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Characterization of a Newly Established Human Urinary Bladder Cancer Cell Line (BT-1)

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Summary. A new human transitonal cell carcinoma cell line has been established in long term tissue culture. The BT-1 cells were derived from a poorly differentiated human bladder cancer. The tumor cell line produces tumors in nude mice. BT-1 has been characterized by cytogenetic and flow cytometric analysis and by isoenzyme typing. As in direct preparations of human bladder tumors, an isochromosome 5p is a consistent marker of the newly established line. The BT-1 cells produce transforming growth factors but do not respond to exogeneous EGF.

Key words: Bladder cancer – Isochromosome 5p – Flowcytometry – Growth factors

The establishment of cell lines from human urinary bladder transitional cell carcinoma (TCC) has been reported by several investigators [22]. Such cell lines have been used extensively in order to screen various chemotherapeutic agents for their antineoplastic activity, to investigate immunological mechanisms and to elucidate other mechanisms which might regulate cellular proliferation, and oncogene behaviour. We describe an additional established cell line from a human transitional cell carcinoma that has distinctive morphologic, cytogenetic and proliferative characteristics.

Materials and Methods

Origin of the Cell Line

A 49 year old white woman was found to have a poorly differentiated transitional cell carcinoma of the bladder. Following external beam radiation of the bladder and of the adjacent pelvic lymph nodes with 4,000 rads a pelvic lymphadenectomy and radical cystectomy with urinary diversion was performed.

Cell Culture. Tumor tissue removed in the operating theatre was placed in DMEM media supplemented with 20% FCS (GIBCO). In the tissue culture laboratory, the tissue was gently minced into 1 to 2 mm^3 pieces using crossed scalpels. 10–12 of the small pieces were placed into 25 cm^2 plastic culture flasks (Falcon) and covered with small volumes of DMEM supplemented with 20% FCS, 100 IU/ml Penicillin, 100 µg/ml Streptomycin and 50 µg/ml Gentamicin.

After overnight incubation at 37 °C in a humidified 95% air-5% CO_2 atmosphere, fresh medium was added dropwise. The culture medium was changed twice a week thereafter. Growth appeared after three days.

Once the cells were confluent, cell sheets were washed once with magnesium-and-calcium-free phosphate buffered saline (GIBCO) and dispersed with 0.25% Trypsin, 1 mM EDTA in PBS at 37 °C for 3 min. After addition of serum-containing medium, the cells were collected by centrifugation, resuspended in fresh culture medium and replated at the desired split ratio.

Cell Growth. Population doubling time of the tumor cells were estimated by plating 5×10^4 cells in to each of a series of 35 mm^2 petri dishes. The number of cells per plate was determined in triplicate at daily intervals. For storage in liquid nitrogen, trypsinized cell aliquots were suspended in culture medium containing 10% DMSO. Anchorage-dependence of cell growth was examined by the method of McPherson and Montagnier [13].

Tumorgenicity in Nude Mice

Aliquots of 5×10^6 cells/0.25 ml were inoculated subcutaneously in 6 week old male athymic homozygous (nu/nu) nude mice (balb c). Subcutaneous tumor growth was measured twice of week. The actual tumor volume was calculated as the cube root of the product of three ruler measurements.

Tumor Histology

Small pieces of the tumor takes in the nude mice were fixed in 10% neutral formalin, embedded, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Monolayers cultured on microscope slides were fixed in Bouin's solution, dehydrated through an ethanol series and stained with hematoxylin/eosin.

Electron Microscopy

Cells grown in tissue culture flasks were fixed with 2.5% glutaraldehyde in 0.005 M cacodylate buffer (pH = 7.2), postfixed for 2 h in 1.5% osmium tetroxid and processed by standard methods for analysis in an EM10 (Zeiss, Oberkochen, FRG) electron microscope.

Chromosomal Analysis

Semi-confluent cultures were treated with 0.1 ng/ml colchicin for 60 min. The cultures were then removed from the culture flasks by 0.25% trypsin and exposed to 0.8% sodiumcitrate (37 °C/30 min). The cells were fixed in a 3:1 mixture of methanol and acetic acid, dropped on precleaned slides, air dried, and subjected to RBG banding with Giemsa stain [21].

Flow Cytometry

For flow cytometric measurements the cells were harvested by trypsinisation, pelleted by centrifugation and resuspended in staining buffer [11]. Flow cytometric analysis was performed with an epiilumination flow system of conventional design (ICP 22, Ortho Diagnostic Systems, Rariton, N.J.) using human diploid fibroblasts as internal standard [11]. Histograms were acquired with a model 2103 multichannel analyzer (Ortho Diagnostic Systems) and transmitted to a PDP11/23 microcomputer (Digital Quipment Corp.). The determination of fluorescence means and fluorescence ratios was done by automated curve-fitting using custom computer software kindly provided by Dr. P. S. Rabinovitch, University of Washington, Seattle.

Isozyme Pattern

The glucose-6-phosphate dehydrogenase isozyme pattern was determined by starch gel electrophoresis using 13% starch and the TRIS-EDTA-borate buffer system at pH 8.6. After an overnight run (5 V/cm, 22 h, 4 °C) the gels were sliced and stained with a standard tetrazolium mixture [8]. The G6PD type was determined by a comparison of the isoenzyme mobility of the target cell line with known Hela cell homogenates [5].

Periodically, frozen stocks of the cell line were shown to be free of mycoplasma contamination by culture methods [4], and by absence of cytoplasmic staining with the Hoechst 33258 dye [2].

Demonstration of EGF-Binding

In order to demonstrate the EGF binding sites at the surface of the BT-1 cells, binding studies were performed with confluent monolayers of BT-1 cells plated into 24 well culture dishes. After overnight seeding of 1×10^5 cells/well the monolayers were treated as described previously [18]. For Scatchard analysis ^{125}J-EGF was used over the range of 1 nM to 5 nM. Nonspecific binding was measured in the presence of unlabeled EGF (100 ng/ml). Nonspecific binding was consistently less than 10% of the total binding. All experiments were performed at room temperature (22 °C). The ratio of bound to free EGF was plotted as a function of the bound nanograms EGF to generate the Scatchard plots [20]. To demonstrate any age-dependent loss of EGF-binding sites on BT-1, the number of cells of BT-1, passage 10 was compared with the results of BT-1, passage 60. To study the time course of binding, the BT-1 cells were prepared as before and ^{125}J-EGF (2 ng/ml) were added to each well. Every third well received 500 ng/ml unlabeled EGF. The incubation was carried out at three different temperatures (4 °C, 22 °C and 37 °C). At the end of each time point, the BT-1 monolayers were washed, solubilized and counted in a gamma-counter.

In order to evaluate the effects of exogeneous EGF and tumor cell extracts, the extracts were prepared by the acid-ethanol procedure as described by Roberts et al. [17] and characterized by soft agar assays in EGF competition assays as described by Nickell et al. [15]. Cell extracts and/or EGF were added in varying concentrations to the BT-1 cells cultured at a density of 1×10^4 cells/well in 24 well culture plates. The cells were counted every other day after trypsinisation in a hemocytometer.

Results

Most pieces of tumor explants were surrounded by cell outgrowth after four weeks in culture. After the first subculture, the growth rate increased and the cells were split 1:5 at weekly intervals. The culture never underwent a growth crisis, and has been kept in continuous culture for 4 years. Tests for mycoplasma were consistently negative.

Morphologic Characteristic

The BT-1 cells adhered well to either glass or plastic culture flasks and formed multiple layers after prolonged growth. Individual cells were uniformly epitheloid-like and contain large, round to oval nuclei together with numerous nucleoli. The cell cytoplasmn has frequent vacuoles (Fig. 3).

Growth Rate, Anchorage Independent Growth, and Tumorgenicity

The doubling time of the BT-1 cell line was 28 h at passage 14 and 34 h at passage 42. The addition of either 50 ng/ml EGF, 100 ng/ml EGF, or of BT-1 cell extracts to the BT-1 cells did not change their growth rate (Fig. 8). The cells grew in soft agar, with a plating efficiency of 0.74%. After subcutaneous injection into nude mice, 6 out of 10 animals developed tumors at the side of inoculation within 4 weeks after injection. As expected, the tumor takes were not sex linked. After cervical dislocation, the tumor nodules were excised for histological examination. Subcutaneous spreading of the tumor, and of metastases, was not observed.

Histology and Electron Microscopy

The histology of the excised tumors revealed a poorly differentiated transitional cell carcinoma of the bladder associated with a squamous cell carcinoma pattern (Fig. 2) closely resembling that of the original tumor (Fig. 1).

Electron microscopy revealed characteristics of the epithelial origin of these cells with abundance of desmosomes,

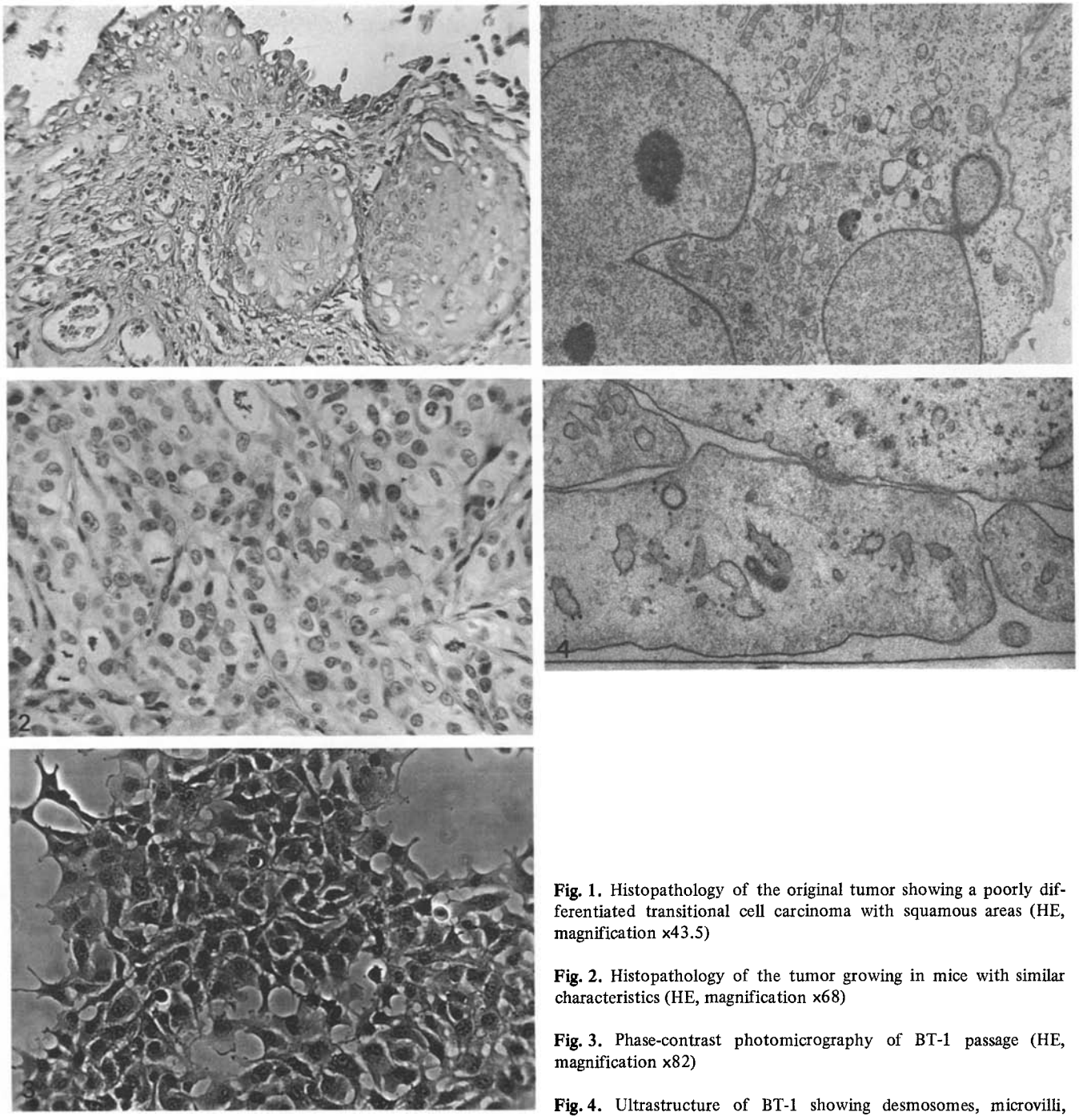

Fig. 1. Histopathology of the original tumor showing a poorly differentiated transitional cell carcinoma with squamous areas (HE, magnification x43.5)

Fig. 2. Histopathology of the tumor growing in mice with similar characteristics (HE, magnification x68)

Fig. 3. Phase-contrast photomicrography of BT-1 passage (HE, magnification x82)

Fig. 4. Ultrastructure of BT-1 showing desmosomes, microvilli, tight junctions and numerous mitochondria

tight junctions and microvilli. The cystoplasm of most cells contained numerous mitochondria and abundant endoplasmic reticulum (Fig. 4).

Cytogenetic and Flowcytometric Analysis

At passage 27 the chromosome counts of the BT-1 cell line ranged from 72 to 80 chromosomes. The modal count was 76, with polysomy for chromosomes 1, 5, 6, 7, 14, 17, 20, 21, 22, and twenty-two marker chromosomes. In order to define the stemline chromosomal pattern of our cell line, the karyotypes of several independent harvests were studied by RBG-banding. As shown in Fig. 5, there is surprisingly little difference between the complements of two such independent harvests. With the exception of a single discordant marker (arrowhead in Fig. 5), structural discordancies between the number two pair, and numerical differences involving chromosomes 16 and 19, the complements are identical. One group of markers (shown at the left bottom position of each karyotype) consists of presumptive isochromosomes, notably isochromosomes 8q and

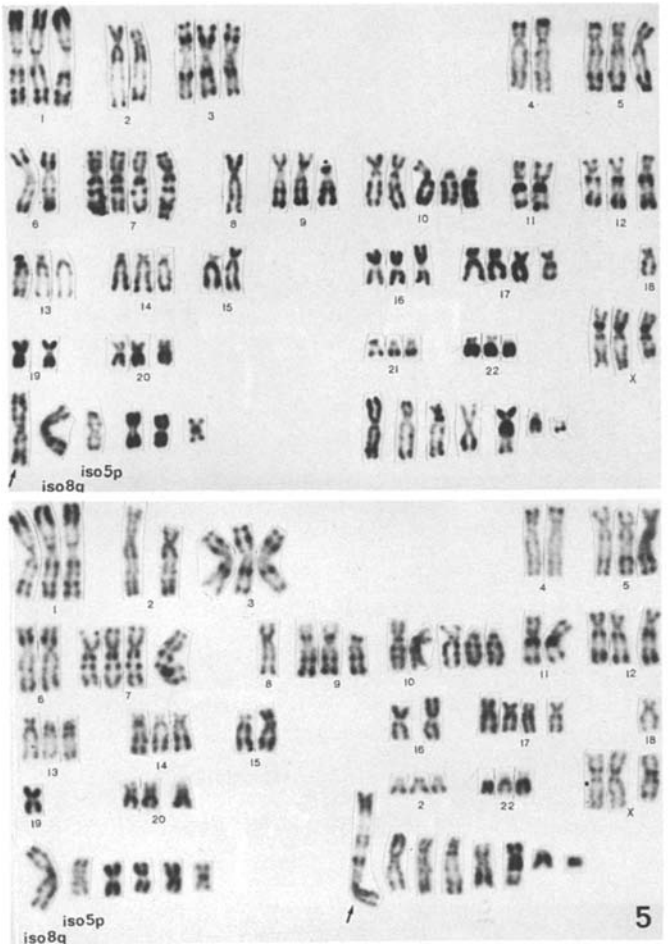

Fig. 5. RBG-banded karyotypes of independent harvests of the BT-1 mass culture. Arrows denote discordant marker chromosomes. Note tetrasomy 7. presumptive pentasomy 10q and presumptive tetrasomy 17. The darky stained metacentrics next to iso 5p are presumptive isochromosomes 22q. The derivation of the bottom right hand series of markers is uncertain. See text for details

Fig. 6. Representative sets of rearranged and marker chromosomes from the mass culture and three clonal isolates (A, E, F) of the BT-1 line. Concordant chromosomal phenotypes are connected by lines. Note constant presence of markers isochromosomes 8q and 5p, the latter as double copies in the mass culture

5p. The derivation of the right-hand group of markers is less clear.

In order to see whether the mass culture cytogenetic pattern would be preserved under cloning conditions, a series of clones were prepared by dilute plating and isolation by the cloning cylinder technique. The clonal isolates were harvested for chromosome analysis when they had undergone an estimated 20 to 23 population doublings from the time of dilute plating. Figure 6 shows a comparative panel of partial karyotypes, depicting selectively all altered chromosomes and unidentified markers. The panel confirms an impressive degree of clonal preservation of the mass culture pattern characteristic for the BT-1 culture. This pattern clearly defines a stemline complement that consists of a series of chromosomal phenotypes which are specific for the BT-1 cell line.

Because of the variation in preparation it is difficult to investigate subtle changes of total genomic size in human hyperdiploid cells by conventional cytogenetics. We therefore employed flowcytometric DNA content determinations in order to pursue the question of genomic stability during longterm serial passage of the BT-1 line. In a series of clones and subclones derived from the BT-1 mass culture, flowcytometry revealed a substantial degree of DNA content variation (Fig. 7). The extent of this variation is depicted as a frequency distribution of individual DNA content measurements of 23 primary isolates (solid bars at the right hand vertical axis of Fig. 7). Subclones derived from each of these primary isolates (open circle symbols in Fig. 7) appear to both mimic and amplify the suggestive bimodal pattern of DNA content variation observed among the primary clones. Seven of the various subclones show a fluorescence ratio of less than 1.4; this very low DNA fluorescence has no obvious equivalent among the series of parental primary clones. Altogether, the data displayed in Fig. 7 illustrate that the DNA fluorescence varies from 0 to a maximum of 7% among independent primary clonal isolates from our cell line: If one measures the relative DNA fluorescence of subclones derived from these primary isolates, the intersample variation becomes twice that large.

Isoenzyme Analysis

The G6PD isozyme pattern was identical with the B type (usual caucasian).

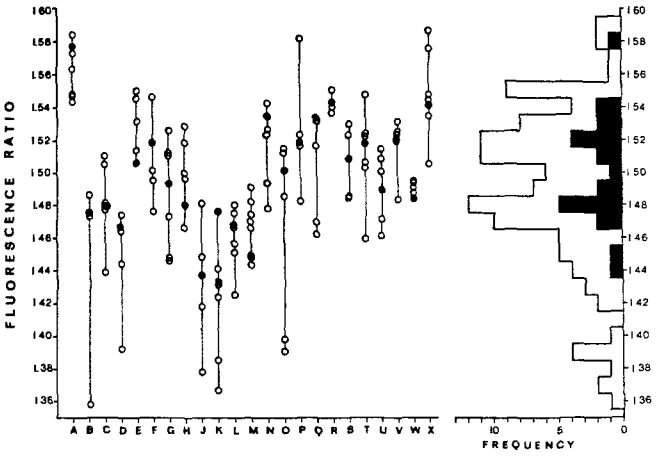

Fig. 7. DNA content variation among clones and subclones of line BT-1. The relative DNA content is expressed as fluorescence ratio of BT-1 clones to cocultivated human diploid fibroblasts-like cells (highly stable karyotype 46.XX). Relative to this standard, a precisely triploid tumor cell clone would have a fluorescence ratio of 1.5. Note that the frequency distribution of fluorescence ratios is bimodal for clones *(solid bars)* and multimodal for subclones *(open bars)*. Individual clonal isolates are identified by capital letters, their corresponding subclones *(open circles)* are connected to the parenteral clones *(solid circles)* by lines. See text for details

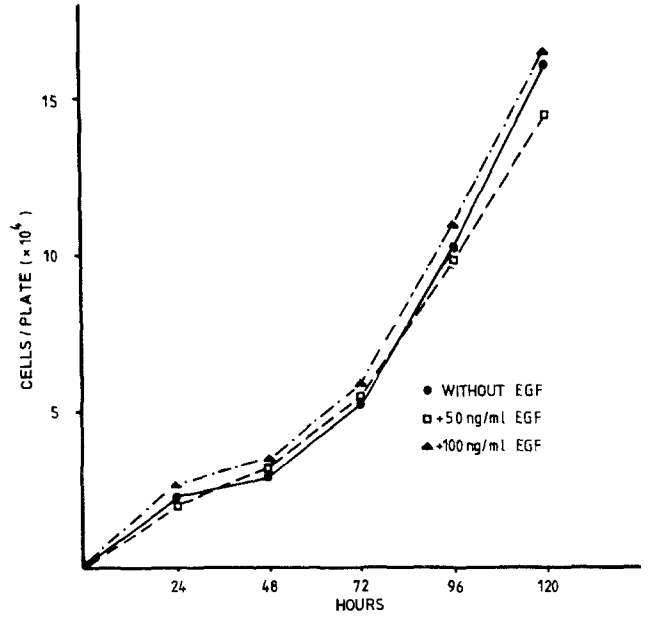

Fig. 8. Growth curves of the BT-1 cells at passage 34 cultured in DMEM/10% FCS without or with 50 or 100 ng/ml EGF

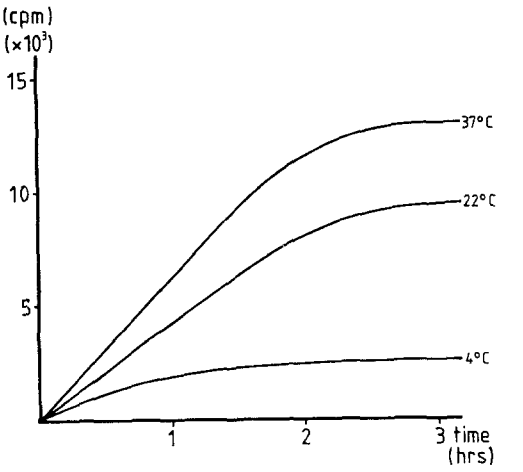

Fig. 9. Analysis of temperature dependent binding of ^{125}J-EGF to the EGF-reeceptors of the BT-1 cells

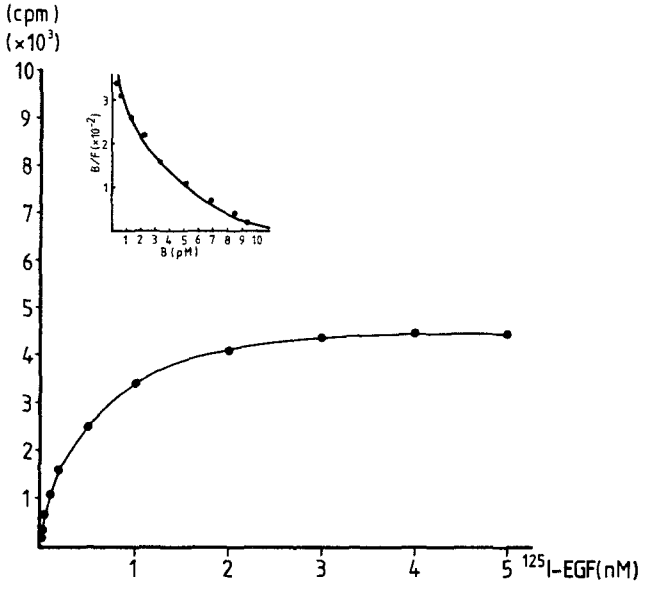

Fig. 10. Analysis of ^{125}J-EGF binding at various concentrations to BT-1. The specific binding was calculated by subtracting the unspecific binding from the mean of duplicate total binding values. Inset, Scatchard plot to the specific binding

EGF-Receptors on the BT-1 Cell Line

Provisional studies comparing the binding of EGF at 4 °C, 22 °C and 37 °C in the BT-1 cell line demonstrated an increased binding with increasing temperature. An equilibium was reached within 3 h (Fig. 9). The BT-1 cells have high- and low-affinity binding sites for EGF. Scatchard plots of the specific binding of ^{125}J-EGF to the BT-1 cells generated a curved line indicating two classes of binding sites with dissociation constants (KD) of 4.4×10^{-9} and 2.7×10^{-10} (Fig. 10). With increasing passage there was a insignificant decrease of the EGF binding sites (data not shown).

Discussion

Attempts to initiate permanent epithelial cell cultures from surgical tumor specimens have generally met with limited success. Since the original report of Williams [22] a number of cell lines of human transitional cell carcinoma origin have, however, been established.

Our newly established cell line BT-1 has a remakably stable phenotype as evidenced by chromosomal and flow cytometric analysis at passages 14 through 56. By cytometric criteria and G6PD pattern, BT-1 is not a Hela contaminant.

From a cytogenetic point of view, the newly described cell line has a unique and highly consistent chromosome pattern. There is no indication whatsoever for its non-human or Hela cell origin. The numerous marker chromosomes characterizing the BT-1 line are exeedingly stable, as evidenced by their global persistence throughout the expansion of single cells to clonal cultures.

Three of the observed cytogenetic alterations deserve a special comment. One is that all clonal and mass culture harvests contained 4 complete copies of chromosome 7. The quadruple dose of this chromosome is interesting, since the EGF receptor gene has been mapped to 7p11-p31, and since polysomy for chromosome 7 has been described both in transitional carcinoma of the bladder and in transitional cell carcinoma of the ureter [7, 19]. Other chromosomes that are present in excess of trisomy are chromosomes 10q, 17 and 22 (the latter possibly as double isochromosomes). According to the 1985 human gene mapping conference [9], each of these chromosomes carries gene loci that could play a role in the control of cellular proliferation (e.g. adenosine kinase on chromosome 10q: c-sis on chromosome 21; and the p53 tumor antigen, the c-erb-A1 oncogene and the thymidine kinase gene on chromosome 17).

The second remarkable finding is the constant presence, in all our BT-1 harvests, of an isochromosome for the short arm of chromosome 5. Although our figures do not show this abnormality clearly, we have no doubt that an iso 5p chromosome is a highly consistant marker of our cell line. Occasional harvests contained two complete copies of this marker (e.g. Fig. 6).

Since the iso 5p marker has been described as the most consistent nonrandom chromosomal change in direct preparations of transitional carcinoma of the bladder [7, 16], this finding lends further credit to the origin of our cell line from the bladder transtitional epithelium. Other, although less specific, changes described in direct preparations of transitional bladder carcinoma (e.g. rearrangements involving chromosome 11: Gibas et al. [7]) were not as obvious in the BT-1 line.

Finally, all the cytogenetic examinations suggest that BT-1 may be monosomic for the short arm of chromosome 8, whereas the long arm of this chromosome is trisomic by way of the formation of an 8q isochromosome. The 1985 gene map for chromosome 8q, yields no obvious clues as to why monosomy 8q could be important for the establishment, immortality or tumorgenicity of the BT-1 line.

In contrast to their remarkable stability under mass culture conditions, cloned BT-1 cells showed a considerable degree of genomic divergence as determined by their mean DNA fluorescence relative to an invariant diploid cell standard. Under carefully controlled conditions of cell preparation and cell staining, the measurement of nuclear fluorescence is highly correlated to the chromosome constitution of cultured cells [3, 11]. Our extensive flowcytometric studies revealed that clonal derivatives from the mass culture differ up to 7% in their mean DNA content, although independent mass culture harvests had nearly constant DNA values (Data not shown). This indicates that genomic variants may arise more frequently under cloning and subcloning conditions. A similar observation of more frequent DNA content variation among clonal isolates than among replicate harvests of the mass culture has been reported for spontaneously transformed cell lines of murine origin [12].

The finding of growth factor activity in this bladder cancer cell line is consistent (a) with the detection of transforming growth factors in the urine of patients with neoplastic disease [10], (b) the fact that a human bladder cancer cell line can stimulate its own growth [14] and (c) the isolation of ras oncogene in the bladder cancer cell limes T-24 and EJ. It is also consistant with the proposed regulation of cell growth by the ras p21 product and other growth factors via specific receptors and transducers of the postreceptor signals [16].

EGF, which can replace TGF-α, is known to be mitogenic in various cell systems. In tumor cells, the detection of EGF receptors on the cell surface could be envisaged as a potential mechanism whereby the tumor shows increased sensitivity to EGF in its environment. The addition of exogenous EGF, or the partially purified BT-1 cell extract to the BT-1 cells, did not change the growth characteristic of this particular cell line, although the concentrations of EGF used in the binding and growth studies reflected the range normally found in human urine. The amounts of EGF added were equal or exceeded those required for in vitro biological activity on a variety of human cell lines [14]. Even though the BT-1 cells carry intact EGF-receptors, their failure to be stimulated does not disprove the possibility of an autocrine mode of growth stimulation for this particular type of tumor cell. The reason for the absent response to EGF might be a failure in the postreceptor signal pathway, or be due to a high net production of negative stimulating factors which antagonizes cell stimulation. Another possibility would be a lesion in the receptor of the positive and negative signalling pathways [16]. Like other human bladder cancer cell lines the newly described BT-1 line proved sensitive to α-IFN in vitro [1].

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