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In Vitro Chemosensitivity of J-82 Human Bladder Cancer Cells*

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Summary. While chemotherapy offers a valuable adjunct to surgery in the management of intravesical bladder cancer, an accurate in vitro predictive test for chemosensitivity has yet to be developed. Drug sensitivity of the human bladder cancer cell line J-82 was assessed using monolayer, stem cell and [^{3}H]thymidine incorporation assays. The 72-h monolayer assay provided a rapid reflection of in vitro drug sensitivity and when combined with the labeling index the results generally paralleled those obtained with the soft agar stem cell assay without the associated large commitment of time and labor. It is suggested that 72-h monolayer assay alone or in combination with [^{3}H]thymidine labeling index may offer valuable insight into the chemotherapeutic response of bladder tumors.

Key words: Bladder cancer, In vitro chemosensitivity, J-82, Stem cells.

Introduction

Intravesicular chemotherapy is a viable adjunct to the endoscopic management of bladder tumors [4, 21] and has been used prophylactically to prevent tumor recurrence [1, 10]. The entire urothelial lining can be exposed to a high concentration of drug when instilled intravesically, with minimal systemic toxicity [9]. Recent attempts to screen chemotherapeutic agents for individual patients have focused on the in vitro chemosensitivity of primary cultures obtained via bladder washings or transurethral resection. In vitro sensitivity has previously been evaluated using parameters such as cellular morphology [24, 26], plating efficiency [27], vital dye exclusion [25], inhibition of cell proliferation or metabolism [7, 11], monolayer colony formation [12] and radiolabeled DNA precursor incorporation [8, 17, 22]. Additionally, Hamburger and Salmon introduced the soft-agar clonogenic assay to evaluate the drug sensitivity of human tumors [6, 16]. In this assay the impact of an agent on tumor stem cell growth is reflected by inhibition of colony formation. While, interpretation of results obtained from monolayer culture assays may be complicated by presence of heterogenous cell types in primary cultures [20], inherent disadvantages in clonogenic assay include difficulty in obtaining single cell suspension from solid tumors, extremely low plating efficiencies, prolonged incubation periods and laborious colony counting [13].

In this study we have utilized an established human bladder cancer cell line (J-82) and six antineoplastic agents to compare the various in vitro assays in an attempt to determine which, if any, produce the best assessment of chemotherapeutic efficacy against cultured cells, and to characterize the chemosensitivity of J-82 to each.

Materials and Methods

Cell Line Maintenance

The human bladder cancer cell line J-82 (Cat. # HTB-4, American Type Cell Culture Collection, Rockville, MD) was maintained as a monolayer culture at 37 °C in an atmosphere of 95% air-5% CO_2 with a 95% relative humidity. All cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 1.0 μg/ml corticosterone, 0.5 μg/ml insulin, 1.0 μg/ml ovine prolactin, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.025 μg/ml Fungizone).

Monolayer Assay

J-82 cells were plated in 24 well multiwell plates (16.7 mm^2; Falcon Plastics, Oxnard CA) at a density of 5.5 x 10^4 cells/well in 0.5 ml of supplemented EMEM. For determination of the 24-h dose response curves, cis-platinum (CP), thiotepa (THT), 5-fluorouracil (5-FU), mitomycin C (MC), adriamycin (ADM), and melphalan

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(MEL) were used at concentrations ranging from 10^{-3} M to 10^{-9} M in supplemented EMEM. All drugs were administered for 24 h to cultures in the log phase of growth. The cells were washed three times with Ca^{++}-Mg^{++}-free phosphate buffered saline to remove deteached nonviable cells. Attached viable cells were removed from the culture plates by use of a trypsin-Versene mixture (M.A. Bioproducts, Walterksville, MD). Cell numbers were determined in a Coulter counter (Coulter Electronics, Hialeah, FL) and results were expressed as a percentage of control values. Cell growth was determined using the following equation:

$$\text{Percent of cell growth} = T - C_0/C - C_0 \times 100\%$$

where T is the number of cells following treatment, C_0 represents the number of cells at the onset of treatment, and C equals the number of cellc in non-treated controls at the termination of the drug treatment [28].

72 h doese responses were determined similarly, except that after 24 h of treatment drug containing media was removed and the remaining attached cells were refed with drug-free supplemented EMEM for an interval of 48 h. Cell number was determined as previously and the results expressed as a percentage of control valures.

Thymidine Labeling Index

For labeling index, monolayer cultures were treated with agents for 24 h followed by a 30 min pulse of 1 μCi/ml [^{3}H]thymidine (6.7 Ci/mM, New England Nuclear, Boston, MA). All cells were washed three times with Ca^{++}-Mg^{++}-free phosphate buffered saline and fixed with 10% neutral buffered formalin. Cutures were dehydrated through graded ethanols (30% to 100%) and coated with diluted nuclear track emulsion (NTB-3, Eastman Kodak, Rochester, NY), exposed for 6 days at 4 °C [2, 14]. After development cultures were stained and the number of labeled nuclei counted. A minimum of 3,000 cells was counted in each culture and the number of S-phase cells present after drug treatment was expressed as a percentage of controls. Labeled cells were defined as those containing 7 or more nuclear grains. Adequate rinsing removed any unincorporated label, thus reducing background to an average of 7–10 grains per 50 μm^2 [18].

Clonogenic Assay

Single-cell suspension of J-82 cells at 5.0 x 10^4 cells/ml density were exposed to agents at concentraions of 10^{-4} M and 10^{-6} M for 1 h at 37 °C. Following drug exposure cell suspension were washed with drug-free EMEM and plated in Difco-Bacto agar (0.3% w/v) prepared in supplemented CMRL 1066 medium as previously described [15]. Colonies containing 30 or more cells appeared in 14–21 days and were counted using an inverted microscope equipped with phase contrast optics. The impact of drug on colony formation was expressed as a percentage on controls.

Results

Monolayer Cell Culture: 24, 72 Hour Assay

At a seeding density of 5.5 x 10^4 cells/wells, J-82 cells exhibited a doubling time of 1.5 days. After 24 h of continuous drug exposure, J-82 cells proved to be resistant to both THT and 5-FU at all molar concentrations tested (<

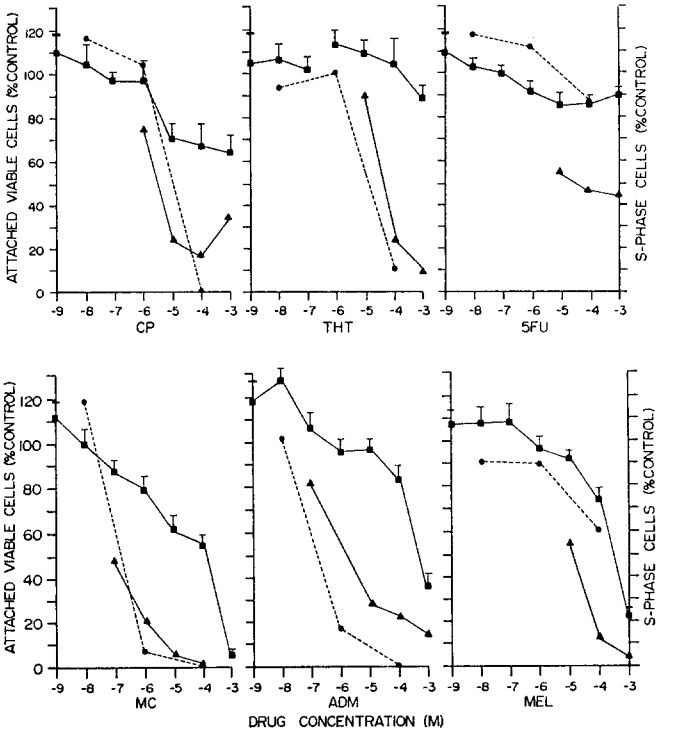

Fig. 1. Dose response curves. Monolayer cultures of J-82 human bladder tumor cells were treated with concentrations of cis-platinum (CP), thiotepa (THT), 5-fluorouracil (5FU), mitomycin C (MC), Adriamycin (ADM) or melphalan (MEL) ranging from 10^{-3}–10^{-9} M. Cultures were treated for either 24 h (▪) or for 24 h followed by a 48 h drug-free interval (▲). The tyhmidine labeling indices of the 24-h assay is also presented (●). All data points represents the results of triplicate counts from three separate experiments

10% growth inhibition), however, less resistance was recorded with CP which induced a 30% reduction between 10^{-5} to 10^{-3} molar concentrations (Fig. 1). Although ADM and MEL were effective at very high concentrations (10^{-3} M), their effectiveness decreased as the concentrations reaches clinically achievable range (10^{-6} M to 10^{-9} M). Mitomycin C appeared to be the most effective agent which demonstrated a dose dependent cytotoxicity and a LD_{50} at approximately 10^{-4} M (Fig. 1).

In the 72-h exposure study, drug-induced damages including metabolic and reproductive death were demonstrated. At 10^{-3} M all agents except 5-FU demonstrated greater than 85% cell kill (Fig. 1). Mitomycin C, deemed the most cytotoxic by the 24-h monolayer assay, exhibited a LD_{50} at 10^{-7} M concentration. A similar degree of cell lethality was also achieved by ADM (10^{-7} M), however, CP, THT, and MEL were less effective requiring a 10–100 fold higher drug concentrations to achieve a similar degree of growth inhibition (Fig. 1).

Thymidine Labeling Index

At 10^{-4} M concentrations or higher, CP, MC or ADM exposure resulted in complete inhibition of DNA synthesis.

Table 1. Colony Forming Assay

Agents	Percent of Inhibition	
	10^{-4} M	10^{-6} M
5FU	17%	5%
THT	27%	6%
CP	49%	9%
ADM	92%	20%
MEL	97%	12%
MC	97%	82%

Note: Percent of inhibition represents the inhibition of colony growth as compared to notreated control cultures. All data points were determined from three dishes/trial and from triplicate trials

At a clinically achievalbe concentrations (10^{-6} M) the number of S-phase cells in cultures exposed to both MC and ADM resulted in a reduction of 80–90%. The remaining drugs had less impact on DNA precursor incorporation between 10^{-4} M and 10^{-6} M concentrations (Fig. 1).

Clonogenic Assay

Reproductive integrity of tumor stem cells is reflected by the number of colonies formed after treatment. At 10^{-4} M or higher concentrations MC, MEL, and ADM severely impacted the proliferative capactity of J-82 human bladder cancer cells resulting in 97%, 97% and 92% colony inhibition, respectively (Table 1). While CP demonstrated moderate inhibition (49% reduction) at 10^{-4} M concentration, THT and 5FU were ineffective at similar molar concentrations. All agents with the exception of MC were ineffective in inhibiting colony formation ($<$ 80% inhibitions) at or lower than 10^{-6} M concentrations (Table 1).

Discussion

Sensitivity of the human bladder cancer cell line J-82 to six chemotherapeutic agents was assessed by four different in vitro assays. The monolayer assay is based upon the fact that non-viable cells can be removed easily through rinsing procedure [24]. Hence, the differences in cell number of treated versus non-treated controls indirectly reflect cell viability [3]. When comparing the 72-h monolayer assay to either the [^{3}H]tyhmidine labeling indices or the stem cell assay, good correlation generally prevailed. However, the 24-h monolayer assay correlated poorly with both the [^{3}H]tyhmidine labeling index and the clonogenic assay. It is likely that only the acute cytotoxcity was evident by the 24-h assay. When extended to 72 h, eventual metabolic death and the subsequent cessation of reproduction was revealed. This is corroborated by the paralleled decrease of radiolabeled DNA precursor incorporation at similar molar drug concentrations [19]. While significant cell lethality of MC and ADM was reflected by all three assays modest correlation for the less cytotoxic agents (CP, THT, 5FU, and MEL) was observed at similar molar concentrations.

The data indicate that a 72-h monolayer assay alone or in conjunction with the [^{3}H]thymidine labeling indices offers a rapid, accurate means of assessing the efficacy of chemotherapeutic agent against cultured cells in vitro. The 72-h monolayer assay also generally supported the results obtained with the clonogenic assay. The ideal assay of chemotherapeutic efficacy should combine both the ease of administration and economy of time and materials while accurately reflecting drug sensitivity [5, 23]. Our data demonstrate that a 72-h monolayer assay reflects a response to drug exposure paralleling [^{3}H]thymidine incorporation, a parameter recently shown to reflect the potential clinical value of a given agent [22]. This assay may well be adequate in determining the efficacy of chemotherapeutic agents in the screening antineoplasic agents for individual patients. While the degree of correlation between predicted responses and actual clinical course has yet to be demonstrated using this anchorage dependent system, it is hoped that this simple method will eventually aid in the management of urologic neoplastic disease.

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