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Subrenal Capsule Assay of Fresh Human Tumors: Problems and Pitfalls

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Abstract—*The* 6 day *subrenal capsule (SRC) assay was performed in normal mice using* 20 *fresh human non-small cell lung cancers and nine fresh ovarian cancers. Different multi-agent chemotherapy regimens administered intravenously on days* 2 + 3 *of the assay were evaluated for activity against these two tumor types. Macroscopic results measured via an ocular microscope showed high activity for some combinations as well as for single agents. However, when subjected to microscopic examination, the SRC implants in the untreated control animals did not show viable tumor growth on* day 6. *Detailed histologic evaluation of over* 800 *SRC grafts reveals an intense inflammatory and fibrotic reaction in the majority of the grafts. These results indicate that drug sensitivity patterns obtained with this assay using only macroscopic criteria do not correlate with actual tumor regression. Microscopic analysis of the grafts prior to transplant shows absence or only minimal presence of tumor in many cases which also contributes to the poor growth observed in these two tumors.*

INTRODUCTION

SEVERAL studies suggest that the 6 day subrenal capsule (SRC) assay in immunocompetent mice can measure chemotherapeutic sensitivity to human tumors taken from fresh surgical explants or from human xenografts grown in athymic mice [1–4]. Accurate, rapid, cost effective preclinical models which predict chemosensitivity of multi-agent therapy against human tumors are lacking. Yet development of drug resistance has necessitated in almost all responsive tumors the use of combination chemotherapy to achieve durable results. We intended to determine if the SRC assay could reliably select synergistic drug combinations using fresh human tumor specimens.

Non-small cell lung cancer and epithelial ovarian cancer were chosen for study since controversy exists regarding the benefits of combination vs. single agent treatment in these diseases. Clinically active drugs were tested alone and in combination against these two human tumors using the SRC assay. Because several investigators have noted a rapid host inflammatory reaction to the SRC graft [3–6], we performed histologic analysis in order to verify if the tumor responses recorded "macroscopically" correlated with the microscopic appearance.

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MATERIALS AND METHODS

SRC assay

Surgically removed human epithelial ovarian cancers and non-small cell lung cancers, contributed from neighboring hospitals, served as fresh human tumor xenografts for implantation under the renal capsule of immunocompetent mice (male BDF_1 [C57BL/6 × DBA/2]F_1 or $B_6C_3F_1$ [C57BL/6 × C_3 H]F_1). Human tumors were preserved in Hanks media with supplemental antibiotics (penicillin and streptomycin) during transport. The maximal interval from surgery to implantation was 24 hr.

Initially the surgical specimen was separated into fragments measuring approx. 1 mm^3 under a stereoscopic microscope, fitted with an ocular micrometer. Following the technique popularized by Bogden *et al.* [7], the mouse flanks were shaved and the animals were anaesthesized with a 10% Avertine (2,2,2 tribromoethanol) solution injected intraperitoneally. The technical details of the assay have been previously reported by our group [3].

Later in the study a minor modification was introduced to allow histologic evaluation of the quality of the graft prior to treatment (day 0). Instead of cutting the tumor specimen into 1 mm^3 pieces, the specimen was divided into fragments

measuring 2 mm^3. One half of this fragment was kept in formalin for histology and the paired remnant, measuring about 1 mm^3, was implanted into a specifically designated mouse to permit sequential follow-up. The primary tumor sample, from which the implanted fragments were taken, was also preserved in buffered formalin. A team of three technicians performed all the implants in 1–1 1/2 hr per experiment. The mice were provided by Charles River Breeding Laboratories (Massachusetts, U.S.A.).

Evaluation of treatment results

Change in tumor size (ΔTS), expressed as the mean tumor diameter on day 6 minus day 0, was calculated for each graft. The variation in tumor size was also expressed as a percentage relative to initial day 0 size as below:

$$\frac{\Delta TS}{TS_0} \times 100$$

$$= \frac{\text{tumor size on day 6} - \text{tumor size on day 0}}{\text{tumor size on day 0}} \times 100.$$

For an experiment to be considered evaluable, control animals needed to show a $\Delta TS \geqslant -0.5$ ocular micrometer units (o.m.u.). In addition, animals with weight loss > 20% of initial body wt during the 6 day assay period were inevaluable due to toxicity. For the treated groups, regression $\geqslant$ 20% of day 1 size was considered a response to therapy.

Chemotherapy

Based on studies performed by Aamdal *et al.* [8] showing equal activity for chemotherapy given for 2 days vs. a day 1–5 schedule in the SRC assay, we opted for a shorter course. To determine optimal dosage, we systematically tested each combination at different levels in non-operated mice to find maximal yet acceptable toxicity (weight loss < 20%). Surprisingly, these dosage levels when used in mice after implant surgery on day 1 + 2 resulted in unacceptable toxicity. However, delaying treatment 1 day to allow recovery from surgery permitted us to use these optimal dosages on a day 2 + 3 schedule. The mice were weighed on day 2 prior to treatment and again on day 6.

Since the selected dosages were close to the toxic level, we decided to use each combination at both the maximally established dose and at slightly lower concentrations to avoid erratic toxicity (Table 1). This also potentially allowed for discovery of a dose–response relationship. To permit comparison with the combination treatments, each agent was also administered alone at the maximal dosage level determined for the combination (Table 1).

There were 3–4 mice per treatment group and 8–12 mice served as controls. Treatments wre given intravenously via the tail vein to assure prompt distribution, except when venous access was not possible. In such cases, the same dose was given subcutaneously—however, this was necessary in less than 5% of all treatments. The drugs used in this study (Table 1) were a generous gift from the NCI (Bethesda, MD, U.S.A.).

Histological evaluation of the graft

Serial sections (3μ thick) were cut through the entire subrenal graft at intervals of 100–300μ and stained with hematoxylin and eosin. All day 6 grafts of treated mice and untreated controls were examined by our pathologist (D.J.). An estimation was made of the areas of each graft constituted by human tumor, mouse cell infiltration and fibrosis by additioning representative serial sections. A single mean value of these estimates was calculated for controls and for each treatment group. In the last 14 experiments, tumor specimens 1 mm^3 taken adjacent to the implanted graft were also examined to verify the quality of the graft and to allow comparison between day 0 and day 6 histologic findings.

Table 1. *Single agent and combination chemotherapy administered i.v. day* 2 *and* 3

Lung	Dose (mg/kg per injection)		Ovary	Dose (mg/kg per injection)	
1. Cisplatin (Cispt)		3	1. Cisplatin		3
2. Carboplatin (CBDCA)		50	2. Carboplatin		50
3. Etoposide (VP-16)		30	3. Melphalan (L-PAM)		4
4. Cispt + VP-16	Hi-	3/30	4. Cispt + L-PAM	Hi-	4/4
	Low-	2/20		Low-	3/3
5. CBDCA + VP-16	Hi-	50/15	5. CBDCA + L-PAM	Hi-	50/4
	Low-	40/10		Low-	40/3

RESULTS

Macroscopic day 6 results

Twenty previously untreated patients with non-small cell lung cancer served as donors. Of the 20 transplanted tumors, 11 cases showed adequate macroscopic growth ($\Delta TS \geq -0.5$) in the controls producing an evaluable rate of 55%. In these immunocompetent mice, growth of the untreated xenografts averaged +1.4 o.m.u.

Using a response criterion of greater than 20% regression in tumor size when compared to day 0 implant size (% ΔTS), Table 2 illustrates the response rates to the various single agents and combinations in those assays with acceptable control growth. Etoposide (VP-16) appears to be highly active as a single agent (73% response rate) (Table 3). The addition of a second drug, either cisplatin or carboplatin (CBDCA) does not improve on this response rate although the number of experiments is small and significant differences could be missed. Of note, the least active single agent is cisplatin (18% response rate).

A similar analysis, shown in Table 4, was performed on nine ovarian SRC transplants. All cases had adequate control growth giving an evaluable rate of 100%. Growth of controls averaged + 1.0 o.m.u. Cisplatin and melphalan were equally effective as single agents (44% response rates) and the higher dose combination regimens appeared slightly more effective (Table 5).

Microscopic day 6 results

Table 6 provides parallel microscopic analyses of day 6 results in the control and treated lung and ovarian SRC grafts in all implants where macroscopic control growth was deemed adequate

Table 2. Macroscopic evaluation of lung tumor regression or growth (%ΔTS) on day 6 of SRC assay

Drug		SCR numbers 1	8	10	11	12	17	26	27	28	29	30
CBDCA VP-16	(Hi)	−18	−28	−38	−61	−35	−4					
CBDCA VP-16	(Low)	−31	−24	−53	−32	−60	−8	−86	−6	0	−11	+6
Cispt VP-16	(Hi)	−29	−24	−79	−27	−40	−10					
Cispt VP-16	(Low)	−43	−15	−28	−42	−35	−19	−37	−10	+7	−5	−20
CBDCA		−5	−14	−21	−19	−46	−15	+23	+14	0	+28	+33
Cispt		−15	−17	−44	−8	−26	−18	+6	+49	+21	−1	+7
VP-16		−60	−26	−42	−48	−50	−15	−3	−25	toxic	−23	−38
Controls		+15 (+2−0)*	−2 (−0.4)	+16 (+2.0)	−1 (−0.2)	+28 +(3.4)	+5 (+0.6)	+13 (+1.1)	+15 (+1.8)	+21 (+2.3)	+22 (+2.8)	−1 (−0.2)

*Numbers in parentheses represent actual change in tumor size (ΔTS) measured in ocular micrometer units.

Table 3. Response rate to chemotherapy in SRC assay of fresh human lung tumors

Drug tested	No. of assays performed	No. of assays rejected*	No. of assays evaluated	No. of assays with 20% tumor regression	Experimental response %
CBDCA	20	9	11	5	45%
Cispt	20	9	11	2	18%
VP-16	20	9	11	8	73%
Hi Dose Vispt + VP-16	8	2	6	5	83%
Low Dose Cispt + VP-16	20	9	11	6	55%
Hi Dose CBDCA + VP-16	8	2	6	4	66%
Low Dose CBDCA + VP-16	20	9	11	6	55%

*Assays rejected when control growth is ≤ 0.5 o.m.u.

Table 4. Macroscopic evaluation of ovarian tumor regression or growth (%ΔTS) on day 6 SRC assay

Drug	SRC numbers 3	5	6	7	9	13	14	20	22	
Cispt L-PAM	(Hi)	+3	−27	−7	−26	−17	−26	−31		
Cispt L-PAM	(Low)	+3	−38	−10	−21	−15	−25	−48	−8	−18
CBDCA L-PAM	(Hi)	+8	−26	+8	−32	−15	−25	−28		
CBDCA L-PAM	(Low)	+19	−16	−3	−22	+1	−13	−12	−11	−11
CBDCA		+9	−29	+13	−12	−15	−15	−17	−8	−30
Cispt		0	−20	−10	−46	−22	−12	−10	−23	−16
L-PAM		+10	−16	−5	−47	−27	−10	029	−4	−27
Controls		+19 (+2.0)*	+8 (+1.2)	+19 (+2.8)	+12 (+1.6)	0 (0)	+4 (+0.5)	0 (0)	+6 (+0.8)	+4 (+0.5)

*Numbers in parentheses represent actual change in tumor size (Δ*TS*) measured in ocular micrometer units.

Table 5. Response rate to chemotherapy in SRC assay of fresh human ovarian tumors

Drug tested	No. of assays performed	No. of assays rejected	No. of assays evaluated	No. of assays with 20% tumor regression	Experimental response %
CISPT	9	0	9	4	44%
L-PAM	9	0	9	4	44%
CBDCA	9	0	9	2	22%
Low Dose CISPT-L-PAM	9	0	9	4	44%
Hi Dose CISPT-L-PAM	7	0	7	4	57%
Low Dose CBDCA-L-PAM	9	0	9	1	11%
Hi Dose CBDCA-L-PAM	7	0	7	4	57%

($\Delta TS \geq -0.5$ o.m.u.). Unexpectedly, half of the lung tumor controls show no recognizable tumor. In the remaining five control cases, tumor growth never accounts for more than 50% of the implant surface area. Inflammatory cells and occasionally fibrotic reaction constitute the bulk of the implants examined. Even in cases where macroscopic control growth was clearly positive, such as nos. 10 and 29 from Table 2 (ΔTS = +2.0 and +2.8 respectively), the microscopic evaluation (Table 6) could find no tumor present at all in the implant. Likewise, when macroscopic results in treated animals indicate drug resistance, as in cases 27 and 28 (Table 2) treated with cisplatin (ΔTS = +49% and +21% respectively), the histologic evaluation (Table 6) shows an intense inflammatory infiltrate without tumor present. Conversely, in implants 1 and 12 treated with VP-16, (Table 2), a very positive chemotherapeutic response is indicated macroscopically (ΔTS = −60 and −50% respectively). Yet the microscopic analysis (Table 6) shows tumor present in 25–50% of the surface area of the implant, comparable to those cases in which optimal macroscopic tumor growth occurred.

The results of the ovarian SRC microscopic analysis show similar disparity. Despite the 100% "take rate" macroscopically, only four cases (Table 7) have tumor present in the controls, and only no. 5 has tumor present in 25–50% of the surface area. Again, several inconsistencies are apparent from Table 7. In no. 6 treated with CBDCA and no. 3 treated with L–PAM, macroscopic results (Table 4) suggest drug resistance with tumor growth but the histologic evaluation of these implants (Table 7) reveals no tumor present.

Microscopic day 0 results

To examine the quality of the xenograft, seven fresh human lung and two ovarian tumors were

Table 6. Day 6 microscopic analysis of lung SRC grafts with acceptable macroscopic control growth

Exp.	Micro*	Control	Cispt	CBDCA	VP-16	CBDCA + VP-16 (Hi)	CBDCA + VP-16 20 (Low)	Cispt + VP-16 (Low)	Cispt + VP-16 (Hi)	Surgical specimen
1	T	++		+	+			++	+	
	I	++	+++	+++	+++	++	+++	++	++	Squamous CA
	F		+			++	+		+	
8	T	++	++				+		++	
	I	++	++	++	++	++	++	++	+	Squamous CA
	F			++	++	++	+	++	+	
10	T								No	
	I	++++	++++	++++	+++	++++	+	+	Graft	Adeno CA
	F				+		+++	+++	Seen	
11	T		+		+			+	+	
	I	++	++	++++	+	++	++	+	+	Squamous CA
	F	++	+		++	++	++	++	++	
12	T	+	+	+	++	+	+	+	++	
	I	+++	+++	+++	++	+++	+++	+++	++	Squamous CA
	F		+							
26	T			++	++	Not	++		Not	
	I	++	++++	++	++	Tested	++	++++	Tested	Adeno CA
	F	++								
27	T	+				Not		+	Not	
	I	+++	++	+++	++	Tested	++	++	Tested	Squamous CA
	F		++	+	++		++	+		
28	T	+				Not		+	Not	
	I	++	+++	+	+	Tested		+	Tested	Adeno CA
	F	+	+	+++	+++		++++	++		
29	T			+		Not			Not	
	I	+++	++	++	+++	Tested	++	++	Tested	Adeno CA
	F	+	++	+	+		++	++		
30	T					Not			Not	
	I	+++	++	+++	++	Tested	++	++	Tested	Adeno CA
	F	+	++	+	++		++	++		

*Microscopic code: T = tumor; I = inflammation by host cells; F = fibrosis.
+ = 0–25% total area; ++ = 25–50% total area; +++ = 50–75% total area; ++++ = 75–100% total area.

examined before implantation using a paired remnant of 1 mm^3 taken adjacent to the 1 mm^3 specimen used in the SRC assay. This remnant was reviewed for the presence of tumor and the results are shown in Table 8. Of 225 total specimens examined, only 73 (32%) clearly showed tumor present. In the paired control of the lung xenografts, only cases 27 and 28 showed tumor present in over half the controls. Most of the other cases had no tumor (nos. 30 and 31) or only occasional tumor present (26 and 29).

However, even when tumor was present in the day 1 control, the paired day 6 control implant often did not show any tumor present. This is clearly illustrated in ovarian tumor no. 22 (Table 8) where tumor was present in all (100%) day 0 paired specimens. Despite these good results, the day 6 control results (Table 7) indicate poor tumor growth and an implant heavily infiltrated by inflammatory cells and fibrosis. This result is not explained by poor sampling of tumor at the time of surgery since the surgical specimen submitted for pathologic diagnosis in all the lung and ovarian cases showed definite tumor present (Tables 6 and 7).

DISCUSSION

The modification of the SRC assay from its original conception in athymic mice to a 6 day format using immune competent mice was an attempt to reduce the high cost and expand the availability of this technique. Bogden *et al.* [7] felt that the immunologic reaction to foreign antigens would take from 7 to 12 days to develop and fostered the 6 day assay based on macroscopic controls. Proof of the validity of this 6 day tech-

Table 7. Day 6 microscopic analysis of ovarian SRC grafts with acceptable macroscopic control growth

Exp.	Micro*	Control	Cispt	L-PAM	CBDCA	Cispt + L-PAM (Hi)	Cispt + L-PAM (Low)	CBDCA + L-PAM (Hi)	CBDCA + L-PAM (Low)	Surgical specimens
3	T		No		+	+		+	+	
	I	++	Graft	++	++	++	++	++	++	Adeno CA
	F	++	Seen	++	+	+	++	+	+	
5	T	++	++	+	++		++	++	++	Papillary
	I	++	++	+++	++			++	+	Adeno CA
	F					++++	++		+	
6	T		+	+				++	+	Papillary
	I	++	+	+	++	++	++	++	+	Adeno CA
	F	++	++	++	++	++	++		++	
7	T					+			++	
	I	++++	+	++	+	++	+	+++	++	Cystadeno CA
	F		+++	++	+++	+	+++	+		
9	T	+	+	No	No			+		Undifferentiated
	I	+++	+++	Graft	Graft	+	+	+++	+++	Adeno CA
	F			Seen	Seen	+++	+++		+	
13	T			No		No	+	0		Papillary
	I	+++	++	Graft	++	Graft	++	++	++	Adeno CA
	F	+	++	Seen	++	Seen	++	++	++	
14	T		+			+				
	I	++	++	++	++		+	++	++	Adeno CA
	F	++	+	++	++	+++	+++	++	++	
20	T	+		+		Not	+ Not	+		
	I	++	++	++	++	Tested	++	Tested	+	Adeno CA
	F	+	++	+	++		+		++	
22	T	+	+			Not		Not		
	I	+	+	+	++	Tested		Tested		Adeno CA
	F	++	++	+++	++		++++		++++	

*Microscopic code: T = tumor; I = inflammation by host cells; F = fibrosis.
+ = 0–25% total area; ++ = 25–50% total area; +++ = 50–75% total area; ++++ = 75–100% total area.

Table 8. Ratio of histologic proven tumor compared to all day 0 paired specimens submitted for microscopic exam

Lung CA SRC No.	Control	Cispt	CBDCA	VP-16	Cispt + VP-16	CBDCA + VP-16
26	3/11	0/2	1/2	2/3	2/3	2/2
27	7/10	3/3	3/3	3/3	1/3	0/3
28	3/4	2/2	2/2	1/3	0/2	0/3
29	1/12	0/3	0/3	0/3	0/3	0/3
30	0/11	0/3	0/3	0/3	0/3	0/3
31	0/12	0/3	0/2	0/3	0/3	0/3
Ovarian CA SCR No.	**Control**	**Cispt**	**CBDCA**	**L-PAM**	**Cispt + L-PAM**	**CBDCA + L-PAM**
20	0/12	0/3	1/3	1/3	3/3	2/3
22	12/12	3/3	3/3	3/3	3/3	3/3

nique is based on their studies comparing macroscopic growth in identical human tumor lines transplanted simultaneously in athymic mice and in normal mice. Since growth curves were somewhat similar in configuration macroscopically during the first 6 days in both groups, they felt this substantiated the 6 day model. However their own results using fresh surgical explants in normal and athymic mice encourage closer inspection. They showed that in 31 primary surgical specimens implanted in either athymic or normal mice, 6/20 (30%) showed positive growth in the athymic mice and 9/11 (82%) showed positive growth in the normal mouse. Calling these results "surprising", the authors interpreted their findings as proof that even in surgical explants, the 6 day assay was acceptable and even superior to the athymic model [7].

Based on the success reported in using this assay to test for single agent chemotherapeutic activity against human tumor explants [9], we intended to evaluate the SRC assay for its ability to discriminate among different drug combinations. The macroscopic results obtained in this study are certainly seductive with tumor growth in the majority of controls and tumor regression in most treated animals (Tables 4 and 6). Our evaluation assay rate of 55% for non-small cell lung cancer and 100% for ovarian cancer also compares favorably with other reported results [1, 9].

However, microscopic data from our experiment clearly demonstrate the danger in concluding that macroscopic growth of the xenograft represents actual tumor growth. In every one of our 30 implant controls, representing nearly 200 individual SRC assays, tumor growth on careful histological review was minimal at best and completely absent in many cases (Tables 6 and 7). This leads us to conclude that the macroscopic measurements simply reflect the greater intensity of the inflammatory infiltrate in the untreated controls compared to the chemotherapy treated animals. Such a mechanism would explain why CBDCA and VP-16, highly myelosuppressive agents, would be more active against non-small cell lung cancer in the SRC assay than cisplatin (Table 3), a drug with less myelotoxicity. Agents that effectively reduce the white blood cell count can thereby decrease the inflammatory reaction which leads to a smaller SRC infiltrate.

Several groups have now independently shown that the immunologic response in normal mice develops before the 7th day, often as early as 3 days post-transplant. Edelstein *et al.* [5] were the first to question the accuracy of this technique in normal mice. They showed lymphocyte infiltration of both fresh surgical explants and established human tumor lines by day 6. To attenuate this immune reaction, they proposed pretreatment of normal mice with irradiaton 2–4 hr before implantation [6]. An even more promising immunosuppressive regimen has been developed using cyclosporine A (CSA) [11]. Such treatment has been shown to permit increasing tumor growth up to 12 days in the SRC assay. More importantly, CSA treated mice with allogeneic tumor implants have developed metastases and local invasion into the kidney.

A final issue raised by our experiment is the optimal origin of the tumor material in this assay. Fresh human tumor specimens certainly represent the surest way of detecting variations in chemotherapeutic sensitivity due to tumor heterogeneity. Yet, as Aamdal *et al.* [4] point out, human tumor lines offer the advantage of a reliable quantity allowing repeated chemotherapeutic experiments under different conditions with many drugs. A further argument for employing human tumor lines comes from the sample itself. Although we processed our specimens rapidly, and kept them in the recommended sterile media with antibiotics, day 1 pre-implant specimens showed great variability in tumor content (Table 8) and often tumor necrosis. Fresh surgical explants therefore exhibit marked heterogeneity. This fact has been extensively studied by Edelstein *et al.* [6] and our results corroborate their findings.

Slagel *et al.* [12] have recently asserted that human tumor specimens, submitted for SRC assay as late as 72 hr post-op, are still capable of tumor growths. Once again, this contention is based entirely on the macroscopic examination. Our findings provide additional confirmation of the need for histologic verification of all fresh tumor specimens used in the SRC assay. Results without microscopic analyses must be held suspect, especially in view of the wide variations in tumor samples submitted for SRC assay noted in fresh human lung and ovarian cancers.

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