Establishment and Characterization of Four Human Bladder Tumor Cell Lines and Sublines with Different Degrees of Malignancy

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ABSTRACT
We have established four human bladder tumor cultures, designated MGH-U1 to -U4 (also known as EJ, HM, RN, and RB in some previous reports). All have been grown in culture for over 30 passages and were free of Mycoplasma contamination.

Characterizations of these cell lines were performed. These include isozyme profile, morphology with light and scanning electron microscopes, karyotype, growth rate, DNA content by flow cytometry, presence of cell surface ABH isoantigens, tumorigenicity in nude mice, lactic acid dehydrogenase isozymes, and colony formation in soft agar.

Results obtained from these characterizations confirm that MGH-U1 and -U2 are sublines of a previously established bladder tumor cell line, T-24. These results also show that MGH-U3 and -U4, derived respectively from a grade 1 tumor and an urothelium biopsy with severe atypia, are likely to be independent human bladder cell lines and different from other transitional cell bladder carcinoma cell lines reported. The study further demonstrates that these four cell lines/sublines have different degrees of malignancy and a close correlation, in biological and malignant characteristics, between the cells in culture and those in the original tumors. Therefore these cultures may represent cells at different stages of malignant progression. These can be useful models for studies of the development and progression of bladder tumors and detection and treatment of bladder tumors of different grades and stages.

INTRODUCTION
Tumor cell lines are valuable tools for cancer research. In human TCC of the bladder, over 30 cell lines have been established (2, 3). Recent utilities of these cell lines include the production of monoclonal antibodies against human bladder tumors (4–6), identification of nonrandom cytogenetic changes,4 identification and isolation of oncogenes (7, 8), and as models for chemotherapeutic treatments (29).

It is important that these in vitro models be extensively characterized to assure the validity and reproducibility of the experimental results. For example MGH-U1 (EJ) is now widely used as an experimental system for many investigations (6–9), but detailed characterization has not been performed and the origin of the cell is still in question. A study by O’Toole et al. (10) found that several cultures, including MGH-U1 and MGH-U2 (HM), previously thought to be independent cell lines, have isozyme profiles and histocompatibility types identical to T-24, a cell line established in 1973 (11). This indicates that they may have the same origin. In the present study we attempted to clarify some of these confusion by performing extensive morphological and biological characterizations of these two sublines, together with the other two new TCC cell lines established in our laboratory.

Results indicated that these four cell lines/sublines have different degrees of malignancy and may represent cells at different stages of tumor progression.

MATERIALS AND METHODS
Origin of Cell Lines. Previously MGH-U1 and -U2 were thought to be independent cell lines established from two different bladder tumors (12). However, results described later in this study and those of O’Toole et al. (10) indicate that they are likely to be sublines subcultured from T-24. The latter was introduced to our laboratory in 1974. These two sublines have been grown continuously in our laboratory since then.

The other two cell lines, MGH-U3 (RN) and MGH-U4 (RB), were established from tumor biopsies from two different patients. The donor of MGH-U3 was a 76-year-old Caucasian male with 19 years of recurrent low grade, noninvasive TCC of the bladder. The biopsy specimen from which the culture originated was obtained in March 1977 with a pathological diagnosis as papillary TCC, grade 1 (Fig. 1A). The passage level was around 70 when the current characterization study was performed. The donor’s ABO blood group was type O negative.

The donor of MGH-U4 was a 57-year-old Caucasian male who had a history of carcinoma in situ and severe atypia of the bladder. The biopsy specimen from which the cell line was established was obtained in December 1978. The pathological diagnosis of the specimen was focal severe urothelial atypia (Fig. 1B). The passage level was around 30 when the current study was performed. The blood type of the donor was A positive.

Cell Culture. Cultures of the tumor tissues (MGH-U3 and -U4) were initiated by mincing the biopsies in 2 to 3 drops of 100% FBS in a 60-mm culture dish. The minced tissue was incubated at 37°C for 4 h to allow the cells to settle and attach. Eagle’s minimum essential medium (3 ml) supplemented with 10% FBS, sodium pyruvate, and nonessential amino acids was then added, and the dish was incubated at 37°C in humidified air with 5% CO2. The culture was routinely fed weekly and passaged with trypsin and EDTA when cell growth reached confluency. After the culture was established as a permanent cell line, it was then switched to McCoy’s Medium 5A with 5% FCS. During the current characterization study, cell cultures were free of Mycoplasma contamination as tested by the method of Madoff and Pachas (13).

Scanning Electron Microscopy. Cells were grown on glass chips, washed three times in serum free medium, and fixed in 2% glutaraldehyde in PBS. The cells were then prepared for scanning electron microscopy as described earlier (14) and scanned with a JEOL JSM-U3 instrument.

Isozyme Phenotypic Profile, Karyotype, and Chromosomal Number. The expression of eight genetically stable isozyme phenotypes was analyzed to provide an identification for each individual cell line. The
analysis was performed by a procedure described previously (15). The
isoenzyme profile generated by this analysis has a minimal frequency
product of 0.05.

Karyotypes of MGH-U1, -U2, and -U3 cells have been performed and
reported earlier (12). The karyotyping of cell line MGH-U4 was done by
Dr. Peterson according to the method described previously (16). In this
method Giemsa-banding preparations were performed on the cells, the
banded chromosomes from each metaphase were arranged, and the
karyotypes were analyzed (17).

For determining chromosomal number log phase cells were treated
with colchicine ($10^{-7}$ M) for 3 to 4 h before harvesting. Cells were then
treated and stained according to the method of Hsu (18) and 100
metaphases were counted for determining chromosomal numbers and
distribution.

Growth Rate and Plating Efficiency. Growth rates of the cells were
determined by seeding $10^6$ (MGH-U1, -U2, and -U3) or 5 x $10^6$ (MGH-
U4) cells/35-mm dish and counting the number of cells daily for 11 days.

Plating efficiencies of the cells were determined in two ways: by the
rate of colony growth; and by the number of cells attached after plating.
The rates of colony growth were determined in triplicate by seeding 100
(MGH-U1 and -U2) or 6400 (MGH-U3 and -U4) single cells per 60-mm
dish in 3 ml McCoy's Medium 5A with 5% FBS. Dishes were incubated for
10 days to allow cells to grow into colonies. Cells were then stained
with methylene blue and colonies with 50 cells or larger were counted.
Attachment efficiencies were determined also in triplicate by seeding 3.3
x $10^3$ cells/100-mm dish in 10 ml of culturing medium. Cell number per
dish was determined after 12 h of incubation.

Tumorigenicity in Nude Mice. Tumorigenicity of each cell line was
determined by s.c. injection of $10^7$ cells in 0.2 ml into each 60-day-old
BALB/c-nu/nu female nude mouse (Charles River Breeding Laboratory,
Wilmington, MA). After 33 days animals were killed and each tumor was
dissected, weighed, and fixed for histological examination.

Growth in Soft Agar. The ability of anchorage independent growth of
the cell lines was determined by the formation of colonies in soft agar.
Single cells, $10^3$/60-mm dish, were dispersed in quadruplicate in a
solution containing 0.36% Noble agar (Difco Laboratories, Detroit, MI) in
McCoy's Medium 5A supplemented with 20% FBS, penicillin, and strep-

tomycin. This was layered over a 0.6% agar solution in McCoy's Medium
5A also supplemented with 20% FBS and antibiotics. Dishes were incubated at 37°C and fed biweekly with McCoy's Medium 5A supple-
mented with 5% FCS. After 5 weeks colonies greater than 50 cells were
counted visually at $\times 100$.

LDH Isozymes. Confluent cells were rinsed three times with phos-
phate buffered saline, removed from dishes by scraping, and resus-
pended in distilled water. Cell lysates were prepared by sonication (three
10-sec disruptions with 1 min cooling in between) and centrifugation
(2000 rpm, 10 min). Separation of the LDH isozymes of the cell lysates
was done by polyacrylamide gel electrophoresis following the procedure
of Dietz and Lubrano (19), and quantitation of the isozymes was done on
a colorimetric densitometer.

Blood Group Isoantigens. Detection of the presence of blood group
ABH isoantigens on cell surface was performed by an indirect immuno-
fluorescence method (20). Tumor cells were grown on plastic dishes as
monolayers. Cells were washed once with phosphate buffered saline,
fixed in 10% formalin for 20 min, and incubated with anti-A, anti-B
antiserum, or diluted Ulex europaeus agglutinin I for 30 min at room
temperature. Fluorescein-conjugated goat anti-human IgM (Miles Lab-
oratories, Naperville, IL) or rabbit anti-U. europaeus agglutinin I (E-Y
Laboratories, San Mateo, CA) was then added. After the cells were
washed they were observed for the presence of the blood group isoan-
tigen under a Zeiss fluorescence microscope.

RESULTS

Isozyme Phenotypes. Results of analyses of the isozyme
phenotypes of the cell lines are presented in Table 1. All cultures
had human isozyme phenotypes. Both MGH-U1 and MGH-U2
had isozyme patterns identical to that reported for T-24 (9, 10),
confirming that these cultures had an identical origin. The isozyme
patterns of MGH-U3 and MGH-U4 were different from
those of the 14 other bladder tumor cell lines analyzed (21, 22)
and different from those of T-24 and HeLa cells (9, 22), indicating
that they were likely to be independent cell lines and that they
were not contaminated by T-24 and HeLa cells.

Growth Characteristics and Cell Morphology. There was a
vast difference in growth rates between MGH-U1 and -U2 and
MGH-U3 and -U4 (Chart 1). The doubling times for MGH-U1 and
-U2 were about 3 times faster than those of MGH-U3 and -U4.
Both MGH-U1 and MGH-U2 had the tendency to form multiple
layer growth at high cell density, thus attaining higher confluency
than those of the 14 other bladder tumor cell lines analyzed (21, 22)
and different from those of T-24 and HeLa cells (9, 22), indicating
that they were likely to be independent cell lines and that they
were not contaminated by T-24 and HeLa cells.

These cultures also had wide variations in morphology (Fig.
2). MGH-U1 cells, similar to those originally described by Kato
et al. (12) and Hepburn and Masters (2), were characterized by a
typical epithelial morphology with mixed populations of polygonal
and spindle shaped cells, consisting of large nuclei and multiple
nuclei. These cells also had well defined margins, especially at
high density growth, during which time most cells were exhibiting
an elongated spindle morphology.

MGH-U2 cells were characterized by larger cell sizes and by
large, round, hyperchromatic nuclei with multiple nucleoli.

MGH-U3 cells were relatively smaller and flat and had an
uniform polygonal epithelioid morphology. These cells had a
round, singular nucleus and most had two to four nucleoli. One
prominent feature of these cells was the swirling pattern of cell
arrangement at confluence. The cell margins were not as well
cells. The morphologies of these cells under the scanning electron microscope were also quite different (Fig. 3). This was particularly apparent in the structure, distribution, and extent of development of the microvilli (Fig. 4). MGH-U1 cells had long and thin microvilli with microridges present in some areas of the cell surface (Fig. 4A). MGH-U2 cells had long and thin microvilli, distributed non-uniformly and sparsely. MGH-U3 cells had short and round microvilli, distributed evenly throughout the cell surface, whereas MGH-U4 cells had numerous, long, and uniformly distributed pleomorphic microvilli.

**Chromosomal Analyses.** Results of chromosomal analyses (Chart 2) showed that MGH-U1 and -U2 were mostly tetraploid while MGH-U3 and -U4 were mostly diploid. Among these four cell lines MGH-U2 had the highest chromosomal number while MGH-U3 had the widest variation of chromosomal distribution. MGH-U4 cells, however, had chromosomal numbers distributed within a narrow range of 40 to 50. Results of these analyses were consistent with the degree of malignancy of the original tumors. These results were also in agreement with those of DMA determined by colony growth and by cell attachment rate after plating (Table 2). About 60% of MGH-U1 and -U2 cells formed colonies whereas only about 1% of MGH-U3 and -U4 cells did so. This was in contrast to the results of attachment efficiency where all cell lines show close to or over 60% attachment at 12 h after plating.

MGH-U1, -U2, and -U3 formed tumors in nude mice whereas MGH-U4 failed to do so. The average tumor sizes at the end of the experiment (33 days) were: MGH-U1, 1.26 ± 0.66 (SD) g; MGH-U2, 1.16 ± 0.16 g; and MGH-U3, 0.83 ± 0.25 g. In MGH-U3 where both histologies of the original tumor and that grown in nude mice were available for comparison, there was a close resemblance in histopathological appearances (data not shown).

MGH-U1 and -U2 cells could grow without anchorage; both have a 25% colony formation rate in soft agar (Table 2). In contrast both MGH-U3 and MGH-U4 failed to grow in the same system, apparently lacking the ability for anchorage independent growth.

**LDH Isozymes and ABH Isoantigens.** Since it has been shown in bladder tumors that the increase of the M component of the LDH isozymes correlated with the increase of the histological grade of the tumor (24, 25), the isozyme patterns were examined in these cell lines as part of the characterization for their malignant properties. Results (Fig. 6; Table 2) indicated that MGH-U1 and -U2 had high levels of isozyme 5 and low levels of isozyme 1, whereas MGH-U3 and -U4 had very low levels of isozyme 5. In fact MGH-U2 had no detectable isozyme 1, whereas MGH-U4 had no detectable isozyme 5.

Another characteristic that correlates with malignancy of the bladder tumor is the blood group isoantigens. Loss of the isoantigens on the cell surface could be demonstrated by loss of the blood group antigens in cells from the original tumors. In these cell lines the ABH isoantigens were present with high levels of either AB or O type. In the MGH-U1 and -U2 cell lines the AB antigen was found on all cells; in MGH-U3 and -U4 the cells were AB negative.

**Table 2**

<table>
<thead>
<tr>
<th>Other designation</th>
<th>Histology of original tumor</th>
<th>ABH isoantigen</th>
<th>LDH isozyme 5/1 ratio</th>
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<tr>
<td>Cell line</td>
<td>EJ</td>
<td>HM</td>
<td>RN</td>
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<tr>
<td>MGH-U1*</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>MGH-U2&quot;</td>
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<td>4/4</td>
<td>4/4</td>
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<tr>
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<tr>
<td>MGH-U4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Growth in soft agar (%)</th>
<th>ABH isoantigen</th>
<th>LDH isozyme 5/1 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1</td>
<td>25%</td>
<td>high</td>
</tr>
<tr>
<td>MGH-U2</td>
<td>25%</td>
<td>high</td>
</tr>
<tr>
<td>MGH-U3</td>
<td>0%</td>
<td>low</td>
</tr>
<tr>
<td>MGH-U4</td>
<td>0%</td>
<td>low</td>
</tr>
</tbody>
</table>

* Other designations for T-24, grade 3 TCC (3).
* Sublines of T-24, grade 3 TCC (3).
cells have the same origin as T-24, a cell line established from a grade 1 TCC, thus joins several other low grade transitional cell bladder carcinoma cell lines reported. MGH-U3, derived from a grade 1 TCC, has mostly diploid chromosomes, has a doubling time around 50 h, and can form tumors in immunodeficient animals. However, the ability for anchorage independent growth in soft agar varies among these cell lines.

MGH-U4 is a somewhat unusual cell line. It was derived from a bladder biopsy specimen diagnosed as severe atypia of the urothelium. The patient, who had a previous history of grade 1 bladder carcinoma and carcinoma in situ, two years later developed invasive cancer with metastases extending to liver and bone. The cell line, although it grew rapidly in culture and had a number of chromosomal abnormalities, failed to produce tumor in nude mice and could not form colonies in soft agar. It also had blood group antigen present on the cell surface and had no detectable isozyme 5 of LDH. All these are considered, to various extents, to be indications of neoplastic transformation (1, 30, 34, 35). Severe atypia or dysplasia in bladder carcinoma bears a resemblance to the same condition of the uterine cervix which is considered as a premalignant abnormality (36). The in vitro properties of this cell line seem to reflect such a status.

From the overall characterizations including growth, morphology, chromosomal analyses, and other properties listed in Table 2, these four cell lines/sublines seem to represent cells with different degrees of malignancy, with MGH-U1 and -U2 being highly malignant, MGH-U3 mildly malignant, and MGH-U4 possibly premalignant. There is also a close correlation and resemblance of their in vitro characteristics with properties of the tumors in vivo, indicating the preservation of key malignant characteristics in these cells despite being cultured in vitro for a long period of time. Collectively these cells may be a valuable model for a wide variety of studies in bladder cancer.

Another difference between MGH-U1 and MGH-U2 is in the morphology under the scanning electron microscope, particularly on the structure of the microvilli. In MGH-U1 cells microvilli were present in parts of the cell surface while MGH-U2 cells had long, thin, and unevenly distributed microvilli. Most recently it has also been noted that MGH-U1 has the capability to form spheroids (29) while MGH-U2 lacks this capability.

There are also considerable morphological and biological differences between MGH-U1, MGH-U2, and their parent cell line T-24. In addition to the fact that the two sublines are morphologically less heterogeneous than is T-24, the present results and those obtained earlier (23, 30) show that the sublines have higher colony forming efficiencies on agar and greater capabilities of forming tumors in nude mice. It is therefore important to note that although MGH-U1, MGH-U2, and T-24 have an identical origin they are by no means identical cell lines. The frequent contaminations of cell lines and frequent subculturing of subpopulations from different laboratories underscore the importance of the establishment of, and periodical monitoring of, individual identities of cell lines, by either isozyme analyses or other genetically stable markers.

Results of isozyme analyses indicate that MGH-U3 and -U4 are most probably independent cell lines different from other transitional cell bladder carcinoma cell lines reported. MGH-U3, derived from a grade 1 TCC, thus joins several other low grade bladder tumor cell lines reported, including RT-4 (31), 682B, 751G, 1016T (32), and KK-47 (33). It is interesting to note that the majority of these cell lines, including MGH-U3, have mostly diploid chromosomes, have a doubling time around 50 h, and can form tumors in immunodeficient animals. However, the ability for anchorage independent growth in soft agar varies among these cell lines.

Previously MGH-U1 and -U2 were thought to be independent cell lines derived from two separate bladder tumor biopsies (12). However, results of isozyme analyses from the current study confirm the earlier observations by O'Toole et al. (10) that these cells have the same origin as T-24, a cell line established from a high grade transitional cell bladder carcinoma in 1973 (11). O'Toole et al. (10) also showed that T-24 contaminates several other cell lines. Thus MGH-U1 and -U2 should be considered as sublines of T-24. The similarities between these two cultures in growth, chromosomal characteristics, and malignant properties such as tumorigenicity and anchorage independent growth also support the notion that they have the same origin.

The accidental subculturing of MGH-U1 from T-24 might have occurred as early as 1974 and that for MGH-U2 around 1975, since morphologies of these cells at early passages were similar to the current cultures. These two sublines are probably derived from selection of subpopulations of T-24 rather than from adaptation to different culturing conditions, since both were grown under identical conditions in the same laboratory. The original report by Bubenik et al. (11) pointed out that T-24 is heterogeneous and consists of two morphologically distinct cell populations: cells with large, round nuclei; and elongated cells with oval, dark nuclei. In our observation the morphology of MGH-U2 seems to resemble the former whereas MGH-U1 resembles the latter.

DISCUSSION

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\[5\] C. Erlichman, personal communication.
HUMAN BLADDER TUMOR CELL LINES

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Histological sections of the original tumors from which MGH-U3 (A) and MGH-U4 (B) cell lines were established. H & E, × 400.
Fig. 2. Morphology of MGH-U1 (A), -U2 (B), -U3 (C), and -U4 (D) cells at confluency. H & E, x 200.
Fig. 3. Scanning electron microscopic morphology of MGH-U1 (A), -U2 (B), -U3 (C), and -U4 (D) cells. A, B, and C, x 1500; D, x 2500.
Fig. 4. Microvilli of MGH-U1 (A), -U2 (B), -U3 (C), and -U4 (D) cells as revealed by scanning electron microscopy and indicating differences in shape, size, and distribution pattern of the microvilli among the cells. A, × 9000; B, × 4000; C, × 9000; D, × 9000.
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