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Colony Formation Assay for In Vitro Chemosensitivity Test of Urologic Malignancies

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Abstract Over the past years we have attemptted to grow 79 urologic tumors using a soft agar colony formation assay similar to that originally described by Salmon and colleagues.

Formation of colonies in vitro occured in 45 of 79 primary urologic tumors (57%), including 25 of 41 uroepitherial cancer specimens, 12 of 19 renal cancer specimens and 5 of 12 testicular cancer specimens. Growth sufficient for chemosensitivity testing ranged from 45% of the testicular cancer cultured to 87% of the uroepitherial cancer cultured. Seven of uroepitherial cancer and four of testicular cancer were demonstrated in the dose of corresponding colony survival curve for Cisplatinum. Nine of renal cancer were demonstrated in the dose corresponding colony survival curve for Interferon. Seventeen patients were used by the identical drugs which were estimated from in vitro chemosensitivity testing. To predict clinical correlation, twelve drugs in ten cases were evaluated from the objective effects of the treatment. The predictability results were 67-75% true positive and 75-83% true negative, with 75% overall predictability.

This assay can therfore be used to study differences of biological characters including drug sensitivity.

Key Words : Colony formation assay, In vitro chemosensitivity test, Urologic malignancies,

Introduction

Soft agar colony formation assay had been used in vitro chemosensitivity testing many different institutions.

But, for clinical application, this assay system must dissolve problems such as cell aggregation, low growth rate, drug exposure time, cut off point in vitro, highly false positive predictability etc.. Nevertheless, there is a great interest in understanding the effective drugs in vitro using conventional soft agar colony formation assay.

At the time of data analysis, determining the cut off point and creating the dose corresponding curve are two of the most important factors among several exsisting problem. Therefore We discuss here the drug sensitivity testing based upon the pharmaceutical datas and the dose corresponding curve resulted from soft agar colony formation assay.

Materials and methods

Samples of experiments were obtained from surgical specimens of solid trumors, TUR specimens and Fluids of of bladder barbotage. After mincing tumor tissues with scalpels, fragments were digested by 0.2% collagenase (Sigma). Tumor cells suspension were incubated with anticancer drugs for one hour at 37℃.

After washing twicely, cells were prepared for culture.

But cells in a petridish were exposed continuously to the Interferon at the concentration of 50 IU, respectively.

The under layer consisting of 1 ml of Dulbeco -MEM contained agar and fetal calf serum at the final concentration of 0.3% and 25%, respectively. The under layer contained 1-5 x 10^5 nucleated cells in the same costitution medium as base layer. Plates were incubated at 37℃ in 5% CO_2 with 100% humidified incubator more than two weeks. The number of tumor cell colonies containing more than 30 cells was counted by the inverted phase contrast microscopy (Nikon). Three different concentration of each drug were tested in triplicated petridish. The in vitro results were expressed as linear survival concentration curve and analyzed at two different cut off points. The high dose cut off points were determined based upon achievable plasma peak concentration for clinical used as shown the following, 2.5ug/ml for CDDP, 2.5ug/ml for ADM, 1.0ug/ml for MMC, 5.0ug/ml for MTX, 50ug/ml for VP-16, 0.2ug/ml for Act-D, 0.1ug/ml for VCR, 0.2ug/ml for VLB, 5.0ug/ml for BLM 500IU/ml for Interferon, respectively.

The low dose cut off points were determined as 1/10 of high dose cut off levels. The sensitive case in vitro was difined more than 70% colony growth inhibition. The sensitive case in vivo was difined as more than partial response.

Results

A total of 79 urological tumors was used for colony forming assay. Formation of colonies in vitro was observed in 20 of 36 (56%) bladder cancer, 5 of 5 ureteral cancer, 5 of 12 (42%) testicular cancer, 12 of 19 (63%) renal cancer, and 3 of 7 (43%) other urological malignancies including a prostate rhabdomyosarcoma and leiomyosarcoma. The evaluable growth for chemosensitivity test (more than 30 colonies in control plate) was observed in 13 of 41 (32%) uroepitherial cancer, 5 of 11 (45%) testicular cancer and 9 of 19 (47%) renal cancer. Finaly, a total of 36% of all examined cases was estimated for in vitro chemosensitivity test. The plating efficiency ranged from 0.007 to 0.67. The number of colonies in plate ranged from 4 to 1700 (Table 1).

Colony formation occured in 62% of low grade and 82% of high grade in uroepitherial cancer. But there was not statistically significance between clony forming rate and tumor grade. The specimens from bladder barbotage created a relatively high success rate in making clonies (71%). Sufficient colony growth in vitro for chemosensitivity test was observed in 13 anticancerous drugs. In the average, three drugs per case tested in vitro. Concerning one hour exposured trials with drugs except Interferon, only one trial of 38 trials had been shownd in vitro activity at the low dose cut off criteria.

On the other hand, average 40% of tested drugs had been shown in vitro activity at the high dose cut off criteria. (Table 2). Forty-five cases were used in vitro chemosensitivity test for more than one drug. Significant colony formation to evaluate sensitivity was observed 29 of 45 (64%) examined cases. Seven cases with uroepitherial cancer and four cases with testicular cancer had shown the significant in vitro dose corresponding curve for Cisplatinum. From analyzing linear survival curve in uroepitherial cancer, there was no sensitive case at the low dose cut off criteria (0.25ug/ml), but three of seven cases proved sensitive in vitro at the high dose cut

Table 1 Success percentage and colony range using soft agar colony formation assay

Tumor	Which grew/ Trials (%)	Which eveluate/(%) Sensitivity trials	C	(P.E. range)	C	(Colony range/ plate)
Bladder	20/36 (56)	9/11 (78)	0.03	(0.015-0.06)	60	(4 -125)
Ureter	5/5 (100)	4/4 (100)	0.01	(0.007-0.07)	12	(6 -240)
testis	5/12 (42)	5/11 (45)	0.14	(0.04 -0.67)	152	(120 -292)
Renal	12/19 (63)	9/14 (64)	0.05	(0.01 -0.34)	62	(12 -1700)
Others	3/7 (43)	3/7 (43)	0.025	(0.002-0.15)	40	(5 -770)
Total	45/79 (57)	29/45 (64)		(0.007-0.67)		(4 -1700)

In vitro sensitivty rate for urological malignancies (Table 2)

Durgs	No. of which evaluated	Sensitive rate Low dose cut off (%)	High dose cut off(%)
ADM	10	1/10 (10%)	2/10 (20%)
CDDP	12	0/12 (10%)	4/12 (33%)
MMC	2	0/2 (0%)	0/2 (0%)
VP-16	3	0/3 (0%)	2/3 (67%)
MTX	3	0/3 (0%)	1/3 (33%)
Act D	4	0/4 (0%)	2/4 (50%)
VBL	2	0/2 (0%)	1/2 (50%)
VCR	1	0/1 (0%)	0/1 (0%)
BLM	1	0/1 (0%)	1/1 (100%)
INF α_2	12	2/12 (17%)	3/12 (25%)
INF α	12	4/12 (33%)	4/12 (33%)
INF β	9	3/8 (38%)	3/9 (33%)
INF γ	5	1/5 (20%)	3/5 (60%)

(α_2 : Ro 22-8181, α : MoR-22, β : GKT-β, γ : TRP-2)

off criteria (2.5ug/ml). From analyzing linear survival curve in testicular cancer, three of four cases showed sensitivity neither in the criteria of low dose cut off point (0.25ug/ml), nor in the criteria of high dose cut off point (2.5ug/ml). (Fig. 1). Nine of nineteen trials with renal cancer had shown a significant in vitro dose corresponding curve for α_2 type Interferon. One of nine cases was sensitive and six of nine cases effective in the both criteria of high and low dose cut off. Two cases seemed to be stimulated in the colony formation compared with control. Seven of nineteen trials had observed a significant dose corresponding curve for α type Interferon. Two of seven cases were sensitive and three cases were effective at the criteria of high and low dose cut off. On the other hand one case seemed to be stimulated. (Fig. 2).

There were twenty one instances wherein seventeen patients had been treated clinically with a specific drug tested in vitro. The primary tumors had been removed completely from seven of seventeen patients before starting chemotherapy. Twelve drugs in ten patients with measurable disease were evaluated for the antitumor effects using CT scaning, X-ray and cytology etc..

Drug concentration was measured in six patients to understand the correlation between the therapeutic effects in vivo and the results of colony forming assay in vitro.

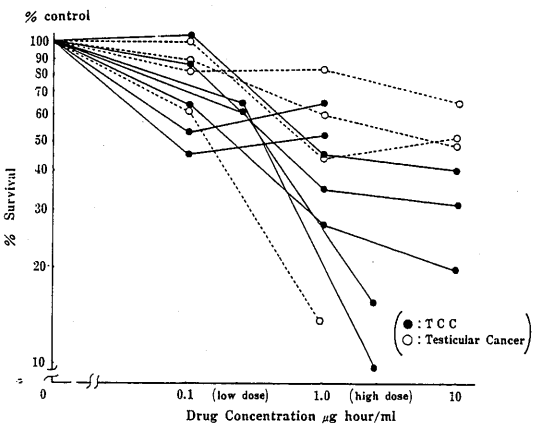

Fig.1 In vitro sensitivity to Cisplatinum in transitional cell cancer and testicular cancer. There was no sensitive case at the low dose cut off criteria (0.25 ug/ml), but three cases showed sensitive in vitro at the high dose cut off criteria (2.5 ug/ml). In testicular cancer, one of four cases did show sensitivity at the high dose cut off criteria.

Four drugs in three patients were able to evaluate the results in vitro based upon pharmacokinetic data. (Table 3).

Case 4 was nineteen years old male with prostate rhabdomyosarcoma. He was treated with the combination chemotherapy using 200

mg doses of Cisplatinum on the 5th day after the administration of five consecutive day 150 mg doses of VP-16. He was able to achieve more than partial response. The linear survival curve in vitro of Cisplatinum showed only 20% colony inhibition of control at the plasma concentration of 2.5 ug/ml which has measured actually on the treatment. on the other hand, linear survival curve of VP-16 showed more than 90% colony inhibition of control at the concentration of 5.0 ug/ml which can be achieved easily by judging from actually measured value (14 ug/ml).

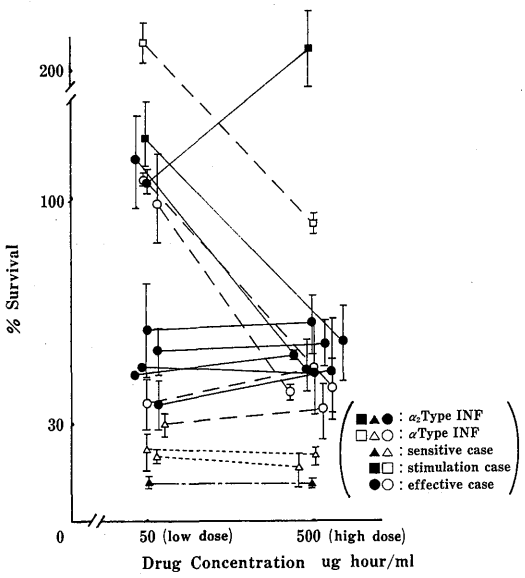

Fig.2 In vitro sensitivity to Interferon in renal cancer. Concerning α_2 type of Interferon, one of nine cases was sensitive (▲) and six cases were effective (●) in the criteria of high and low dose cut off, but two cases seemed to be stimulated (■) compared with control.
Concerning α type of Interferon, two of seven cases were sensitive (△) and three cases were effective (○) in the both criteria. On the other hand one case seemed to be stimulated (□).

We changed the administration schedule to use mainly VP-16 from judging based upon in vitro datas and pharmacokinetic datas.

Case 5 was sixty seven years old male who had been continuing high grade urinary cytology (class V) from bilateral catheterized urine samples. Cystoscopic finding and random biopsy suggested the existence of carcinoma in situ on the mucosa of bladder.

The results of colony forming assay for Cisplatinum using bladder barbotage urine showed 80% of colony inhibition at the concentration of 2.5 ug/ml. On the other hand, it showed only 30% colony inhibition at the concentration of 0.25 ug/ml. We decided to administer 100 mg/M² doses of Cisplatinum for this case. Maximum Cisplatinum concentration in blood and urine were measured 2.1 ug/ml and 26 ug/ml, respectively. After the chemotherapy, his bladder tumors disappeared and urine cytology improved to be nomal. The size of metastatic lymphnode in the position of common iliac artery decreased 45% of pretreatment size.

Case 6 was thirtyfour years old male with multiple bladder cancer. Intrabladder instillation of Adriamycin at the concentration of 1000 ug/ml over 60 minutes was repeated several times. The decresing of tumor size corresponded well to this treatment. The predictability resulted in 75% true positive

Relationship between in vitro and in vivo results based upon pharmacokinetic datas (Table 3)

Case	Histology	Drugs	Dose	Pharmacokinetic datas Cmax (ug/ml)	βmax (ug/ml)	AUC (ug.hour/ml)	in vitro/ in vivo
1.W.M.	ch+E+S	MTX	6 g/M²	470		2x10⁴	-/S
2.F.A.	S+E	CDDP	120mg/M²	6	2.5	38	-/S
3.M.H.	S	CDDP	120mg/M²	1.9	1.6	30	-/S
4.T.T.	Rhabdomyo sarcoma	CDDP	120mg/M²	2.5	1.9	30	R/S
		VP-16	200mg/body	14	5.0	33	S/S
5.W.T.	T.C.C.	CDDP	100mg/M²	2.1	1.0	35	S/S
N.M.	T.C.C.	ADM	30mg*	1000		1000	S/S

(S : seminoma, E : embryonal ca., Ch : choriocarcinoma, T : teratoma, * : bladder instilation)

and in 75% (6/8) true negative by the criteria of low dose cut off in vitro. On the other hand, the predictability resulted in 67% (4/6) true positive and in 83% (5/6) true negative by the criteria of high dose cut off in vitro. The difference of cut off criteria in vitro affected only two cases to judge the predictability in our series. Overall predictability was same 75% in the both criteria of low dose and high dose cot off in vitro. (Table 4).

In vitro predictability judged from the patient
who has measurable disesase (Table 4)

Dose predictability	True pos.	True neg.	Over all
High dose	4/6 (67%)	5/6 (83%)	9/12 (75%)
Low dose	3/4 (75%)	6/8 (75%)	9/12 (75%)

Discussion

The colony formation rate was obtained more than 60% in renal cancer and uroepitherial cancer, in 56% of all series. The results of chemosensitivity in vitro were obtained in 64% of tested cases. The colony formation rate in urologic tumors was reported 50% of renal cancer by Lieber[1], 76% of 164 urological tumors by Sarosdy[2], 77% of 91 urological tumors by Hashimura[3].

Our culture system had quite a high concentration of fetal calf serum (25%) without any enriched nutrition. The number of colonies were counted using the phase contrast microscopy (Nikon) without anu vital staining. But recently, we are using INT vital staining[4] which enable us to distinguish dead cells and living cells.

None of the seven cases showed sensitivity in vitro for Cisplatinum in the low concentration (0.25 ug/ml), but three of them showed sensitivity in vitro for Cisplatinum in the high concentation (2.5 ug/ml). Concerning Cisplatinum, because the patient is able to excrete relatively high concentration of total administered Cisplatinum through urinary tract, a high dose cut off point is prefered for judgung datas in vitro. On the other hand, we did experienced a bladder cancer patient who responed very well to the bolous administration of 100 mg/M^2 Cisplatinum which dose was decided to use from in vitro results. Sarosdy[2] reported that five of thirteen teticular cancer patients were effective in the low concentration of Cisplatinum (0.25 ug/ml). Three of four testicular tumors in our tested series did not show sensitivity even in the high dose cut off criteria. Our in vitro results for Cisplanum indicated a less effective rate than Sarosdy's report for Cisplatinum for testicular tumors. The reason could be that the examined tumor specimens were exposed already to more than 1000 mg of Cisplatinum in the accumulated dosage. Our in vitro results concerning testicular tumor may indicate the possibility of the acquired resistance for Cisplatinum after the administration of long period with low dosage.

Lieber[1] reported that fifty percent of renal cancer (80/159) showed the colonies formation in vitro, approximately two third of them were resistance to all drugs tested, only five tumors (6%) had sensitivity to numerous anticancer drugs in vitro. We succeeded in creating colonies in twelve of nineteen renal cancer cases (63%). Interferon is thought one of the effective anticancerous drugs against renal cancer. The experiment ensuring the antitumor activity ot Interferon has many difficult problems such as species specificity, time and financial support for clinical phase study. Cocerning the direct antitumor activity of Interferon for renal cancer, prospective in vitro phase study using the soft agar colony formation assay is a valuable method. The direct antitumor effects of Interferon from our experiments resulted in eleven percent (1/9) effectiveness for α_2 type Interferon, fourty three percent (3/7) effectiveness for α type Interferon. On the other hand, the stimulation of colony growth was observed one case in α type of Interferon and two cases of α_2 type of Interferon. Ludwing[7] reported that tumor growth stimulation in vitro by Interferon was observed in thirteen percent of 225 malignant tumors, in twenty percent of renal cancer. Therefore, it could

be better to pay attention before starting Interferon treatment.

Recently, the predictability using this colony forming assay system was reported 60-70% in true positive rate and 80-90% in true negative rate by many reporters[5]. In our small number of urologic malignancies which were examined the correlation between in vitro and in vivo, the predictability resulted 67-75% in true positive rate and 75-83% in true negative rate, 75% overall. The predictability did not show significant difference between low dose cut off and high dose cut off in vitro, because it was too small number to distinguish the difference between the both criterias. We prefer to use high dose cut off criteria for reporting in vitro results, because the drugs selected by higher cut off criteria may have the possibility of effectiveness when the drugs are administered by the large dosage.

Recently, many institutions have changed the drug exposure time to continuous exposure instead of one hour exposure, because it is more simple and more easy to find out the effective drugs. From the results of colony forming assay, we are trying to find out adequate dosage and best administration way based upon pharmacokinetic datas. We belive the usefulness of this assay system to study the differences of biological character in individual tumor and the in vitro screening of new anticanceros drug like Interferon.

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