Spontaneous Transformation of Rat Ovarian Surface Epithelial Cells
Results in Well to Poorly Differentiated Tumors with a Parallel Range of Cytogenetic Complexity

Joseph R. Testa,^2 Lori A. Getts, Hernando Salazar, Zemin Liu, Laura M. Handel, Andrew K. Godwin, and Thomas C. Hamilton^2


ABSTRACT

We previously used rat ovarian surface epithelial cells subjected to repetitious growth in vitro to provide experimental evidence in support of a role for incessant ovulation in the etiology of ovarian cancer. We have now initiated a series of 30 independent rat ovarian surface epithelial cell lines. This report describes findings in eight of the cell lines that, based on tumor formation in athymic mice, have undergone malignant transformation. Each of the tumors exhibited chromosomal alterations. Two well to moderately differentiated tumors had only one or two cytogenetic changes, and they had in common the presence of numerical gains. Each of five poorly differentiated tumors had complex karyotypes with three to eight clonal aberrations, prominent among them being unbalanced rearrangements and numerical losses. Several poorly differentiated tumors also had marker chromosomes, double minutes, or homogeneously staining regions. These findings demonstrate that the malignant tumors produced by spontaneously transformed rat ovarian surface epithelial cell lines range in degree of differentiation, which is paralleled by the cytogenetic complexity. Thus, this model system may fill an important void in future efforts to define the genetic basis of common epithelial tumors of the ovary and many features characteristic of these neoplasms.

INTRODUCTION

It is generally accepted that most malignant ovarian tumors arise from the single layer of cells which covers the ovarian surface. It has intrigued pathologists for generations that these cells, which in their normal setting are phenotypically unremarkable, upon malignant transformation grow to produce tumors with diverse histological features (1, 2). These tumors, in order of their frequency, are classified as serous, mucinous, endometroid, clear cell, and other, based on cellular and structural features reminiscent of normal adult tissues of Mullerian origin (3). The diverse characteristics of these so-called common epithelial tumors of the ovary, which are believed to be derived from a single cell type, pose many questions. It is of particular interest to consider whether the molecular etiology may be distinct between these tumor subcategories and whether additional factors are functionally related to grade and stage of disease. In humans, cytogenetic and molecular genetic approaches are beginning to be applied to these problems. These investigations have produced some potentially valuable leads regarding chromosomal sites that may harbor genes which impinge on disease initiation and progression.

In human ovarian cancer, many regions of the genome have been implicated by cytogenetic and loss of heterozygosity studies. Cytogenetic analysis has documented complex karyotypes in most ovarian carcinomas. Such investigations have revealed several recurrent chromosomal alterations, including rearrangements resulting in partial losses affecting chromosome arms 1p, 3p, 6q, 11p, and 19p (4—10). Loss of heterozygosity studies of human ovarian carcinomas have shown frequent allelic losses from various chromosomes including 3p, 4p, 6, 7p, 8q, 11p, 12, 13q, 16, 17, 19, and Xp (11—14). Recent reports suggest that the long arm of chromosome 17 may be the location of one or more tumor suppressor genes of importance in ovarian oncogenesis. A predisposing gene for familial breast and ovarian cancer (BRCA1) has been mapped by genetic linkage analysis to 17q12—q21. Linkage to this locus has been found in approximately 80% of families with a high incidence of breast and ovarian cancer (15). Amplification and/or overexpression of proto-oncogenes (MYC, Kras, Hras, Fms, Erbb2, and Akt2) have also been implicated in the pathogenesis and aggressiveness of some ovarian cancers (16—32).

The fact that ovarian cancer is most frequently discovered late in its clinical course has resulted in the majority of the cytogenetic and molecular genetic studies being performed on Stage III and IV specimens, sometimes after radiation and/or chemotherapy. This has made it difficult to segregate changes potentially related to disease initiation from those related to progression, drug resistance, or exposure to clastogenic agents. This complex situation suggests that a model system that would allow the accumulation of molecular and cytogenetic changes to be determined as ovarian surface epithelial cells progress from normal through the various steps of transformation could assist in efforts to separate genetic changes into specific categories. Such a model might also provide direct experimental proof as to the cell type responsible for common epithelial tumors of the ovary. We have previously used rat ovarian surface epithelial cells subjected to repetitious growth in vitro in order to provide evidence supporting a role for "incessant ovulation" in ovarian cancer etiology (33). In our earlier study, we noted losses of rat chromosome 5. To determine whether this is a consistent feature of malignantly transformed rat ovarian surface epithelial cells, we carried out an expanded study as reported here. We describe the frequency for various levels of transformation of a large series of independent rat ovarian surface epithelial cell lines and present evidence that the malignant tumors produced by some of these cell lines range in degree of differentiation which is paralleled by the complexity of karyotypes. Hence, this model system may fill an important void in our efforts to define the genetic basis for many features of common epithelial tumors of the ovary.

MATERIALS AND METHODS

Initiation of Rat Ovarian Surface Epithelial Cell Lines. A detailed description of the methods used to initiate these rat cell lines has been reported previously (33). Briefly, ovaries were aseptically removed from 50 mature female Fischer rats (12—16 weeks of age) and incubated in trypsin to selectively remove the surface epithelial cells. Cell suspensions derived from groups of 10 ovaries were transfected to individual tissue culture flasks (i.e., cells from 100 ovaries yielded 10 cultures), incubated at 37°C until cells approached confluence, and then harvested with trypsin. Pooled cells derived from the expansion of surface epithelial cells from 100 rat ovaries were transfected to 30 tissue culture flasks. The cells in these individual flasks were designated cell lines 1 to 30 and, after this passage, were maintained as independent cultures.

Received 11/22/93; accepted 3/18/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by National Cancer Institute Grants CA-56916 and CA-69627, by the Evy Lessin Fund, and by an appropriation from the Commonwealth of Pennsylvania.

2 To whom requests for reprints should be addressed, at Fox Chase Cancer Center, Department of Medical Oncology, 7701 Burholme Avenue, Philadelphia, PA 19111.
Individual cell lines were subcultured with trypsin at 1- to 2-week intervals at split ratios of 1:5 (early passage, i.e., <10 subcultures) and 1:10 (late passage, i.e., 10 or more subcultures).

**Determination of Substrate-independent Growth Capacity.** Individual cell lines were examined for substrate-independent growth early (~passage 5) in their culture history and after 35–40 subcultures. A two-layer agarose system was used to determine substrate-independent growth. Cells (100,000/ml) were suspended in agarose (0.3% w/v; Sigma Type VII; Sigma Chemical Co., St. Louis, MO), prepared in complete medium, and maintained at 37°C. The cell suspension (1 ml) was layered over a cold (4°C), solidified underlayer of agarose (0.6% w/v) which was prepared as above and added as 1-ml amounts to 10-cm² culture dishes. After the upper, cell-containing layer of agarose had solidified, dishes were incubated as for routine tissue culture (see above) for 10–21 days. When the ability of EGF (30 ng/ml; Collaborative Research) to influence substrate-independent growth was examined, the growth factor was added to the upper, cell-containing agarose layer. Colony formation was quantitated with an ARTEK FASIS Image Analysis System (Dynatech Laboratories, Inc., Chantilly, VA). Cells were deemed to have the capacity for substrate-independent growth if they grew into colonies of >60 µm in diameter (values of <20 colonies/5 cm² of dish area were considered artifactual and equivalent to no capacity for substrate-independent growth).

**Determination of Tumorigenicity and Initiation of Cell Line from Tumors.** All rat ovarian surface epithelial cell lines were evaluated for tumorigenicity by bilateral s.c. injection of cells (5 × 10⁶ suspended in 0.1 ml of Matrigel) into the subscapular areas of three female athymic mice. Tumors 0.5–1 cm in diameter were removed for histopathological evaluation and initiation of tumor-derived cell lines. Histopathological characterization was done by standard techniques. To produce tumor cell lines, the tumor was minced and suspended in complete medium containing collagenase (250 units/ml; Sigma Type IV), incubated for 4 h at 37°C with constant rocking, and then aggregates of cells were isolated by gravity. These aggregates were resuspended in complete medium, placed in tissue culture flasks, and incubated at 37°C. These cell lines were subcultured at 1-2-week intervals.

**Metaphase Chromosome Preparations.** Detailed karyotypic analyses were carried out on early passage cell lines and rat cell lines derived from tumors growing in athymic mice. In the case of cell lines derived from tumors, karyotypes were prepared as soon as adequate numbers of cells were available. This was done to minimize the possible impact of additional in vitro growth on karyotypes. Cell cultures in logarithmic growth phase were treated with Colcemid (0.03 µg/ml), trypsinized, transferred to hypotonic KC1 (0.075 M) for 15 min at 37°C, and then fixed in several changes of methanol:acetic acid (3:1). Metaphase spreads were steam-dried and then G-banded according to our usual method (33). Chromosome identification and breakpoint designations were done in accordance with published idiograms of rat chromosomes (34, 35). The cytogenetic nomenclature used followed the recommendations of the International System for Human Cytogenetic Nomenclature (1991; Ref. 36).

**RESULTS**

**General Characteristics.** Surface epithelial cells isolated by selective trypsinization of rat ovaries, when placed in tissue culture, grew with an epithelioid morphology, covering the culture surface, and then exhibited the contact inhibited phenotype. All 30 cultures (Table 1) initiated failed to become senescent. Hence, they were considered spontaneously immortalized cell lines in that they have continued to grow at the maximum passage levels thus far tested, i.e., passage 65. In addition, the cells lost their contact inhibited phenotype after approximately 10 in vitro passages. Eight of these cell lines formed tumors with high efficiency in athymic mice. Such features are not characteristic of cultured rat epithelial cells, in contrast to mouse cells (33).

**Capacity for Substrate-independent Growth.** In no case did early passage cells (<passage 6) show the capacity for colony formation in semi-solid agarose. By our criteria, i.e., a colony forming efficiency of >0.4%, we observed substrate-independent growth in the absence of exogenously added growth factor in 40% (12 of 30) of the cell lines (Table 1). When the effect of exogenously added EGF (30 ng/ml) on substrate-independent growth was evaluated, we observed that in all cases, except cell lines 2, 23, and 26, EGF enhanced the capacity for growth in agarose. Additionally, 11 cell lines that did not clone in the absence of exogenous EGF showed colony formation in its presence.

**Tumorigenicity.** In previous studies, we have never observed tumorigenicity of early passage rat ovarian surface epithelial cells in athymic nude mice. Therefore, only representative cell lines of the current panel of 30 lines which were tumorigenic in late passage were examined for tumorigenicity in early passage. Consistent with previous findings, these early passage cell lines were not tumorigenic. When cell lines in late passage were examined for tumorigenicity, 57% (17 of 30) cell lines were tumorigenic. Eight of these lines (i.e., 8, 9, 10, 12, 14, 19, 23, and 26) formed tumors at a high percentage of injection sites and were >0.5 cm in diameter by 3–6 weeks after injection of 5 × 10⁶ cells/site. This group of cell lines and the tumors which they formed were therefore selected for detailed histopathological and cytogenetic analysis.

**Histopathological Findings.** Histologically, the tumors always appeared relatively well circumscribed but frequently invaded s.c. tissue, including underlying skeletal muscle. All tumors studied were adenocarcinomas that we subdivided, according to the degree of glandular differentiation, into two distinct groups, well to moderately differentiated and poorly differentiated. The majority of the tumors were poorly differentiated adenocarcinomas (cell lines 8, 9, 10, 19, 23, and 26). One tumor (cell line 12) appeared to be a well-differentiated adenocarcinoma with a well-defined tubulo-glandular pattern, and another tumor (cell line 14) was moderately differentiated.

---

2 The abbreviations used are: EGF, epidermal growth factor; dmin, double minute chromosome; hsr, homogeneously staining region; G-banded, Giemsa-banded.
Fig. 1. A, well-differentiated adenocarcinoma (derived from cell line 12) with well-formed glands of various sizes and shapes lined by single epithelium with "enteric" pattern containing mucous "goblet" cells. The glands infiltrate a fibrous stroma (× 250). B, higher power view of neoplastic glands invading fibro-collagenous stroma. The glands are dilated and contain mucous and desquamated cells. Note the goblet-type mucous cells within the pseudostratified epithelium with other secretory, clear, and intercalated cells and some mitoses. The stroma contains fibroblasts and mononuclear inflammatory cells (× 400). C, panoramic view of a moderately differentiated adenocarcinoma (cell line 14) in which most of the epithelial elements formed solid sheaths or packed cords of small polygonal cells, in some areas forming small glands with mucous goblet cells, with or without lumen, but in most areas giving just the suggestion of poorly differentiated glands. The connective tissue stroma is scant (× 100). D, Higher power view of the same tumor above showing ill-formed neoplastic glands with mitotic activity and narrow lumens among poorly differentiated masses of epithelial cells with adenoid appearance including individual mucous cells not forming glandular structures. Note the pleomorphism of the tumor cells (× 400). E, undifferentiated carcinoma (cell line 10) formed by diffuse masses of disorganized neoplastic epithelial cells infiltrating and destroying skeletal muscle (remnants of muscle fibers at lower left) and with focal areas of necrosis (lower right; × 100). F, high power view of same tumor above showing the highly pleomorphic neoplastic epithelial cells with obvious variation in size, shape, and orientation, some forming small groups and cords separated by fine strands of collagenous stroma. Note larger cells with irregular nuclei and large nucleoli, some of which are hyperchromatic (× 400).
Table 2. Cytogenetic findings in eight tumors produced in athymic mice following inoculation of rat ovarian surface epithelial cell lines

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>42–43,XX,der(3;12)(p11;p11),ins(6;7)(q16;?),−16,+2−3mar (one mar contains an hsr)[5]</td>
</tr>
<tr>
<td>9</td>
<td>42,XX,der(2;7)(q10;q13 or q21.1),−7,add(14)(q11.2),der(16)add(1X?q31)(1;16)(q22;q12.3),+mar,1−2dmin[2]/84−85,ide(2)[2]</td>
</tr>
<tr>
<td>10</td>
<td>42–43,XX,ins(6;12q10),add(13)(q26),del(16)(k1;16)(q22;q12.3),−17,+1−3mar[5]</td>
</tr>
<tr>
<td>19</td>
<td>42,XX,+4,del(4)(q24.1q47),−17[5]</td>
</tr>
<tr>
<td>23</td>
<td>43,XX,add(3)p11,add(3)(p11),−4,der(5)(4;5)(q22;q10),−12,+3mar[5]</td>
</tr>
<tr>
<td>26</td>
<td>42–43,XX,del(6)(q24.1q32),−15,+19,+der(7)(4;7;11),hsr(4;11)[5]</td>
</tr>
<tr>
<td>Well to Moderately Differentiated</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>43,XX,der(16)(1;16)(q22q12.3),+19[5]</td>
</tr>
<tr>
<td>14</td>
<td>43,XX,+12[5]</td>
</tr>
</tbody>
</table>

In the well-differentiated adenocarcinoma (cell line 12), the predominant neoplastic elements were glands of various sizes (Fig. 1A). The glands were formed by a single layer of polygonal or cuboidal cells with eosinophilic, clear, or condensed cytoplasm and ovoid, alternating clear, or hyperchromatic nuclei, as well as isolated or short rows of "goblet" type epithelial cells with abundant mucous apical cytoplasm (mucicarmine positive) and compressed basal nuclei, giving an "enteric" appearance to the glandular epithelium. Numerous mitoses were observed. The tumor invaded the deep dermis, s.c. adipose tissue, and underlying skeletal muscle (Fig. 1, A and B).

The moderately differentiated tumor (cell line 14) was composed of a mixture of solid sheets, cords, or masses of neoplastic epithelial cells with scant fibrous stroma and scattered groups of small glands formed by epithelial cells similar to those of the well-differentiated group, including "goblet" or "signet-ring" mucous cells (Fig. 1C). Some of the neoplastic cells had a high nuclear/cytoplasmic ratio with scant eosinophilic cytoplasm and pleomorphic hyperchromatic nuclei, usually ovoid, with a prominent nucleolus. Mitoses were not numerous. Foci of necrosis and stromal hyalinization were observed (Fig. 1D).

The poorly differentiated tumors (cell lines 8, 9, 10, 19, 23, and 26), which represent the most frequent subtype observed, were formed by solid sheets, masses, and closely packed cords of undifferentiated epithelial cells that invaded the skin, s.c. adipose tissue, and underlying muscle. Fibroblastic or collagenous stroma was very scant (Fig. 1E), although foci of hyalinization were observed. The tumor cells appeared disorganized and pleomorphic and displayed ovoid or angular nuclei with one or more nucleoli, thick nuclear membrane, and scant pale cytoplasm (Fig. 1F). Although no well-formed glands were identified, occasional signet-ring-like cells...
Fig. 3. Karyotype of a G-banded metaphase spread from a poorly differentiated tumor produced in an athymic mouse following inoculation of cell line 9. The karyotype displays multiple cytogenetic changes (arrows).

were present among the generally undifferentiated sheets of malignant epithelioid cells.

Cytogenetic Features. Cytogenetic analysis revealed abnormal karyotypes in cells from every tumor (Table 2). The two more differentiated tumors (numbers 12 and 14) had only one or two cytogenetic changes. In tumor 14, trisomy 12 was the only alteration observed (Fig. 2). Tumor 12 had an extra copy of chromosome 19 and a der(16)(1;16)(q22;q12.3).

Each of the poorly differentiated tumors had complex karyotypes with five to eight clonal aberrations, except tumor 19, which showed only three abnormalities: +4, 4q−, and −17. An example of such a complex karyotype is shown in Fig. 3. Unbalanced rearrangements and numerical losses were found in every one of the poorly differentiated tumors. Three of these tumors had one or more marker chromosomes of unknown origin, and three tumors had either dmin (Fig. 4A) or hsr (Fig. 4, B and C). Two poorly differentiated tumors (numbers 9 and 23) had both near-diploid and near-tetraploid populations. In each case, the near-tetraploid subline represented a polyploid version of the near-diploid clone. Occasional metaphase spreads with even higher ploidies were also observed. Histological examination of tumors 9 and 23 revealed a prominent subpopulation of cells containing large nuclei.

DISCUSSION

The histopathological features of these rat ovarian surface epithelial tumors are similar to those observed in clinical human ovarian cancer. The data derived from this rat model of ovarian carcinoma suggest that chromosome abnormalities play an integral role in ovarian surface epithelial oncogenesis. Moreover, the degree of tumor differentiation parallels the cytogenetic complexity observed. Every one of the tumors examined displayed cytogenetic alterations, although none of the tumors from this larger series had losses of chromosome 5. The histopathological findings appear to correlate with the karyotypic pattern, with only simple changes being observed in well to moderately differentiated tumors and increased chromosomal complexity occurring in the less differentiated tumors. The two more differentiated tumors examined had only one or two cytogenetic changes, and they had in common the presence of numerical gains (i.e., +12 or +19). One of the more differentiated rat tumors (number 12) also had a der(16)(1;16)(q22;q12.3). However, this rearrangement may have originated early in cell culture, as this same abnormality was also found in several rat ovarian surface epithelial cell lines examined at subculture passage 5. The poorly differentiated tumors generally displayed complicated karyotypes with at least five clonal changes. The exception was tumor 19, which showed an intermediate karyotype with three chromosome abnormalities.

In humans, Pejovic et al. (10) observed simple karyotypic changes (i.e., numerical changes only or a single structural aberration) in 6 of 52 ovarian carcinomas. The remainder had complex karyotypes. Five of their six carcinomas with simple karyotypes were well differentiated, leading the authors to conclude that simple karyotypic changes are generally characteristic of less aggressive ovarian tumors. Our data from this rat model support this contention and indicate the potential relevance of this model to the study of clinical ovarian cancer. Furthermore, simple karyotypic changes appear to be much more common in benign epithelial tumors of the ovary than in ovarian carcinomas. For example, in one study, clonal abnormalities were identified in 7 of 42 benign ovarian tumors; all 7 cases with chromosome changes had simple karyotypes (37). Five cases had trisomy 12,
...some of which are indicated by arrows. B and C, partial metaphase spreads showing hsr (arrows) observed in tumor 8 (B) and tumor 26 (C). Insets in B and C show additional examples of the hsr-containing chromosomes from cells of these two tumors with brackets demarcating each hsr.

Fig. 4. A, portion of polyploid metaphase spread from tumor 9 showing numerous dmin, some of which are indicated by arrows. B and C, partial metaphase spreads showing hsr (arrows) observed in tumor 8 (B) and tumor 26 (C). Insets in B and C show additional examples of the hsr-containing chromosomes from cells of these two tumors with brackets demarcating each hsr.

including three in which this was the only change observed. Crickard et al. (38) reported trisomies of 2 and 7 as the only changes in a serous tumor of low malignant potential (“borderline”). Among 13 reported examples of the hsr-containing chromosomes from cells of the two tumors with brackets demarcating each hsr.

...in the poorly differentiated rat tumors. Such chromosome aberrations have been correlated with advanced disease (17, 42, 43). In ovarian cancer, sporadic amplification of KRAS (16, 21, 22), MYC (17, 19, 27), HRAS (19), ERBB2 (23, 24, 29), and AKT2 (30) has been reported. We intend to determine whether any of these genes is amplified in the hsr and dmin seen in our rodent tumors. Alternatively, it may be possible to identify the amplified DNA sequence by microdissection cloning.

Although the karyotypes observed in the poorly differentiated rat tumors are complex, many human ovarian carcinomas have even more cytogenetic changes than we observed in our rat model. Many of the changes observed in human ovarian carcinomas probably represent late events associated with tumor progression. In human ovarian cancer, there are often many unidentified markers, and the chromosome banding quality may be suboptimal. These obstacles have hindered efforts to identify primary cytogenetic changes that might have clinical relevance. Such problems appear to be less pronounced in our rodent model. Consequently, it may be possible to focus attention on a few chromosomes that are repeatedly altered in the rat tumors. While no single change has been consistently observed in our model, several chromosomes were altered in two or more tumors. For example, trisomy 19 and monosomy 17 were each observed in two tumors. Monosomy 16 occurred in one tumor, and partial loss of 16q due to a der(16)(q16) was observed in two others. Various rearrangements of chromosome 6, including two with breakpoints at band q16 (tumors 8 and 10), were found in three poorly differentiated tumors.

In conclusion, the experimental malignant tumors produced by these rat ovarian surface epithelial cell lines range in degree of differentiation which is paralleled by the complexity of the karyotype. Thus, this model system could facilitate efforts to define the genetic basis for many features of common epithelial tumors of the human ovary.

REFERENCES


2783


Spontaneous Transformation of Rat Ovarian Surface Epithelial Cells Results in Well to Poorly Differentiated Tumors with a Parallel Range of Cytogenetic Complexity

Joseph R. Testa, Lori A. Getts, Hernando Salazar, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/10/2778

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.