Bladder Cancer Xenografts: A Model of Tumor Cell Heterogeneity

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ABSTRACT

Twenty bladder biopsies from patients with primary transitional cell carcinoma were inoculated into nude mice. To date, eleven of these have grown as primary implants and three serially transplantable xenograft lines (UCRU-BL-12, UCRU-BL-13, UCRU-BL-14) have been established. The histological and ultrastructural features of human transitional cell carcinoma have been maintained in each line. Despite a relatively uniform histological appearance, several indices of occult tumor heterogeneity have been revealed. Immunocytochemical staining was negative for β-subunit human chorionic gonadotrophin but positive for carcinoembryonic antigen only in areas of squamous differentiation. All three tumors bound peanut lectin. Flow cytometric DNA analysis of UCRU-BL-13 showed multiple aneuploid peaks, separate populations being demonstrated in different xenografts of the same generation. However, the morphologies of these tumors remained identical. On initial implantation UCRU-BL-12 and UCRU-BL-14 were near diploid but aneuploid populations became apparent with increasing passage number. Each xenograft line caused cachexia in the host mice. Treatment with the cisplatin analogue, isopropyl platinum, ameliorated the cachexia disorder in mice carrying UCRU-BL-14 but did not cause tumor regression. UCRU-BL-12, when tested with cisplatin, isopropyl platinum, and carboplatin, showed equivalent growth retardation with each drug. These xenografted human bladder cancers may be useful models for the study of heterogeneity of the tumor populations in bladder cancer and for the evaluation of new approaches to treatment.

INTRODUCTION

An important problem in the management of patients with bladder cancer arises because of the differences in the natural history and responses to treatment of tumors. In many instances, these variations can be attributed to the histological cell type (transitional cell carcinoma, adenocarcinoma, squamous cell carcinoma, or combinations of these occurring within one tumor); frequently, however, tumors with identical features respond differently, perhaps reflecting the heterogeneity of the constituent tumor cells.

Heterotransplantation of various human tumors into immunosuppressed hosts has been used to generate tissue for the study of aspects of tumor biology, such as patterns of growth and differentiation, the expression of tumor antigens, and response to treatment (1–3).

To assess the utility of this model for the study of the diversity of bladder cancer cells, we have established a series of xenografted primary bladder cancer lines and have characterized them extensively, relating light morphology to ultrastructure, tumor marker production, and DNA flow cytometry. We report the characteristics of three of these lines.

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3 Abbreviations used are: TCC, transitional cell carcinoma; CEA, carcinoembryonic antigen; β-HCG, β subunit, human chorionic gonadotrophin; PNA, peanut lectin; FCM, DNA flow cytometry; RoPNA, rabbit anti-PNA.

MATERIALS AND METHODS

Patients. The details of the patients whose tumors yielded initial "takes" are listed in Table 1. In each case, the biopsied tumors were primary grade 1–III transitional cell carcinomas, sometimes with regions of squamous or glandular differentiation. The majority of patients had not received prior radiotherapy or cytotoxic drug treatment. Both superficial and invasive tumors were represented.

Mice. Male BALb/c nu/nu ("nude") mice, aged 8–12 wk, were obtained from the Australian Atomic Energy Commission, Lucas Heights, New South Wales, Australia, and were housed in standard cages fitted with filter tops and handled in laminar flow hoods. They were fed irradiated standard mouse diet and sterile acidified water ad libitum.

Bladder Xenografts. Biopsies from twenty patients with primary TCC were transported immediately from the operating theatre and were minced finely. Tumor fragments of approximately 2 mm³ were inoculated s.c. through small incisions in the scapular regions. When tumor nodules reached 5–10 mm in diameter they were removed from host mice and 2-mm³ fragments were serially transplanted after dissection to remove stromal capsule and necrotic tissue. Tumor growth was assessed at weekly intervals starting when the tumor was approximately 5 x 5 mm, measuring with calipers 2 diameters (d₁, d₂) at right angles to each other. The tumor volume was estimated from the formula

\[ V = \frac{\pi}{6} (d_1 \cdot d_2)^{3/2} \]

Mean doubling times were calculated from a semilogarithmic plot of volume versus time.

Light and Electron Microscopy. Specimens for light microscopy from the original tumors and xenograft passages were fixed in buffered 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin, periodic acid-Schiff plus diastase, mucicarmine and Alcian Blue for mucin. Tissue blocks of xenograft specimens of approximately 1 mm³ were fixed in cacodylate-buffered 2.5% glutaraldehyde and processed for electron microscopy as previously described (4).

FCM. The cellular DNA content of specimens of the original tissue and of xenografts was measured by flow cytometry. Tissue specimens were mechanically disaggregated in a staining solution (5), containing propidium iodide (Calbiochem-Behring Corp., San Diego, CA) at a final concentration of 50 μg/ml in 5% v/v Triton X-100 (Packard Instrument Co., Downers Grove, IL), with RNase, 1 mg/ml (Calbiochem-Behring Corp.) in 0.9% NaCl. Freshly prepared and washed chicken erythrocytes were added at a concentration of 3 x 10⁶/ml as an internal DNA standard. The cell suspension was then filtered through an 85-μm nominal pore size nylon mesh (Henry Simon, Sydney, Australia).

FCM analysis of total DNA content per cell was carried out using a Cytovorouorograf System 50H cell sorter (Ortho Instruments, Westwood, MA) with an argon ion laser excitation source at 488 nm. A minimum of 5 x 10⁶ cells were counted and the resultant frequency distribution histograms of DNA content were analyzed as described by Hillen et al. (6). The number of cells in the DNA S phase of the cell cycle could only be determined in specimens which did not have aneuploid cells present. The DNA content of aneuploid populations was expressed as a proportional increase in DNA over the diploid (2n) level. When insufficient fresh material was available for analysis, FCM measure-
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Table 1  Patient details, histological findings, and DNA flow cytometry profiles of tumor biopsies which grew in nude mice

<table>
<thead>
<tr>
<th>Urological Cancer Research Unit BL line</th>
<th>Current passage no.</th>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Prior treatment</th>
<th>TCC grade</th>
<th>Stage</th>
<th>FCM profiles</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ploidy % G0/G1 cells*</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>M</td>
<td>73</td>
<td>Nil</td>
<td>I</td>
<td>T1</td>
<td></td>
<td>2.4n 80</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>M</td>
<td>55</td>
<td>Thiopepa; radiotherapy</td>
<td>II</td>
<td>T2</td>
<td>2.8n 20</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>M</td>
<td>62</td>
<td>Nil</td>
<td>II</td>
<td>T3</td>
<td>2n</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>M</td>
<td>55</td>
<td>Nil</td>
<td>II—IIIa</td>
<td>T1</td>
<td>2n—2.9n 70</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>M</td>
<td>63</td>
<td>Cisplatin III</td>
<td>II</td>
<td>T1</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>M</td>
<td>43</td>
<td>Nil</td>
<td>II</td>
<td>T1</td>
<td>2n</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>F</td>
<td>69</td>
<td>Nil</td>
<td>III†</td>
<td>T1</td>
<td>2n</td>
<td>90</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>M</td>
<td>56</td>
<td>Nil</td>
<td>II</td>
<td>T1</td>
<td>2n</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>F</td>
<td>85</td>
<td>Nil</td>
<td>III</td>
<td>T1</td>
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<td>F</td>
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<td>II</td>
<td>T1</td>
<td>2n</td>
<td>52</td>
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<tr>
<td>21</td>
<td>2</td>
<td>M</td>
<td>75</td>
<td>Radiotherapy</td>
<td>II—III</td>
<td>T1</td>
<td>Not determined</td>
<td></td>
</tr>
</tbody>
</table>

* Percentage level of each ploidy.
† Plus signet ring adenocarcinoma.
§ Plus squamous and glandular carcinoma.

ments were carried out on nuclei from paraffin embedded material as described elsewhere (7).

Tumor Markers. Immunoperoxidase staining for CEA, β-HCG, and PNA was carried out using the unlabeled antibody enzyme method (peroxidase antiperoxidase) (8) on dewaxed paraffin sections. Antibodies to CEA and β-HCG and conjugates were obtained from Dakopatts, Copenhagen, Denmark. PNA and rabbit anti-PNA were obtained from Sigma Chemical Co. For PNA binding, sections were incubated for 30 min in phosphate-buffered saline with PNA, 25 μg/ml, then for 30 min in RNase (diluted 1/6000). This was followed by sequential application of sheep anti-rabbit immunoglobulin and peroxidase antiperoxidase. Peroxidase activity was revealed using diaminobenzidine (Sigma); the counterstain was Harris’ hematoxylin.

PNA staining was also carried out following treatment with neuraminidase (PNA binding after neuraminidase treatment from Clostridium perfringens; catalogue No. N2876, Sigma) to remove terminal sialic acid residues. Briefly, dewaxed paraffin sections were flooded with neuraminidase (0.2 units/ml in phosphate-buffered saline), for 30 min at 37°C, washed and stained for PNA.

Cytogenetic Analysis. A 72-h harvest was carried out on cells cultured in liquid suspension in siliconized McCartney bottles at 1 × 10⁵/ml in RPMI 1640 with 10% fetal calf serum and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. Cells were treated with hypotonic potassium chloride for 20 mm and fixed with Carnoy’s fixative (9). Metaphase spreads were G-banded by a modified method of Seabright (10) and cytogenetic analysis was performed according to the Paris convention (11).

Drug Treatment. The maximum tolerated dose for each cytotoxic drug was administered i.p. Cisplatin (Platinol; Briston Laboratories) was used at a dose of 2.5 mg/kg, carboplatin at 50 mg/kg, and isopropyl platinum at 25 mg/kg (provided by Dr. Ken Harrap, Institute of Cancer Research Sutton, England). Drugs were administered weekly for 3 wk, the first treatment being given when tumors measured 5 × 5 mm. The initial dose of cytotoxic drug was administered at this small size to avoid the problems of interpretation of increasing spontaneous necrosis with increased tumor size.

RESULTS

Establishment of Xenografts. To date, 11 of 20 specimens have grown as primary implants in nude mice, and 3 serially transplantable xenograft lines have been established. The details of patients and tumors are shown in Table 1. Both superficial and invasive TCCs yielded xenografts, although all lines were derived from tumors of Grade II—III differentiation.

The xenografts had lag periods of 4—40 wk before demonstrable initial “takes” were documented, and in the exponential growth phase, doubling times were in the range of 7—11 days. To date, doubling times for each line have been stable during serial passages after the initial “take.” Once established as stable lines, high transplantable take rates have been regularly achieved (>80%). Spontaneous rejection of xenografts was not seen in these tumor lines. Most of the xenografts grew as solid s.c. masses of uniform consistency. In one line (UCRU-BL-13) the xenografts grew as a solid tumor containing fluid-filled sacs. No macroscopic evidence of invasion was seen and metastases were not demonstrated. Each of the serially transplantable lines caused cachexia in the recipient mice, documented clinically and histologically, by analysis of fat pads in the scapular region.

Human karyotypes were demonstrated for the three transplantable lines UCRU-BL-12, -13, and -14. UCRU-BL-13 demonstrated a modal number of 63 chromosomes and several marker chromosomes were present although too distorted for detailed analysis. Abnormal human karyotypes were also seen in UCRU-BL-12 in short-term cultures. Although not all marker chromosomes could be identified, a deleted 6q clonal abnormality was observed.

Light and Electron Microscopy. The histology of the original tumors is compared with that of the xenografted passages in Table 2 and Fig. 1. Each of the transplantable lines has retained the dominant features of TCC, grade II—III. In the case of UCRU-BL-14, the original tumor showed juxtaposition of a TCC, grade II—III and a signet ring adenocarcinoma; only the TCC has been expressed in the xenografts. Although the urothelial tumors appeared to maintain the same histological grade through serial passages, new patterns of somatic differentiation were sometimes seen to emerge in later passages. For example in UCRU-BL-12, small squamous foci were seen; in UCRU-BL-13 some areas of glandular tissue with mucin production became evident. However, no outgrowth of dominant elements other than TCC has been demonstrated.

The ultrastructural findings confirmed those of light microscopy (Table 2). By electron microscopy, the majority of cells had the features of intermediary transitional cells (12) (Fig. 1B). The junction complexes, asymmetrical membrane, ridges,
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Table 2  Morphology and cytochemical staining of original tumors and xenografts derived from them

<table>
<thead>
<tr>
<th></th>
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<th>Light microscopy</th>
<th>Immunoperoxidase staining</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TCC grade</td>
<td>Squamous</td>
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<tr>
<td>UCRU-BL-12</td>
<td>Original</td>
<td>II</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>II</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>II</td>
<td>+ focal</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>P4</td>
<td>II</td>
<td>—</td>
<td>—</td>
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<td></td>
<td>P5</td>
<td>II</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>P6</td>
<td>II</td>
<td>+ focal</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>UCRU-BL-13</td>
<td>Original</td>
<td>II</td>
<td>+ focal</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>P6</td>
<td>II</td>
<td>+ focal</td>
<td>+ focal</td>
<td>+ focal</td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>III</td>
<td>+ focal</td>
<td>+ focal</td>
<td>+ focal</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>III</td>
<td>+ focal</td>
<td>+ focal</td>
<td>+ focal</td>
</tr>
<tr>
<td>UCRU-BL-14</td>
<td>Original</td>
<td>II</td>
<td>—</td>
<td>—</td>
<td>+ focal</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>II</td>
<td>—</td>
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</tbody>
</table>

* Periodic acid-Schiff plus diastase, mucicarmine, and Alcian Blue.
# UCRU-BL-12 was also stained with PNA after neuraminidase treatment. Increased intensity of staining was observed without change in cellular distribution.
^ C. surface; G. cytoplasm; Sq. squamous; G. glandular; P. passage; subscripts i, iii, xenografts from different mice of the same passage number.

and deep vesicles characteristic of superficial cells (12) were not seen. However, in areas of cystic degeneration (UCRU-BL-13), the lining cells sometimes possessed stumpy microvilli with a poorly developed glycocalyx. At the periphery of tumor nodules, the cells generally showed an undulating contour lacking any evidence of surface specialization or the “reversed polarity” described by Tannenbaum (12). An external lamina was absent or at best fragmentary, the neoplastic cells being walled off by a layer of fibroblasts from which they were separated by a thin band of collagenous stroma. In a few instances, macrophages were seen in close contact with tumor cells.

The cytoplasm contained the usual organelles (12—14) (Fig. 1B) including prominent perinuclear filaments and patches of glycogen. Lysosomes and autophagic vacuoles were present in some cells and erythrophagocytosis was sometimes seen in areas of haemorrhage. Poorly differentiated cells (Table 2) often had a comparatively clear cytoplasm containing profuse free ribosomes and polyribosomes, glycogen particles, but few other organelles.

Squamous differentiation (Table 2) was generally apparent only in isolated cells (Fig. 1D). Such elements contained interlacing bundles of tonofilaments and tonofilament-desmosome complexes were common. Obvious extracellular lumina limited by junction complexes as well as so-called intracellular lumina or crypts (Fig. 1F) were seen in areas of glandular differentiation (Table 2). The lumen was lined by microvilli with filamentous cores and covered by an obvious glycocalyx, and frequently contained electron opaque secretory material. Small mucin granules were sometimes seen in the bordering cytoplasm.

No retrovirus particles were seen in any of the material examined.

Tumor Markers. The results of immunocytochemical staining for CEA, β-HCG, and PNA carried out on each xenograft passage and on the original tumor biopsies are summarized in Table 3. There was considerable variability of tumor ploidy with differences emerging in serial passages. In the early passages of UCRU-BL-12 and UCRU-BL-14, a pure diploid complement was found, with an aneuploid population being demonstrated in later passages.

In UCRU-BL-13, multiple aneuploidy was present in the biopsy material; different subpopulations, each present in the original, were expressed in different initial implants, yet these tumors had similar histological patterns (each TCC grade II with some squamous and glandular differentiation).

Treatment of Xenografts. As shown in Figure 2, two of the xenograft lines were used for a comparative evaluation of the antitumor activity of cisplatin, isopropyl platinum, and carboplatin. UCRU-BL-12 appeared to be markedly sensitive to cisplatin and both analogues, with clear evidence of growth delay and tumor necrosis. By contrast, neither platinum nor its analogues caused any growth inhibition for UCRU-BL-14; however, the cachexia of tumor-bearing mice was ameliorated by treatment with isopropyl platinum. This was confirmed histologically; adipose tissue was found s.c. only in treated mice.

In UCRU-BL-14 there was no evidence of damage to the tumor on light or electron microscopy. However flow cytometry demonstrated the apparent loss of an aneuploid peak in UCRU-BL-14 after treatment with isopropyl platinum.

DISCUSSION

The extent of occult heterogeneity present within morphologically similar tumors may explain the diverse natural history of bladder cancer. Detailed study of the biology of this disease has been restricted somewhat by the paucity of tumor tissue available from clinical practice for experimental study, and by the limitations of the available tumor models (15). To overcome some of these problems, the xenograft model has been applied to the study of TCC of the bladder, with emphasis to date including establishment and validation of the model (16, 17), the study of chromosomal patterns (18), the radiobiology of bladder cancer (19), and drug-radiation interactions (20). Most of these studies, however, have emphasized the similarities of the constituent tumor populations. To explore the possibility...
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that occult heterogeneity present within morphologically similar tumors may explain the diverse natural history of this disease, we have studied a series of xenografts of similar morphology, comparing such features as ultrastructure, DNA complement, tumor marker production, and sensitivity to cytotoxic drugs.

The consistency of the histological patterns seen in the original tumors and in serial passages of their xenografts (Table 2) supports the validity of this model for future studies, while the emergence of foci of squamous and glandular differentiation with increasing passage parallels the clinical experience. Xenografts derived from superficial or invasive TCC, when grown in the s.c. plane, exhibit similar morphology, which could imply an inherent biological similarity between superficial and invasive bladder cancer or may reflect selection of tumor cells able to grow in an alien environment. This will be the subject of further studies in our laboratory.

The emergence of aneuploid populations in both of the diploid lines, UCRU-BL-12 and UCRU-BL-14, on serial passage (Table 3) may have reflected a natural evolution of the tumors or may have been due to selection of preexisting populations with aneuploid content that were masked in the original samples. Whether prior treatment with intravesical thiotepa or with radiotherapy (Table 1) could have had an impact on the ploidy of UCRU-BL-12 is not clear. However, we believe this to be unlikely because the xenografts remained diploid through the first 3 passages (over more than 12 mo). The finding of subpopulations expressing different degrees of aneuploidy in each of the initial implants from the invasive tumor, UCRU-BL-13, suggests the outgrowth of different cells from a hetero-

geneous tumor. The ability of tumors to grow as xenografts in nude mice did not appear to correlate with their DNA profiles (Table 1). The presence of multiple aneuploidy in the invasive tumor, UCRU-BL-13, and the diploid profiles of the superficial tumors, UCRU-BL-12 and -14, are consistent with other studies relating DNA flow cytometric profiles to the natural history of bladder cancer (21, 22). This also correlates with the prognostic significance and the presence of marker chromosomes in cytogenetic studies of bladder cancer (23).

Our immunocytochemical studies suggested that the tumors were composed of subpopulations of functionally dissimilar cells. None of the xenografts expressed HCG. CEA binding correlated with the foci of squamous differentiation as reported in clinical studies by others (24). Similarly, peanut lectin bound to only some of the cells of each transplantable line. PNA binds to the T-antigen, a precursor of the human blood group MN glycoprotein (25), which is sialylated on normal urothelial cells. There is some controversy with regard to the correlation between the expression of T-antigen and invasiveness of bladder cancer (26, 27). Nevertheless, the variable staining pattern represents another index of the occult heterogeneity of these tumors.

The marked differences in sensitivity to cisplatin and analogues between UCRU-BL-12 and UCRU-BL-14 reflect the experience in clinical practice using i.v. cisplatin (28). The apparent sensitivity of UCRU-BL-12 to cisplatin and analogues, contrasted with the cross-resistance demonstrated in UCRU-BL-14, will be further examined in our other xenograft lines. The reduction in cachexia with UCRU-BL-14 in the absence of tumor growth retardation, and the loss of an aneuploid population after treatment with isopropyl platinum is not yet understood and will be further studied.

In summary, we have established and characterized three xenograft lines of primary bladder cancer and have demonstrated that each xenograft reflects the features of its human tumor of origin. In addition, eight other tumors have yielded primary lines. The reduction in cachexia with UCRU-BL-14 in the absence of tumor growth retardation, and the loss of an aneuploid population after treatment with isopropyl platinum is not yet understood and will be further studied.

ACKNOWLEDGMENTS

We thank Dr. Ken Harrap, Institute of Cancer Research, Sutton, England for generously providing supplies of carboplatin and isopropyl platinum. Sally Pittman performed karyotypes on the xenografted material and Stewart Davies provided excellent technical assistance in immunocytochemical staining. We also thank our surgical colleagues in the Urological Cancer Research Unit for providing specimens from routine biopsies for xenografting.

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