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Chemosensitivity study of urological malignancies using a novel dye-exclusion method

Katsusuke Naito, Haruo Hisazumi, Shinya Mihara, Toyoki Asari, Kazunori Kobashi, Toshiyasu Amano, and Tadao Uchibayashi

Department of Urology, School of Medicine, Kanazawa University, 13-1, Takara-machi, Kanazawa 920, Japan

Summary. The chemosensitivity of several urological malignancies was determined using a novel dye-exclusion method. The tests proved to be successful in 22 out of 33 urothelial transitional-cell carcinomas, 21 out of 29 renal-cell carcinomas, and in 8 out of 13 testicular tumors, but in 0 out of 4 Wilms' tumors. At 10% of the peak plasma concentration of anticancer drugs achievable after single bolus injection, the sensitivity rates of urothelial transitional-cell carcinomas for cis-platinum, adriamycin, and carboquone were 27.3%, 13.3%, and 5.6%, respectively. At the peak plasma level, urothelial transitional-cell carcinomas were 'susceptible' to cis-platinum, carboquone, and adriamycin, the sensitivtiy rates being 60%, 50%, and 30%, respectively. The number of these carcinomas susceptible at the peak plasma level was significantly larger than that at 10% of the peak plasma level ($P < 0.01$). However, renal-cell carcinomas exhibited a low sensitivity rate of 38% even when exposed to the peak plasma level. A similar trend was observed when tumor chemosensitivity was determined using a modified human tumor clonogenic assay. These results suggest that urothelial transitional-cell carcinomas may show good responses when anticancer drugs are given at high concentrations using methods such as intra-arterial infusion and intravesical instillation.

Introduction

Since the 1950s, many investiagtors have tried to develop in vitro or in vivo methods to predict the response of individual tumors to anticancer drugs [13, 16]. Such chemosensitivity assays could play an important role in selecting the most efficient drugs with which to treat cancer patients. In addition, such assays would protect patients from adverse reactions resulting from the selection of an unsuitable drug. Recently, Hamburger and Salmon [4, 9] have described a human tumor clonogenic (HTC) assay using a double-layer soft-agar system. Despite early favorable reports [6, 10, 14], this assay is still regarded with considerable scepticism, and several critical appraisals of the procedure have recently been published [1, 8, 11].

Several investigators have used dye-exclusion techniques to estimate cell death after exposure to cytotoxic drugs: viable cells have the ability to exclude dyes such as eosin, nigrosin, trypan blue, and erythrocin B. However, many investigators have reported a poor correlation between dye-exclusion tests and other in vitro tests for measuring the reproductive capacity of cells in culture after exposure to drugs [7]. Weisenthal et al. [17–19] have reported a novel dye-exclusion (NDE) assay using hematoxylin and eosin (H & E) along with Fast Green after a 4-day preincubation. This method makes it possible to distinguish viable tumor cells from dead cells and nontumor cells after a certain period of drug exposure. The purpose of the present study was to apply this method to urological malignancies and to compare the results with previously described clinical responses. Also, the results of applying a modified version of Hamburger and Salmon's clonogenic chemosensitivity test [4] for urological malignancies are compared to those obtained using the NDE assay.

Materials and methods

Fresh tumor specimens from 79 patients with malignancies of the urinary tract were minced into 1- to 2-mm^3 fragments and exposed to an enzyme cocktail [5] for 30 min. The specimens were then passed through a sterile gauze and resuspended in Ham's F12 medium supplemented with 15% heat-inactivated fetal calf serum (FCS) and 50 μg/ml kanamycin. The viability of the cells was determined by 0.5% trypan-blue dye exclusion.

For each drug to be assayed, between 1×10^5 and 1.5×10^5 viable cells were aliquoted into individual polypropyrene tubes (Falcon 2005 tube; Becton Dickinson, USA) to prevent fibroblast growth. The cells were then suspended in 0.9 ml medium. Either 0.1 ml the drug solution or 0.15 *M* NaCl (as a control) was added to samples. After incubation at 37° C for 1 h, the cells were centrifuged, washed twice with fresh medium, resuspended in 1.0 ml fresh medium, and cultivated for 4 days at 37° C in a humidified atmosphere consisting of 5% CO_2 in air. The cells were then concentrated to a density of 10^5 cells per 0.2 ml by centrifugation. After this, 0.2 ml 2% Fast Green dye in 0.15 *M* NaCl was added, and the cell suspension was agitated using a vortex mixer. Exactly 10 min after the addition of the dye, the cell suspension was sedimented onto microscope slides using a centrifugal cell collector (Tominaga, Tokyo, Japan), and counterstained using a modified H & E technique [17]. 'Living' cells stained reddish-pink with H & E, while 'dead' cells stained green. The ratio of 'living' cells versus 'living' cells plus 'dead' cells was deter-

Offprint requests to: Katsusuke Naito

Table 1. The anticancer drugs investigated in vitro

Drug		Concentration range tested (μg/ml)	Single concentration used in study (μg/ml)
Adriamycin	(ADR)	0.005 – 0.5	0.05
Cis-platinum	(CDDP)	0.025 – 2.5	0.25
Carboquone	(CQ)	0.0005 – 0.05	0.005
Vinblastine	(VBL)	0.01 – 1.0	0.1
THP-adriamycin	(THP)	0.0005 – 0.05	0.005
Interferon alpha	(IFN-α)	10 – 1,000[a]	100.0[a]
Peplomycin	(PEP)	0.01 – 1.0	0.1
Vincristine	(VCR)	0.005 – 0.5	0.05
Actinomycin-D	(ACT-D)	0.001 – 0.1	0.01
Etoposide	(VP-16)	0.3 – 30	3.0

[a] International units per milliliter

mined for each slide and then expressed as a percentage of the control value. In Interferon experiments, the drug was not washed out but remained for the entire 4-day culture period.

Table 1 shows the anticancer drugs studied and their concentration upon exposure to tumor cells, i.e., the estimated peak plasma concentration after a bolus injection; 10% and 1% of the plasma concentration were used. However, in some samples, only the 10% concentration was studied, as indicated by the single concentrations given in Table 1.

In an additional experiment, a modified version of the HTC assay described by Hamburger and Salmon [4, 9] was tested in 92 patients with malignancies of the urinary tract. In this modified HTC assay, 1-ml feeder layers of 0.5% agarose in Ham's F12 medium supplemented with 15% FCS were poured into 35-mm Petri dishes (Falcon 3001; Becton Dickinson). The cells tested were suspended in 0.24% agarose in Ham's F12 medium supplemented with 15% FCS to yield a final concentration of 10×10^4 cells/ml, and 1 ml of this mixture was poured over the feeder layer. The dishes were incubated at 37° C in 5% CO_2 in humidified air for 3–4 weeks. All assays were performed in triplicate. The number of colonies was counted using a colony counter (Shiraimatsu CP-2000, Osaka, Japan). Colonies were defined as aggregates consisting of more than 50 cells. At least 30 tumor colonies in each control dish were required to provide an effective evaluation of the drug. The drug exposure time and drug concentration were the same as those in the NDE assay, so that the results could be compared with the NDE-assay results.

A cell survival rate of 30% or less in the NDE assay and a colony-forming inhibition rate of 70% or more in the modified HTC assay were arbitrarily defined as indicating 'susceptibility' to the drug. To analyze the results in both assays, the sensitivities at the peak plasma drug level after a bolus injection (defined as a high concentration) and at one-tenth of that level (defined as a low concentration) were studied.

Table 2. Assay success rates using our novel dye-exclusion method

Tumor type	Specimens studied	Successful assays
Renal-cell carcinoma	29	21 (72.4%)
Urothelial transitional-cell carcinoma	33	22 (66.7%)
Testicular tumor	13	8 (61.5%)
Wilms' tumor	4	0 (0.0%)
Total	79	51 (64.6%)

Results

Cell survival rates adequate for the NDE assay were obtained in 21 of the 29 renal-cell carcinomas, 22 of the 33 urothelial transitional-cell carcinomas, and 8 of the 13 testicular tumors, but in none of the 4 Wilms' tumors (Table 2). The assay was technically successful in 51 of the 79 samples employed. In renal-cell carcinomas, 3 out of 13 tumors were susceptible to adriamycin, 1 out of 17 were susceptible to vinblastine, 3 out of 5 were susceptible to Interferon – α, and 0 out of 9 were susceptible to cis-platinum at the low concentration (Table 3). In 10 of the 49 chemosensitivity tests, susceptibility to the drugs at the low concentration was observed, and in 8 out of 21 tests, susceptibility was observed at the high concentration (Table 3). There was no significant difference between the chemosensitivity rates for the low and high concentrations.

In urothelial transitional-cell carcinomas, 2 out of 15 tumors were susceptible to adriamycin, 6 out of 22 were susceptible to cis-platinum, 1 out of 18 were susceptible to carboquone, and 0 out of 12 were susceptible to THP-adriamycin at the low concentration (Table 4). At the high concentration, 3 out of 7 urothelial transitional-cell carcinomas were susceptible to adriamycin, 6 out of 10 were susceptible to cis-platinum, 5 out of 10 were susceptible to carboquone, and 2 out of 4 were susceptible to THP-adriamycin (Table 4). At the low concentration, only 9 out of 67 chemosensitivity tests revealed susceptibility to the drugs, whereas 16 out of 31 tests demonstrated susceptibility to the drugs at the high concentration. The number of tests results showing susceptibility at the high concentra-

Table 3. Number of renal-cell-carcinoma specimens susceptible[a] to anticancer drugs (NDE assay)[b]

Drug / Drug concentration	ADR	VBL	IFN-α	CDDP
Low	3/13 (23.1%)	1/17 (5.9%)	3/5 (60.0%)	0/9 (0.0%)
High	3/7 (42.9%)	3/8 (37.5%)	2/5 (40.0%)	0/1 (0.0%)

[a] A cell survival rate of 30% or less was arbitrarily defined as indicating susceptibility to the drugs.
[b] Specimens susceptible to the drug / specimens studied

Table 4. Number of urothelial transitional-cell-carcineoma specimens susceptible[a] to anticancer drugs (NDE assay)[b]

Drug / Drug concentration	ADR	CDDP	CQ	THP
Low	2/15 (13.3%)	6/22 (27.3%)	1/18 (5.6%)	0/12 (0.0%)
High	3/7 (42.9%)	6/10 (60.0%)	5/10 (50.0%)	2/4 (50.0%)

[a] A cell survival rate of 30% or less was arbitrarily defined as indicating susceptibility to the drugs.
[b] Specimens susceptible to the drug / specimens studied

Table 5. Success rates using the HTC assay

Tumor type	Specimens studied	Adequate growth for drug testing	Plating efficiency (%)[a]
Renal-cell carcinoma	43	12 (27.9%)	0.052 ± 0.040
Urothelial transitional-cell carcinoma	40	20 (50.0%)	0.075 ± 0.057
Testicular tumor	6	1 (16.7%)	0.033
Wilms' tumor	3	0 (0.0%)	–
Total	92	33 (35.9%)	–

[a] $\frac{\text{Number of colonies}}{\text{Number of plating cells}} \times 100$; mean ± SD

Table 6. Number of renal-cell-carcinoma specimens susceptible[a] to anticancer drugs (HTC assay)[b]

Drug / Drug concentration	ADR	VBL	IFN-α	CDDP
Low	2/9 (22.2%)	4/12 (33.3%)	0/4 (0.0%)	1/4 (25.0%)
High	2/5 (40.0%)	2/6 (33.3%)	0/3 (0.0%)	0/1 (0.0%)

[a] A colony-forming inhibition rate of 70% or more was arbitrarily defined as indicating susceptibility to the drugs.
[b] Specimens susceptible to the drug / specimens studied

Table 7. Number of urothelial transitional-cell-carcinoma specimens susceptible[a] to anticancer drugs (HTC assay)[b]

Drug / Drug concentration	ADR	CDDP	CQ	THP
Low	3/12 (25.0%)	4/19 (21.1%)	4/16 (25.0%)	1/7 (14.3%)
High	6/10 (60.0%)	3/12 (25.0%)	5/12 (41.7%)	1/2 (50.0%)

[a] A colony-forming inhibition rate of 70% or more was arbitrarily defined as indicating susceptibility to the drugs.
[b] Specimens susceptible to the drug / specimens studied

tion was significantly greater than that at the low concentration ($P < 0.01$).

In the NDE assay, 28 out of 79 tumors were not successfully assayed because of staining errors (1 tumor), bacterial contamination (5 tumors), setting-up errors (7 tumors), and the absence of viable cells after the 4-day culture (10 tumors).

In the modified HTC assay, colony growth suitable for drug evaluation was obtained in 12 out of 43 renal-cell carcinomas and in 22 out of 40 urothelial transitional-cell carcinomas (Table 5). The number of colonies and the plating efficiency in controls are shown in Table 5. In renal-cell carcinomas, 7 out of 29 chemosensitivity tests revealed susceptibility to the drugs at the low concentration, and 4 out of 15 tests revealed susceptibility at the high concentration (Table 6). In urothelial transitional-cell carcinomas, 12 out of 54 chemosensitivity tests revealed susceptibility at the low concentration, and 15 out of 36 tests demonstrated susceptibility at the high concentration (Table 7).

The results obtained with the two chemosensitivity tests in 14 tumors are compared in Table 8: in the cases where one test indicated that a tumor was susceptible the other test always indicated resistance, while good agreement was found on resistance in 28/36 tests.

Table 8. A comparison of the results obtained using the two chemosensitivity tests[a]

Tumor type \ Drug	ADR	CDDP	CQ	THP	VBL	IFN-α
RCC	R/R				R/R	
RCC	R/R				R/R	
RCC	R/R				R/R	R/S
RCC		R/R			R/R	
TCC	R/S	R/S	R/R			
TCC	R/R	R/S	R/R			
TCC	R/R	R/R	R/R			
TCC	R/R	S/R	S/R			
TCC		R/S	R/R	R/R		
TCC		R/R	R/R	R/R		
TCC	R/R	R/R	R/R			
TCC	R/R	R/R	R/R			
TCC	R/S	R/R				
TCC		R/R				

RCC, renal-cell carcinoma; TCC, urothelial transitional-cell carcinoma; S, susceptible; R, resistant
[a] HTC assay / NDE assay

Discussion

Our study showed that the NDE assay is a technically feasible method for determining the chemosensitivity of human urinary-tract tumor specimens in vitro. In chemosensitivity tests, the NDE assay had a significantly higher success rate than the modified HTC assay ($P < 0.05$). The chemosensitivity rate obtained for the NDE assay was almost identical to the clinical responses previously described by Yagoda [20] and Swanson [12]. The accuracy of the assay for urinary-tract malignancies should be determined by correlation of the test results and clinical data.

The loss of membrane integrity is a late event in cell death [17]. Some time has to elapse following drug treatment for lethally damaged cells to lose their membrane integrity. However, it has been concluded that dye-exclusion assays are inappropriate for measuring drug-induced cell death [7], because the time required for the loss of membrane integrity is ignored. Weisenthal et al. [17] and Bosanquet et al. [2] allowed 4 days to elapse after drug exposure period before the addition of Fast Green, and they also used a higher drug concentration than that used in the HTC assay to achieve equivalent levels of cell death. Although we used the same drug concentrations as those used in the HTC assay, the chemosensitivity rate obtained in our NDE assays was similar to the previously described clinical response [12, 20] for each drug tested. This result indicates that, at the drug concentrations used, many cells lose their membrane integrity within 4 days.

Weisenthal et al. found that all of their 19 specimens of transitional-cell carcinomas of the bladder showed susceptibility to a concentration of adriamycin (i.e., 100 μg/ml) that can easily be achieved by intravesical administration. Their 100% in vitro response rate exceeded that attained in clinical studies with topical [3] or systemic chemotherapy [20]. The in vitro evaluation of cell suspensions with drugs is problematic, because the environment of the cells in vitro, which differs from that in vivo, may alter their chemosensitivity. Furthermore, the optimal concentrations and exposure times for the tested drugs are unknown.

The data obtained using our NDE assay in renal-cell carcinomas indicated that, even when the drug concentration was increased, susceptibility did not increase. In urothelial transitional-cell carcinomas, however, the number of tests revealing susceptibility significantly increased in accordance with an increase in drug concentration. A similar trend was observed in the modified HTC assay.

These results suggest that urothelial transitional-cell carcinomas may show a good response when high concentrations of drugs are administered by methods such as intra-arterial infusion and intravesical instillation. In this context, it has been reported that a high response rate for transitional-cell carcinoma of the bladder can be obtained by the intra-arterial infusion of chemotherapeutic agents [15] or the intravesical instillation [3] of such drugs. On the other hand, all previous reviews of the application of cytotoxic chemotherapy for renal-cell carcinoma have failed to identify a single drug or combination of drugs that is consistently active against such carcinomas [12].

In chemosensitivity tests, it is necessary to obtain clear results for all cases studied. However, because of the low success rate observed for both chemosensitivity tests, the present results are insufficient to be used as a basis for clinical application. A great deal of further work will be necessary in order to improve assay systems suitable for particular tumors , and this will include the investigation of better growth media.

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