Hsa 4 was introduced into PA1N5, have been associated with reversion to a nontumorigenic phenotype (14, 15) and may result from the cells entering a differentiation pathway. By selecting cells that had undergone morphological changes (flat revertants) upon transfection of a complementary DNA expression library, Kitayama et al. (16) were able to clone Krev-ir/apla, a tumor suppressor gene which suppresses the transforming ability of K-ras in rodent fibroblasts. Although PA-i has an activated N-ras, the tumor suppressor gene that we have identified cannot be K-revl/apla, which has been mapped to Hsa 1 (6). However, since other members of this family have been mapped on Hsa i2 and i3 (6), it is possible that the new tumor suppressor gene located on Hsa 4 could be a member of the rapla family.

Currently, there are no known tumor suppressor genes which map to Hsa 4; however, several lines of evidence suggest that this chromosome may harbor a tumor suppressor gene. Krizman et al. (5) have shown that the loss of Hsa 4 from whole cell hybrids of early and late passage PA-1 was associated with reversion to tumorigenicity. The addition of Hsa 4 in cell hybrids has been correlated with cell senescence (17), a characteristic of nontumorigenic cells. Moreover, specific loss of Hsa 4 has been noted in tumorigenic segregants of whole cell hybrids derived from tumorigenic rodent cells and normal human fibroblasts (18). In fact, loss of Hsa 4, implicating tumor suppressor gene inactivation at this locus, has recently been reported in a primary human ovarian teratocarcinoma and in a metastatic tumor (resected 1 year after removal of the primary tumor; Ref. 19). This finding indicates that loss of a tumor suppressor gene on Hsa 4 may be an early event in the genesis of immature ovarian teratocarcinomas.

Further evidence that Hsa 4 may encode a tumor suppressor gene comes from the finding that several human hepatocellular carcinomas exhibit loss of heterozygosity at chromosome 4q. It has been suggested that inactivation of a tumor suppressor gene at this locus contributed to liver cancers (20). An interstitial deletion of Hsa 4 from q11-q13 has also been detected in the human hepatoma cell line HuH-7 and may have resulted in the loss of tumor suppressor gene activity (21). This situation is very similar to the Hsa 4 rearrangement present in PAB1O8-TD2, which we believe resulted in inactivation of the putative tumor suppressor gene. The inactivation could be due to the addition occurring in the short arm of Hsa 4, which could disrupt the normal transcription of the tumor suppressor gene, similar to mutagenesis induced by retroviral insertion (22). Alternatively, the deletion in the long arm of Hsa 4 could have completely removed the tumor suppressor locus, as is often the case in human cancers (23). It is impossible at this point to determine if the putative tumor suppressor gene implicated in hepatocellular carcinoma is the same as the gene responsible for the loss of tumorigenicity seen in PAB108. We are now mapping the breakpoints present in the rearranged Hsa 4 from PAB108-TD2 in order to clarify this issue. Chromosomal translocation involving Hsa 4 has also been associated with acute lymphoblastic leukemias (24) and malignant T-cell lymphoma (25). Although such translocations often result in activation of oncogenes (26), it is conceivable (as we have suggested for PAB108-TD2) that they could disrupt a tumor suppressor gene and thus interfere with its proper transcription. We are now generating a physical map of the rearranged Hsa 4 and attempting to clone this new tumor suppressor gene in order to resolve these ambiguities.

**Acknowledgments**

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**References**


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