Cellular Heterogeneity in Normal and Neoplastic Human Urothelium

W. J. Mackillop, J. P. Bizzari, and G. K. Ward

McGill Cancer Centre, McGill University, Montreal, Quebec, Canada

ABSTRACT

Cell suspensions derived from 2 specimens of normal human urothelium and 13 human transitional cell carcinomas were studied by discontinuous density gradient centrifugation. The histological and proliferative properties of the fractions were evaluated. In normal urothelium, DNA synthesis was restricted to a subpopulation of small round cells which could be partly separated from the more numerous pyramidal and giant cells on the basis of their higher physical density. Normal urothelium did not form colonies in agar. In well-differentiated tumors, the majority of cells present were low-density, elongated cells which histochromically resembled the differentiated pyramidal cells of normal tissue, but DNA synthesis and the ability to form colonies in agar were restricted to a subpopulation of high-density small round cells. Colony size analysis showed that intermediate-density bands contain cells capable of a few divisions which do not form large colonies. With increasing tumor grade, there was an apparent shift into the high-density clonogenic compartment.

INTRODUCTION

Most human neoplasms arise in self-renewing tissues in which cellular proliferation goes on continuously to compensate for physiological losses. Numerous studies of hemopoietic tissue (1, 2), the germinal epithelium of the testis (3, 4), and several glandular (5, 6) and squamous epithelia (7, 8) have shown that these diverse renewal tissues each contain a subpopulation of cells which retains the potential for proliferation throughout the life of the organism. These "stem cells" have 2 essential functions: to divide and produce the large family of functional differentiated cells necessary to replace those lost in normal tissue function; and to produce stem cell daughters which replace those used up in the production of differentiated cells. This second type of division is referred to as self-renewal and may be regarded as the defining property of a stem cell (10). In normal tissues in steady state, the PSR² is 0.5, so that, although the stem cell pool is continuously depleted to produce differentiated cells, it is replenished at an equal rate by the competing process of self-renewal and is thus maintained constant.

The stem cell model of human tumor growth is based on the hypothesis that a stable heritable change which confers on a stem cell a PSR > 0.5 may result in an expanding clone of stem cells and that such an expanding clone constitutes a neoplasm (10). When PSR = 1.0, all the cells present in the neoplasm will be stem cells; but for all intermediate values of 0.5 < PSR < 1.0, terminally differentiated cells will still be produced. It has therefore been predicted that spontaneous neoplasms should contain 3 cell categories analogous to those present in normal tissues: nonproliferating differentiated end cells; proliferating nonrenewing transit cells; and proliferating self-renewing stem cells. The concept of neoplasia as a caricature of the process of tissue renewal was initially proposed by Pierce et al. (11), who demonstrated that terminal differentiation does occur and can be induced in several experimental neoplasms (12, 13). It has long been suspected that this might also be true of human tumors (14); but until the development of the in vitro clonogenic assay, there was little evidence to support this. Using this system to study human ovarian carcinoma, we identified 3 cell categories consistent with those predicted by the model (16-18), but our attempts to draw parallels with normal tissue were limited by lack of information about the tissue kinetics of the normal superficial epithelium of the ovary. We have therefore proceeded to study human urothelium which is a well-characterized renewal system (19-23) where we have access to both normal and neoplastic tissue.

MATERIALS AND METHODS

Clinical Material. Thirteen tumor biopsies were obtained at the time of cystoscopy and transurethral resection for apparently localized transitional cell carcinoma of the bladder. In each case, the specimens were divided; part was sent for histological examination, and part was submitted for laboratory studies. Of these, 11 were papillary tumors, 6 of which proved to be well-differentiated transitional cell carcinomas and 5 of which proved to be moderately differentiated transitional cell carcinomas. Two tumors were sessile and were histologically proved to be poorly differentiated. Only the poorly differentiated tumors showed muscle invasion. The specimens of normal bladder urothelium were obtained from patients who died of disease unrelated to the urinary tract and were histologically confirmed as normal. All tissue specimens were placed immediately in ice-cold culture medium and transferred to the laboratory within 1 h of removal from the patient.

Cell Suspensions. Tumor specimens were minced finely with scissors and then passed through needles of decreasing size to 23-gauge. No enzymes were used in the preparation of cell suspensions. Residual clumps were removed by allowing the cell suspension to stand for 10 min at unit gravity in a 50-ml test tube and then harvesting the supernatant. Clumps were needled again, and the process was repeated as required. The epithelium was scraped from the normal bladder using the edge of a microscope slide and was then reduced to a single-cell suspension by passing through needles of decreasing size to 23-gauge.

Cytology and Histochemistry. Differential cell counts were carried out on cyt centrifuge preparations stained with Wright's and Giemsa or by the Papanicolaou technique as described previously (18). The Unna-Pappenheim technique was used to stain with MGP for DNA and RNA (24). Lymphocytes, polymorphonuclear leukocytes, and RBC were clearly distinguishable cytologically from the epithelial cells, but mononuclear cells were not and they may have been included in differential counts with the small round epithelial cells. There may also be a few stromal cells present, but we were not able to indentify these.
Primary Culture of Human Tumors. A modification of the 2-layer semisolid culture procedure of Hamburger and Salmon (14) is used to assess the clonogenicity of tumor cell suspensions. Underlayers of 0.5% agarose (type VII; Sigma Chemical Co., St. Louis, MO) α-medium plus 10% fetal calf serum were prepared (1 ml in 35-mm plastic Petri dishes), and the cells to be tested were suspended in a top layer of 0.3% agarose in α-medium plus 10% fetal calf serum. No additions were used to the standard medium, and neither conditioned medium nor helper cells were used. Plates were examined after seeding, any large clumps were counted, and this number was subtracted from final colony counts. Cultures were incubated for 14 days in a humidified atmosphere of 5% CO₂ in air, and colonies containing >40 cells were counted under low power in the inverted microscope.

Colonies were scored using a-overlay microscope to score their growth rate individually.

Cell Size Analysis. Agar plates were examined under low power on the inverted microscope about 3 h after plating and subsequently at 14 days. Single cells within a randomly chosen field were counted; then, the number of cells in each cluster or colony was counted or estimated, and the number of colonies was scored according to binary size intervals; i.e., doublings were scored separately, 3 to 4 cell clusters were lumped together as were 5 to 8 cell clusters, etc. The number of cells in a group was actually counted up to 32 cells, but beyond this size counting became difficult. Two colony diameters were therefore measured using a scaled ocular, and the volume of these larger colonies was calculated initially as that of a sphere of radius equal to one-half the mean measured diameter. The number of cells contained in the larger colonies was calculated by dividing the volume of the colony by the mean volume of the individual cells. Cell volumes were measured using a Coulter Counter linked to a pulse height analyzer in a custom-built system as described previously (18) and were validated by measuring 2 diameters of 50 tumor cells in suspension, using the scaled ocular. When every cell cluster or colony within a low-power field had been assigned a size and counted, another field was chosen at random, and the process was repeated until at least 2000 cells or groups of cells had been scored. The process is time consuming and is feasible only because of the high frequency of single cells. Grid marks scored on the underside of the tissue culture dish assist the observer to score each field systematically. In this way, a colony size distribution can be constructed at varying time points after plating. Day zero counts show the frequency of clumps in the “single”-cell suspension. No attempt was made to identify specific colonies and to score their growth rate individually.

In view of the recent observation of Meyskens et al. (25) that calculations of cell numbers based on the assumption that a colony is spherical may seriously overestimate the number of tumor cells present, we have since carried out direct cell counts on large colonies derived from 2 well-differentiated transitional cell carcinomas. Meyskens’ technique of squashing individual stained colonies flat was used to permit direct counting (25), and the numbers obtained were compared with those estimated by 2 colony diameters as described above. Fifty colonies from each of 2 cases were measured and counted. The mean ratios of real cell count per estimated cell number were 0.67 and 0.96. This is not as large an error as we had expected, and it may be that our estimates of cell size, which were based on mean volume of cells in suspension before plating, were too high, and this produced a compensatory effect. The cell numbers in colonies reported here are not corrected since individual correction factors are clearly required, and these cannot be produced in retrospect. Cell numbers for large colonies may therefore be viewed as possibly overestimating true numbers by as much as 50%.

Replating Experiments (PE2). The basic procedure has been described in detail elsewhere (18). Briefly, cells are plated as for colony growth, replacing agar with methyl cellulose in the top layer. Colonies are harvested after 10 to 14 days and separated from the remaining single cells by allowing them to sediment at unit gravity through 7% bovine serum albumin for 10 min. A 50-μl sample of the colony suspension is counted, and the suspension is diluted to give a final concentration of 5 colonies/ml in culture medium (α-medium plus 10% fetal calf serum). Aliquots (200 μl) are distributed between individual microwells. The wells are inspected under the inverted microscope, and those containing a single colony are identified. Single colonies are dispersed by passing through a 25-gauge needle using a tuberculin syringe. These single cells derived from the individual colonies are finally replated in 0.3% agarose on top of previously prepared 0.5% agarose underlayers in microwells.

Cell Density Determination. Cell numbers for large colonies may therefore be viewed as not absolute and cannot be used to calculate clonogenicity. In view of the recent observation of Meyskens et al. (25) that calculations of cell numbers based on the assumption that a colony is spherical may seriously overestimate the number of tumor cells present, we have since carried out direct cell counts on large colonies derived from 2 well-differentiated transitional cell carcinomas. Meyskens’ technique of squashing individual stained colonies flat was used to permit direct counting (25), and the numbers obtained were compared with those estimated by 2 colony diameters as described above. Fifty colonies from each of 2 cases were measured and counted. The mean ratios of real cell count per estimated cell number were 0.67 and 0.96. This is not as large an error as we had expected, and it may be that our estimates of cell size, which were based on mean volume of cells in suspension before plating, were too high, and this produced a compensatory effect. The cell numbers in colonies reported here are not corrected since individual correction factors are clearly required, and these cannot be produced in retrospect. Cell numbers for large colonies may therefore be viewed as possibly overestimating true numbers by as much as 50%.

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Labeling Index. Cell suspensions were washed twice in PBS, resuspended at 10⁶ cells/ml in α-medium without nucleosides plus thymidine (5 μCi/ml; specific activity, 65 Ci/mmol), and incubated at 37°C for 1 h. The cells were then washed 3 times in PBS, and cytocentrifuge preparations were made. The slides were fixed for 2 min in 95% ethanol. In the darkroom, the slides were dipped in Kodak NTB3 emulsion, diluted 1:1 with distilled water, developed using standard Kodak reagents following 48 h exposure in the dark, and then stained with Wright’s and Giemsa (17).

Equilibrium Density Gradients. Discontinuous density gradients of Percoll were used. Densities of Percoll dilutions were checked by weighing a known volume. Steps of 9 × 1 ml from 1.1 to 1.02 g/ml were layered on manually, and the cell suspension was layered on top in the same way (2 × 10⁶ cells in 1 ml of PBS). The gradients were centrifuged at 800 × g for 20 min. Increasing centrifugation rate or time beyond this did not alter the distribution of cells within the gradient, confirming that a pure equilibrium density separation had been obtained. The cells banded at the interface between the steps and were collected from the top in 10- × 1-ml volumes using a Pasteur pipet. When isolated density fractions were reloaded onto fresh gradients and recentrifuged, >95% of the cells consistently relocated in the original density band.

RESULTS

Cytology. In cell suspensions derived from normal urothelium, we were able to distinguish 3 cell classes already well recognized by cytologists (19). Fig. 1, a and b, shows the intact tissue and single-cell suspension. Differential cell counts are shown in Table 1. Small round cells approximately 10 μm in diameter made up about 20% of the total population. MGP staining showed large amounts of cytoplasmic RNA, and these were also alkaline phosphatase positive. Morphologically and histochemically, this cell type therefore corresponds to the cells lying adjacent to the basement membrane in the intact urothelium (24). Elongated cells, usually MGP negative, with undetectable cytoplasmic alkaline phosphatase activity comprised 60% of the total population. Cytologically, these are thought to correspond to the polygonal cells of the intermediate layer of the intact urothelium. A third class of large, irregular, pleomorphic, often multinucleated cells comprised 17% of the normal urothelium. These giant cells are thought to correspond to the umbrella cells of the superficial layer of the normal urothelium.

The cell suspensions derived from the well-differentiated neoplasms contained 2 clearly distinct cell types similar to the small round cells and polygonal cells of the normal urothelium (see Fig. 1, c and d). Like their normal counterparts, the small round cells were alkaline phosphatase positive and MGP positive. The elongated cells were in many cases longer than their normal counterparts and in some cases had distinct tails. Like the normal polygonal cells, they were negative for alkaline phosphatase and MGP negative. Giant cells, >25 μm in diameter, were also found in some cases, but we have not observed multinucleated cells identical to the umbrella cells of normal urothelium.

Labeling Index. Labeling indices of the 2 specimens of normal urothelium and 10 of the bladder tumors are shown in Table 1.
UROTHELIAL TUMOR CELL HETEROGENEITY

Table 1

<table>
<thead>
<tr>
<th>Case</th>
<th>Gross pathology</th>
<th>Tumor grade</th>
<th>Muscle invasion</th>
<th>Clinical data</th>
<th>% of elongated cells</th>
<th>% of giant cells</th>
<th>% of round cells</th>
<th>Colonies/10⁶ tumor cells</th>
<th>Labeling index</th>
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<td>1</td>
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<td>II</td>
<td>No</td>
<td>New case</td>
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<td>0.17</td>
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<td>52</td>
<td>4</td>
<td>ND</td>
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<td>No</td>
<td>New case</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>103</td>
<td>ND</td>
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<td>New case</td>
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<td>45</td>
<td>0</td>
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<td>53</td>
<td>5</td>
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<tr>
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<td>New case</td>
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<td>2</td>
<td>44</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>Papillary tumor</td>
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<td>No</td>
<td>New case</td>
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<td>2</td>
<td>72</td>
<td>161</td>
<td>0.6</td>
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<tr>
<td>10</td>
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<td>II</td>
<td>No</td>
<td>Recurrence</td>
<td>10</td>
<td>0</td>
<td>90</td>
<td>35</td>
<td>1.1</td>
</tr>
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<td>Papillary tumor</td>
<td>II</td>
<td>No</td>
<td>Recurrence</td>
<td>29</td>
<td>2</td>
<td>70</td>
<td>9</td>
<td>1.2</td>
</tr>
<tr>
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<td>No</td>
<td>New case</td>
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<td>0</td>
<td>97</td>
<td>85</td>
<td>1.4</td>
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<td>Papillary tumor</td>
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<td>No</td>
<td>Recurrence</td>
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<td>ND</td>
<td>ND</td>
<td>6</td>
<td>ND</td>
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<td>Yes</td>
<td>Recurrence</td>
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<td>3</td>
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<td>5</td>
<td>2.7</td>
</tr>
<tr>
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<td>Sessile tumor</td>
<td>IV</td>
<td>Yes</td>
<td>New case</td>
<td>2</td>
<td>0</td>
<td>98</td>
<td>140</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* ND, not determined.

Chart 1. Density distribution of cellular subtypes identified in a suspension derived from normal bladder urothelium.

The mean labeling index of the 2 normal specimens was 0.2%. Median labeling indices of the well-, moderately, and poorly differentiated tumors were 0.9, 1.15, and 4.7%, respectively. The small number of cases studied precludes any meaningful statistical analysis of these data.

Plating Efficiency. Neither of the normal bladder specimens showed any evidence of proliferation in semisolid culture conditions, whereas 11 of the 13 tumors formed colonies as shown in Table 1. The median plating efficiencies of well-, moderately, and poorly differentiated tumors were 5/100,000, 35/100,000, and 72.5/100,000. Although the median plating efficiencies increased with increasing tumor grade, values varied very widely within each small subgroup, and the small numbers preclude any meaningful statistical analysis of the data.

Density Gradients. Chart 1 illustrates the density profile of the normal urothelial cell population. The elongated cells which make up 60% of the total population locate in the density bands corresponding to densities less than 1.045. The umbrella cells, or giant cells, have even lower densities. The greatest proportion of small round cells is in the high-density bands, although small cells are found in all density bands.

The density distribution of cell types present in a moderately differentiated neoplasm (Table 1, Case 9) is shown in Chart 2. The majority of elongated and giant cells lie in the low-density bands, and as in normal urothelium the round cells have a higher density.

Chart 3 shows the proliferative properties of density gradient fractions obtained from a normal human bladder and from a well-differentiated transitional cell carcinoma. Colony formation did not occur with the normal bladder and DNA synthesis was confined to the high-density bands. In the well-differentiated carcinomas, the fraction with the highest labeling index is the 1.055-g/cm³ band, as in the normal urothelium. The cells capable of forming colonies in agar are likewise confined to the high-density bands, and the property of self-renewal, as estimated by PE₂, paralleled the primary plating efficiency.

Colony Size Analysis. Chart 4 shows the colony size distribution obtained in culture from a moderately differentiated transitional cell carcinoma at different time intervals after plating (Table 1, Case 9). The day zero count reflects the number of small clusters found in our “single”-cell suspension despite the unit gravity precipitation step designed to avoid this problem. The absolute number of cells counted on each day varied so that the data have also been expressed in the form of a percentage. With increasing time, the average colony size increases, and the tabular data can be understood as a form of the growth curve. Significant growth did not occur in this system with this tumor...
UROTHELIAL TUMOR CELL HETEROGENEITY

Chart 3. Proliferative properties of density gradient fractions from normal urothelium (A) and a moderately differentiated transitional cell carcinoma of the bladder (B) (Table 1, Case 9). •, labeling index (L.I.); •, clonogenicity in agar; O, PE₂. P.E., plating efficiency.

COlONy SIZE (CELL NUMBER)

<table>
<thead>
<tr>
<th>Day</th>
<th>Total</th>
<th>0.96</th>
<th>0.99</th>
<th>1.02</th>
<th>1.03</th>
<th>1.04</th>
<th>1.05</th>
<th>1.06</th>
<th>1.07</th>
<th>1.08</th>
<th>1.09</th>
<th>1.10</th>
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<tbody>
<tr>
<td>0</td>
<td>1234</td>
<td>567</td>
<td>89</td>
<td>12</td>
<td>34</td>
<td>56</td>
<td>78</td>
<td>90</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>%</td>
<td>80.5%</td>
<td>90.4%</td>
<td>95.6%</td>
<td>99.2%</td>
<td>99.9%</td>
<td>99.6%</td>
<td>99.0%</td>
<td>98.5%</td>
<td>96.5%</td>
<td>93.5%</td>
<td>90.5%</td>
<td>87.5%</td>
</tr>
</tbody>
</table>

Beyond 14 days. Chart 5 expresses graphically the colony size distribution of gradient fractions from the same case. (The density distribution, clonogenicity, and PE₂ are shown in Chart 3.) Only cells in the high-density subcompartment are capable of forming large colonies. In the lowest-density bands, there is no evidence of any growth. In the intermediate-density band, cells are present which are capable of dividing several times before arresting, but no large colonies are formed.

Tumor Grade. Chart 6 illustrates typical density distributions of well, moderately, and poorly differentiated tumors and their clonogenicity profiles. There is an apparent shift towards the high-density bands with increasing tumor grade consistent with the increasing proportion to small round cells observed cytologically. The plating efficiency in agar in each case is maximal in the higher-density bands. Similar observations have been made in 4 other tumors (2 well-differentiated and 2 moderately differentiated) for which data are not presented here. In the single poorly differentiated tumor which we have studied in this way, clonogenicity was not confined to the high-density bands, and clonogenic cells of lower physical densities were also present.

DISCUSSION

We have no assay for the stem cells of normal human urothelium, but we have shown that DNA synthesis is normally confined to a subpopulation of small round cells of relatively high physical density which correspond histochemically to the cells of the basal layer in the intact urothelium. In experimental systems, it is known that regeneration following trauma begins in this layer (23), and it is therefore probable that the stem cells of normal urothelium are contained within this group. Well-differentiated transitional cell carcinomas contain a large proportion of elongated cells which morphologically and histochemically resemble the differentiated intermediate cells of normal urothelium. These do not synthesize DNA and are not capable of forming colonies in agar. The colony-forming cells in these tumors belong to a high-density small round cell population histochemically similar to the putative stem cells of normal urothelium. Some of these cells are capable of self-renewal as demonstrated by the ability of their descendants to form further colonies when replated. The properties of labeling with tritiated thymidine, clonogenicity in agar, and PE₂ were not separable by density gradient centrifugation. These data are consistent with a 2-compartment model in which tumor contains only proliferative stem cells and differentiated end cells. In high-grade tumors, however, clonogenic cells...
The present study indicates that this approach is feasible. A more appropriate approach may be to identify and isolate stem cell subpopulations and to compare the properties of normal and neoplastic tissue are usually directed at total cell populations with the implicit assumption that these are homogeneous. A more appropriate approach may be to identify and isolate stem cell subpopulations and to compare the properties of normal and neoplastic tissue populations with the implicit assumption that these are homogeneous. A more appropriate approach may be to identify and isolate stem cell subpopulations and to compare the properties of normal and neoplastic tissue populations with the implicit assumption that these are homogeneous.

These observations are consistent with those previously reported in ovarian carcinoma in which it was shown that not all tumor cells were clonogenic and that the clonogenic subpopulation could be partially separated from the remainder by physical separation procedures (16-18). Those studies were confined to cells from ascites, and the present study extends this work to solid tumors and includes a comparison with normal tissue which was not possible with the ovarian model. The observation that there is an apparent shift into the clonogenic compartment with increasing grade is consistent with the finding of a similar shift in ovarian carcinoma which accompanied tumor progression (26). The more careful analysis of colony size which has been applied may coexist within a tumor, and the implications of this interclonal heterogeneity for clinical oncology have been extensively discussed. With increasing tumor grade, there is a relative shift towards the high-density clonogenic compartment consistent with the increase in PSR with increasing tumor grade predicted by the stem cell model (10).

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Fig. 1. a, normal human urothelium. b, cell suspension derived from normal human urothelium. Arrows (left to right): pyramidal cell type; giant cell type; and small round cell type. c, well-differentiated papillary transitional cell carcinoma. d, cell suspension derived from the same tumor. Arrows (top and bottom): small round cell type and elongated cell type. Bars, 10 µm.
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