Histological Comparison of the Growth of Rat Bladder Carcinoma R-4909 Observed for Two Years in Vitro and in Vivo

Keiji Toyoshima², Nabil Abaza, and Joseph Leighton³

Cancer Bioassay Laboratory, Department of Pathology, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129

SUMMARY

A transplantable bladder tumor (Chapman R-4909) of the rat, when first received in our laboratory, grew with a complex histopathology. The predominant component was transitional cell carcinoma, but there were foci of keratinization, including pearl formation, and foci of a less well-defined cystic appearance.

We report here observations made during the first 2 years of an ongoing study on the divergent histopathology of R-4909 under several conditions of propagation. During the entire period, the tumor has been maintained by serial passage in rats (Fischer 344) and by serial passage in vitro. At intervals, cells of the tissue culture series were inoculated into rats to compare the histopathology of animal- and culture-passed strains. We obtained several clones from the stock cultures and these also were maintained continuously in vitro. At intervals, cells from two of these lineages, clone A and clone B, were inoculated into rats.

After 2 years, cells maintained in stock culture, on injection into new rats, produced growths similar to the original in that all three epithelial patterns, transitional, squamous, and adenomatous, were perpetuated. In contrast, the tumor passed exclusively in vivo lost its squamous component completely. It became anaplastic, with tissue architecture almost entirely adenomatous and cystic. Unlike the stock tissue culture line, the clonal isolates following prolonged culture produced adenomatous tumors only.

In a related preliminary study, we inoculated into rats R-4909 cells that had been cultivated for up to 2 months under aerobic and anaerobic conditions. Tumors grew in most of the animals, and those of the aerobic group were more cystic than the others.

INTRODUCTION

Neoplasms arising in urothelium appear variously as transitional cell carcinoma, squamous cell carcinoma, or adenocarcinoma. In the United States, most bladder cancers are of the papillary transitional cell type; in Egypt, where bladder cancer is the most common tumor of men, most are squamous cell carcinomas. Adenocarcinomas are least frequent and often have, conceptually and clinically, an association with abnormalities of development (1). Tumors that are predominantly transitional cell carcinoma may have foci of squamous features or of gland-like structures, or both. Identification of the developmental mechanisms whereby such histotypic aberrations arise may be an academic exercise, since these foci are not now believed to be clinically significant.

For controlled laboratory study, transplantable urothelial carcinomas of laboratory animals and continuous cell lines derived from rodent and human bladder carcinomas provide the closest available approximation to bladder cancer in the patient. We have found that a squamous cell carcinoma of the rat bladder, NBT-II, produced abundant keratin pearls in control medium, but grew with complete inhibition of keratin formation in the presence of low concentrations of vitamin A (6). At the time the vitamin A studies were initiated, we undertook some baseline studies of a microscopically more complex transplantable rat tumor, the Chapman R-4909. This tumor, when first received from Dr. Chapman, was predominantly of the transitional cell type, but with foci of keratinization, including pearl formation, and foci of a less well-defined cystic appearance. The tumor grows readily either on transplantation in Fischer 344 rats or on cultivation in vitro. It can be easily propagated indefinitely in culture and in laboratory rats.

We have undertaken a series of studies on R-4909 in order to understand better the mechanisms responsible for, and the significance of the several morphological patterns of, the tumor. In this 1st report, we describe the morphological drift, over a 2-year period, of Chapman R-4909 in 2 situations: (a) continuous passage in rats and (b) consecutive passage in culture. Cultured cells were evaluated by inoculating them into rats and studying the histopathology of the resulting tumors. We also explored the possibility that divergence in histopathology might appear when inocula in rats were selected from the aerobic and anaerobic parts of meniscus-gradient cultures after 2 months in vitro.

MATERIALS AND METHODS

Animals. Fischer 344 male rats, weighing approximately 150 g each, were obtained from the NIH. All rats were placed...
in individual hanging stainless steel cages and fed Purina laboratory chow diet and tap water ad libitum.

**Cancer Cells (R-4909) in the Rat.** Dr. Warren H. Chapman (Department of Urology, University of Washington, Seattle, Wash.) graciously provided us, in February 1973, with carcinoma line R-4909 (7) as a s.c. tumor growing in a Fischer 344 rat. Since then, the tumor has been maintained continuously in the same strain of rats. About every 1 to 3 months, excised tumor tissue was finely minced and injected s.c. into new rats.

**Cancer Cells in Culture.** Continuous passage in tissue culture was initiated from a s.c. transplant in the rat in April 1973. The medium used for the initial tissue culture passages consisted of 30% fetal calf serum in Waymouth’s MB 752/1 medium (Microbiological Associates, Inc., Bethesda, Md.) containing L-glutamine (2 mm), kanamycin (50 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and Fungizone (1 μg/ml). About 6 months later, the basic commercial medium was changed to Eagle’s MEM (Microbiological Associates, Inc., Bethesda, Md.), since we found that the quality and rate of growth were the same with either one. As a further economy, we reduced the concentration of serum in 2 steps using, in the initial preparation of subcultures, 20% fetal calf serum in Eagle’s MEM immediately after suspension with EDTA-trypsin (Grand Island Biological Co., Grand Island, N. Y.). Once adhesion to glass had been observed in the subculture bottles, we used 5% fetal calf serum in Eagle’s MEM to support further growth. During the transition period, we saw minimal differences in the appearance of the cells between those fed on Waymouth’s and Eagle’s medium, was cut away and discarded. The remainder was inoculated s.c. into male Fischer rats, 1 to 2 x 10^6 cells/rat. This same number of cells was used for each culture, but in this case the cells were suspended in 2 ml of medium with serum. All cultures were incubated at 36° in a roller drum at a near horizontal position. After confluency was attained, some cultures were moved to a stationary vertical position to produce a gradient, while others as controls remained in the slowly moving roller drum. Depleted medium was replaced with 2 ml of fresh medium, as required by changes in pH. In the vertical cultures, 10 mm of the Gelfilm protruded above the surface of the medium, and 15 mm were immersed. The Gelfilm coverslips were removed and prepared for inoculation into rats at 4, 6, and 8 weeks. One or 2 cultures from each group at each of the 3 intervals were fixed in formalin and stained with hematoxylin and eosin.

**Cell Cloning.** A 2-step cloning method was devised. Plastic coverslips, 0.5 x 3.0 cm, (LUX Scientific Corp., Thousand Oaks, Calif., distributed by Microbiological Associates, Inc., Bethesda, Md.) were cut aseptically with sterile pinking shears to produce serrations on both edges of the plastic rectangle. Cultures were dispersed with EDTA-trypsin, and the cells were suspended in 20% fetal calf serum-Eagle’s MEM at a concentration of approximately 10^5 cells/ml. One ml of this dilute cell suspension was inoculated into each of a series of small Leighton tubes containing a serrated plastic coverslip. The tubes were incubated overnight, and the coverslips were then transferred to fresh tubes with fresh medium. The serrated coverslips were carefully examined with an inverted microscope with phase optics to identify single cells attached in complete isolation from others on the surface of one of the points. These were carefully mapped. Colony formation starting from single cells was observed daily under the microscope.

As the 2nd step in the procedure, a segment of the coverslip bearing a single isolated colony was cut off with scissors and placed in a Petri dish containing 25 ml of 20% fetal calf serum-Eagle’s MEM. Each dish was kept in a humidified box of 5% CO₂ in air at 37° until the single colony had produced isolated daughter colonies in the Petri dish. With the use of capillary pipets under phase contrast observation, single colonies were picked up and each was placed in a fresh tube to provide the population of cloned lines.

**Meniscus-Gradient Cultures on Gelfilm.** Early in 1974, after 8 weeks in meniscus-gradient culture on glass coverslips, a pattern of zonation was seen in stock cultures of the Chapman line that was unique in our experience with gradient cultures of several bladder carcinoma cell lines (3, 4). After 2 months, the cells closest to the gas phase consisted mainly of mononucleated tumor giant cells. We decided to inoculate separately cells from the aerobic and anaerobic parts of the gradient cultures into the s.c. tissue of rats.

To accomplish this, the substrate used in the gradient cultures was flexible gelatin film measuring 35 x 25 mm (Gelfilm; Upjohn Co., Kalamazoo, Mich.). Immediately before use, each Gelfilm rectangle was moistened in Hanks’ balanced salt solution, placed in a 150- x 50-mm standard round culture tube, and positioned against the side of the tube at the closed end. The cultures were fed on 15% fetal calf serum in Waymouth’s MB 752/1 medium. Cells from stock cultures were dispersed with EDTA-trypsin. From this pool of cells, some, for purposes of control, were suspended in Waymouth’s medium without serum and injected s.c. into male Fischer rats, 1 to 2 x 10^6 cells/rat. This same number of cells was used for each culture, but in this case the cells were suspended in 2 ml of medium with serum. All cultures were incubated at 36° in a roller drum at a near horizontal position. After confluency was attained, some cultures were moved to a stationary vertical position to produce a gradient, while others as controls remained in the slowly moving roller drum. Depleted medium was replaced with 2 ml of fresh medium, as required by changes in pH. In the vertical cultures, 10 mm of the Gelfilm protruded above the surface of the medium, and 15 mm were immersed. The Gelfilm coverslips were removed and prepared for inoculation into rats at 4, 6, and 8 weeks. One or 2 cultures from each group at each of the 3 intervals were fixed in formalin and stained with hematoxylin and eosin.

In preparing gradient cultures for inoculation into rats to observe the possible formation of tumor, the uppermost portion of the Gelfilm, the part that had protruded above the medium, was cut away and discarded. The remainder was cut into the aerobic and anaerobic parts, each about 7 mm high. Each segment was inoculated s.c. into the back of a rat. In the controls, the horizontal cultures, the Gelfilm was cut into segments of similar size, and pieces were similarly inoculated into rats.

After either 6 or 12 weeks, rats bearing tumors were killed and the tumors were examined. Under comparison was the morphology of tumors produced following inoculation of cells from the donor stock bottles; from the Gelfilm pieces after 4, 6, and 8 weeks in control horizontal culture; and from the upper and lower segments of the Gelfilm in gradient culture after the same intervals.

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*The abbreviation used is: MEM, minimal essential medium.*
RESULTS

Tumor as Received from Dr. Chapman. The tumor, located s.c. in the back of the rat, appeared to be surrounded by a thin fibrous capsule but was growing invasively. No metastases were found. The tumor was composed primarily of many elongated masses of transitional cell carcinoma in which the cancer cells were in no particular pattern. Necrosis was scanty. The cells at the periphery of the masses had a palisade arrangement. Stroma separating the islands of tumor cells was cellular, and newly formed fine collagen was prominent. Some of the solid masses of tumor cells had foci of squamous features, including keratinization. In other areas, but very infrequent, were cystic formations. Typical well-defined adenomatous patterns were not observed (Fig. 1).

Appearance in Tissue Culture. The tumor taken from the rat grew in vitro in a predominantly epithelial pattern. During 2 years of growth in continuous culture, we observed the consistent presence of randomly distributed mononucleated tumor giant cells and the infrequent occurrence of multicellular epithelial hemicystic structures. The latter resembled structures seen previously in a dog kidney cell line (2). Hemicysts were observed with greatest frequency in 1 clonal population, clone B. Details on the appearance of the cultures through time in the parent line and in the clones, and, in particular, the conditions of cultivation in which bladder carcinoma hemicysts were seen with high frequency, are described in the 2nd paper of this series (5). In clone B, a coincidence of hemicysts in vitro and of adenomatous features in vivo was observed as time passed. We think that they are related.

Appearance after Serial Passage in Rats. There were no discernible changes when the original tumor was compared with growth after the 1st few passages. After 18 months (the 12th passage), the tumor cords, columns, and masses were larger and more irregular than in the donor tumor. Squamous foci were very infrequent, but cystic changes had increased. These changes were observed in many masses of tumor cells with and without accompanying necrosis. Distinct adenomatous changes were also found.

After 28 months and 17 passages, the histopathology of the tumor was further removed from the donor tumor. Squamous changes could not be identified. Much of the tumor consisted of a relatively haphazard arrangement of atypical and anaplastic transitional cells. Zones of tumor necrosis were large and numerous. Adenomatous foci were very frequent, and some were highly organoid (Fig. 2; Table 1).

Inoculation into Rats of Cells from Horizontal Monolayer Cultures. When cells that had been in culture for 3 months were inoculated into rats, the tumors that developed were predominantly of transitional cell type. Distinct foci of squamous epithelium with keratinization and of adenomatous growth were also found. After 5 months, the 3 patterns of the tumor appeared in equal amounts. After longer periods, the squamous pattern was more prominent and mature keratin became a regular feature. This was evident after 17 months and even more so after 26 months (Fig. 3; Table 2).

The tumors produced following injection of clonal isolates differed from those seen following injection of the stock strain, especially after 2 years. Clone A and clone B, injected in rats 2 months after their isolation, produced tumors of mixed morphology similar to those seen from the

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### Table 1

<table>
<thead>
<tr>
<th>Histological changes in R-4909 maintained by passage from rat to rat</th>
<th>Changes at mo. following receipt of tumor-bearing rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajossilated pattern</td>
<td>Mo. 0</td>
</tr>
<tr>
<td>Transitional cell</td>
<td>++*</td>
</tr>
<tr>
<td>Squamous</td>
<td>+</td>
</tr>
<tr>
<td>Keratinized squamous</td>
<td>±</td>
</tr>
<tr>
<td>Cystic or papillary</td>
<td>-</td>
</tr>
<tr>
<td>Adenomatous</td>
<td>-</td>
</tr>
<tr>
<td>No. of rats</td>
<td>1</td>
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</table>
* ++, prominent; +, present; ±, rare; -, none.

### Table 2

<table>
<thead>
<tr>
<th>Histological changes in R-4909 inoculated into rats after being maintained in vitro</th>
<th>Changes at mo. in when inoculated into rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent line</td>
<td>Clone A*</td>
</tr>
<tr>
<td></td>
<td>Mo. 3</td>
</tr>
<tr>
<td>Transitional cell</td>
<td>++*</td>
</tr>
<tr>
<td>Squamous</td>
<td>+</td>
</tr>
<tr>
<td>Keratinized squamous</td>
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<td>Cystic or papillary</td>
<td>+</td>
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<tr>
<td>Adenomatous</td>
<td>+</td>
</tr>
<tr>
<td>No. of rats</td>
<td>6</td>
</tr>
</tbody>
</table>
* Clones A and B each arose from the parent line when the latter had been in culture 17 months.
* See explanation of symbols in Table 1.

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stock line. After 9 months in culture following cloning, a total of 26 months in vitro, tumors produced in rats by the cloned lines were almost exclusively adenomatous (Fig. 4; Table 2).

Segments of Meniscus-Gradient Cultures on Gelfilm Inoculated in Rats. In fixed stained preparations of horizontal and vertical Gelfilm cultures, tumor giant cells were distributed in a random pattern. Even after 8 weeks no concentration of giant cells just below the gas phase was seen on the gelatin substrate, in contrast to our consistent observation of such concentrations near the gas phase when the cells grew on glass coverslips.

Tumors grew in most of the inoculated animals. 26 of 31 from the aerobic segments and 17 of 28 from the anaerobic. The tumors were allowed to reach a large size, to the point of ulceration of the skin, before the animals were killed and the tumors examined. In gross examination of cross-sections of the tumors, areas of necrosis and foci of fluid-filled cysts were seen. No metastases were found either on gross examination or on histological examination of various organs.

Microscopically, the tumors from the gradient were different from the original donor tumor. They showed a decrease in the predominance of the transitional cell pattern, a slight increase in squamous areas, and a greater increase in cystic glandular areas. Cells from the aerobic and anaerobic ends of the gradient gave rise to tumors with similar mixtures of the 3 patterns, except that there was a greater proportion of cystic foci in the tumors derived from aerobic inocula of 8-week-old gradient cultures.

DISCUSSION

The data presented here indicate that the Chapman tumor is a worthy candidate for study of the determinants and regulators of histopathological identity of bladder cancer. Four populations of carcinoma cells, all derived from a single mass of transplantable tumor, were maintained independently for more than 2 years. Divergence of pattern was observed after long intervals when rats received inocula from each population. The most prominent one was the divergence between stock lines passed in vitro. We wonder whether the observations would be the same if 10 sets of the tumor were propagated in vivo and 10 in vitro, all from a common ancestor. When the stock line was fed on an enriched medium and cultured for up to 8 weeks in a meniscus gradient, the differences between tumors produced in rats by inocula from the anaerobic and aerobic parts of the culture were minimal, consisting of a slight accentuation of cystic patterns in the aerobic group. No interpretation of this divergence is warranted, but it is of interest that, in gradient cultures of another cell line, MDCK, the formation of prominent cystic structures in vitro was enhanced when meniscus gradient cultures were grown where 95% O₂ was compared with air in the gas phase (4).

Two mechanisms are usually proposed to explain the growth of a tumor as a combination of transitional cell, squamous cell, and adenocarcinoma. One is that the differences reflect areas of metaplasia in a tumor that is basically a transitional cell carcinoma. The other explanation is that they are the products of growth by different populations of stem cells. Metaplasia is a presumptive factor, since vitamin A readily inhibits keratinization of another rat bladder cancer (6). These 2 views in ensemble may not adequately explain the different histopathological expressions we observed in clonal isolates. Both clonal lines went through a period when they evoked histopathological components in vivo, yet at a later time were completely free of these components. Clone B finally produced an adenomatous picture in vivo and numerous hemicysts in vitro. Our current work is focusing on this adenomatous variant of the Chapman tumor and its proclivity for forming hemicysts.

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REFERENCES


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