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6-Day Subrenal Capsule Assay (SRCA) as a Predictor of the Response of Advanced Cancers to Chemotherapy

ROGER FAVRE, LOUIS MAROTIA, MICHEL DRANCOURT, JOCELYNE JAQUEMIER, JEAN ROBERT DELPERO, GÉRARD GUERINEL and YVES CARCASSONNE

Institut J. Paoli-I. Calmettes, 232 *Bd de Ste Marguerite*, 13009 *Marseille*, *France*

Abstract—*The* 6-day *subrenal capsule assay (SRCA) chemosensitivity prediction test using fresh human tumor xenografts was performed in BALBc mice for* 80 *advanced cancer bearing patients. Among* 97 *SRCA, nine were non-evaluable because patients either received no chemotherapy or non-assayed drugs, and* 12 *were non-interpretable because of inadequate control growth. One hundred and six correlations were established between test results and clinical response,* 56 *retrospective (chemotherapy then test) and* 50 *prospective (test then chemotherapy). Among the* 56 *retrospective correlations there are* 45 *true negative* (−/−) *and three false positive* (+/−) *corresponding to* 48 *poor clinical responders; and eight true positive* (+/+) *and* 0 *false negative* (−/+) *corresponding to eight good clinical responders. Among the* 50 *prospective correlations there are* 26 *true negative* (−/−) *and four false positive* (+/−) *corresponding to* 30 *poor clinical responders; and* 19 *true positive* (+/+) *and one false negative* (−/+) *corresponding to* 20 *good clinical responders. In total among the* 106 *correlations there are* 98% *good negative correlations and* 79% *good positive correlations. The SRCA is practicable and is available for routine clinical use providing results relevant to cancer site and reflecting previous treatment status.*

INTRODUCTION

ONCOLOGISTS are in search of a chemosensitivity test that would assist in optimizing chemotherapy of human tumors when medical treatment is the modality of choice. Predictive chemosensitivity tests can be classified into three groups corresponding to three different methodologies [1]. In the first group are those methods aimed at determining effects on specific intracellular mechanisms which are targets of anticancer drugs, i.e., the kinetic and biochemical tests. Methods in the second group are concerned with the responses of the intact cancer cell to the anticancer agents. Although such tests can use isolated cells, they more often employ tumor cell populations as in the clonogenicity test [2] or as with human tumor xenografts in animals. These xenografts are implanted subcutaneously in athymic nude mice [3], or under the renal capsule of athymic or normal conventional mice [4]. Methods in the last group attempt to evaluate the pharmokinetics of anticancer drugs in each patient, i.e., the metabolic reactions of the individual receiving a chemotherapeutic agent.

These three types of tests are applied under two different circumstances. Firstly, in new drug screening. They also guide therapeutic trials in man to identify which tumor types are sensitive to the new drug. Secondly, they are used to predict individual tumor sensitivity. They permit selecting from among anticancer drugs for individualizing chemotherapy.

We have used a slightly modified version of the 6-day subrenal capsule assay (SRCA) chemosensitivity prediction test, using human tumor xenografts implanted in normal mice, as described by Bogden *et al.* [5]. Its practical use appeared better than *in vitro* methods, as higher evaluable assay rates permitted more correlations with higher predictive values [6, 7].

We report here, the results obtained with advanced cancers from which tumor biopsies were obtainable. Most of the patients were in relapse and had been heavily pretreated.

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MATERIALS AND METHODS

Mice

Normal syngenic BALB/c Jackson mice, both male and female, aged 3–5 months, bred in our laboratory or coming from IFFA credo l'Arbresle

France, were used. Preliminary experiments [8, 9] prompted us to utilize a minimum number of five mice per group treated with anticancer drugs and eight mice for the control group. Sex of graft recipients corresponded to that of the donor patient.

Anaesthetic

Mice, fasted for 12–18 hr before implantation, were given a 70 μg/g Pentobarbital injection.

Trocar

A long tapered bevel Pitkin needle, 40–50 mm long, 12/10th section, permitted accurate placement of tumor graft with its plunger.

Straight and curved tweezers

Sterile at the beginning of manipulation, tweezers were cleaned with 0.9% NaCl solution at least once between each implantation.

Microscope

Tumor grafts are measured at the beginning (day 0) and at the end (day 6) of an assay at the same magnification, by the same person, on a stereoscopic microscope Wild M8 fitted with an ocular micrometer calibrated in omu (Ocular Micrometer Units) where 10 omu = 1.0 mm.

Anticancer drugs

Maximum tolerated doses were determined for a great number of the anticancer drugs. Injections were given on the first, third and fifth day of the assay. Subcutaneous route was employed, in the right-dorso lateral site opposite to the implanted kidney, except for adriamycin, mitomycin C and mitoxantron which were administered intraperitoneally. Each mouse was weighed before the implant and on the 6th day. Loss of weight had to be less than 25% for the test to be considered as valid, and that drug toxicity was acceptable. Ten drugs previously selected on the basis of activity according to tumor histology were used for testing against each histologic type of tumor (Table 1). In this table some cancer types include more than 10 drugs because there have been variations during this study due to the use of additional new drugs.

Statistical analysis: Implants were measured with stereoscopic microscope across two diameters L and W in omu.

For this analysis the change in tumor size formula, i.e., $\Delta TS = (L+W)/2$ final $- (L+W)/2$ initial as used by Bogden [5] was applied. Means and standard deviations, group by group were compared to the control and analyzed for statistically valid difference using Student's t-test. Each result is given with significance. Limits of assay validity were taken into account. Control grafts must show a minimum growth: mean ΔTS control group must be > -0.5 omu or the test was considered uninterpretable. Grafts in treated mice had to show significant regression: mean $\Delta TS < -1.0$ omu. Even if there was significant difference from control group the implant was not considered as

Table 1. *Chemotherapeutic agents, dose levels, used for treating fresh surgical explants in* 6-day *SRC assay according to tumor sites*

Common name	Abbreviation	Dose level (mg/kg/inj)	Tumor site
Actinomycin D	ACT D	0.3	S T
Adriamycin	ADR	5	B C D H L O S T U
Bleomycin	BLEO	32	C H L T U
Cis DD Platinium	Cis DDP	3.5	B C D H O S T U
Cyclophosphamide	CTX	225	B C D H L O S T U
Dacarbazine	DTIC	300	S
5 Fluouracyl	5FU	150	B C D H U
L Disine	VDS	2.5	B C D H L O S U
Melphalan	MLP	20	B C D H O T U
Methotrexate	MTX	7	B C D H L O S T U
Mitomycin C	MIT-C	3.4	B C D H S U
Mitoxantron	MITO	2.8	B C D H L O S T
Navelbine	NVB	6	B D H L S T
Peplomycin	PEPLO	32	H L T U
Methylprednisolon	MPD	450	L
Thiotepa	TTP	20	B O T
Velbe	VLB	1.4	L O S T U
Vincristine	VCR	1.1	B D L O S U
VM 26	VM 26	10	L O U

B, breast; C, cervix; D, digestive; H, head and neck; L, lymphoma; O, ovarian; S, soft tissues and bone; T, testicular; U, uterin.

being sensitive to the chemotherapeutic agent if tumor regression of the treated series was not < -1.0 omu.

Clinical response evaluation: Clinical responses corresponded with measurable tumors according to usual UICC criteria [10]. There were two patient categories. Poor clinical responders who showed either no tumor regression or tumor regression less than 50% of measured total tumor volume. Good clinical responders ranged from tumor regression of more than 50% to complete clinical disappearance of the tumor. Duration of observation must be a minimum of 2 months.

Manipulation schedule

SRCAs were performed on Wednesdays or Thursdays. Sterile human tumor was removed after surgery in the morning. Implantations were generally performed within the following 2 hr. Pathologic examination of the tumor specimens was routinely performed. Such random biopsies help in selecting tumor fragments to be implanted. Quality controlling the remaining of grafted fragments was always performed.

At the laboratory, tumor fragments were cut under sterile conditions into 1 mm cubes, approximately calibrated with paper guides placed under a Petri dish. Each dish contained 80–100 implants, each protected with a drop of 0.9% NaCl solution containing 0.5% Gentamycin. Each trocar was loaded with one of the calibrated implants.

A left dorsal incision was made in anaesthesized and previously weighed BALB/c mice. The kidney was exteriorized and supported with curved forceps. A Pitkin needle containing a tumor fragment was inserted underneath the renal capsule and the xenograft deposited by pushing gently on the plunger. The trocar was removed and the implant was measured *in situ* with the stereoscopic microscope. Deep and external tissues were then sutured. Fifty-eight to sixty implantations were routinely performed in less than 3 hr. The following day (Thursday or Friday), implanted mice were divided at random into 11 groups: one control group and 10 treated, each receiving a different drug selected according to the human tumor histologic type.

Drugs were administered on the first, third and fifth day after engrafting. Control mice receive the same number of 0.9% NaCl injections. On the sixth day (Tuesday or Wednesday) mice were weighed and then killed. Tumor grafts were measured using the same technique by the same person as at the initial implantation.

For rapid results, data were computed on a simple program which takes into account group values, statistical significance and limits of test validity (mΔTS). Standardization of the methods, for technique as well as for calculations, permitted obtaining results with 6 or 7 days for two assays per week and 10 anticancer drugs tested per assay. We have applied this test to a relatively homogeneous group of patients who present relapsed or metastatic cancers, primarily of the head and neck as well as sarcomas, lymphomas, genital or breast cancers.

Both retrospective and prospective studies were made. In the case of retrospective studies SRCA was performed after chemotherapy; in prospective studies, SRCA was done before chemotherapy. Test results (+ : active drugs, − : non active drugs) were systematically correlated with clinical response results (+ : good responders, − : poor responders), providing the following correlation types: −/− : true negative, −/+ : false negative, +/+ : true positive, and +/− : false positive. Good correlations correspond to true negative and true positive, bad correlations to false negative and false positive.

RESULTS

Eighty patients representing 97 tests (Table 2) were studied.

Nine clinical examinations were unevaluable because of patients who either did not receive any chemotherapy, or received non-tested anticancer drugs. This is not a rare occurrence in clinical studies and does not detract from the value of the assay. Better selection of patients, at least for chemotherapy trials, can reduce that number.

Twelve SRCAs were not interpretable (ΔTS control < -0.5 omu), they represent 12% corresponding to 11 patients. One patient, bearing soft tissue sarcoma, had two successive non-interpretable assays. One assay could be repeated for a lymphoma bearing patient with interpretable assays. Finally, among the 80 patients entering this study, there were only 10 for whom we could not obtain an interpretable assay, which still makes 12%.

Nevertheless, 106 correlations were established between test results and clinical therapeutic responses; 56 retrospective and 50 prospective. Among the 56 retrospective cases, there were only three discrepancies which all were false positive (Table 3). There were 100% good negative correlations but only 72% good positive correlations (Table 4); total: 94% (53 of 56).

Among the 50 prospective cases (Table 5) we observed 26 true negative of 30 poor clinical responses. There were only five discrepancies corresponding to four false positive and one false negative. There were 96% good negative correlations and 82% good positive correlations (Table 6); total: 90% (45 of 50).

For both studies there are more than 98% good

Table 2. Overall results

Tumor site	Number patients	Number tests	Unevaluable tests, no chemotherapy or no drugs tested	Non interpretable tests ΔTS control < -0.5	Retrospective correlations	Prospective correlations
Breast	22	26	1	1	19	13
Cervix	2	3	0	1	2	1
Digestive	9	10	3	1	3	4
Head and neck	18	20	1	1	11	15
Lymphomas	9	12	2	3	4	7
Ovarian	5	7	0	1	6	4
Osteosarcomas	3	4	0	1	3	1
Soft tissues sarcomas	6	8	0	3	5	2
Testicular	2	2	0	0	2	1
Uterin	1	2	0	0	0	2
Metastasis	3	3	2	0	1	0
Total	80	97	9	12	56	50

Table 3. Fifty-six retrospective correlations (patient chemotherapy then SRCA)

	Breast	Cervix	Digestive	Head and Neck	Lympho-mas	Ovarian	Osteo-sarcomas	Soft tissue sarcomas	Testicular	Uterin	Metastasis	Total	
True negative −/−	18	2	3	10	0	4	3	2	2	0	1	45	48 Poor clinical responders
False positive +/−	1	0	0	0	1	1	0	0	0	0	0	3	
True positive +/+	0	0	0	1	3	1	0	3	0	0	0	8	8 Good clinical responders
False negative −/+	0	0	0	0	0	0	0	0	0	0	0	0	
Total	19	2	3	11	4	6	3	5	2	0	1	56	

Table 4. Summary of 56 retrospective correlations

	Test	Clinic	Number	
True negative	−	−	45	100% Of good negative correlations
False negative	−	+	0	
True positive	+	+	8	72% Of good positive correlations
False positive	+	−	3	

negative correlations and more than 79% good positive correlations (Table 7); total: 92% good correlations (98 of 106).

DISCUSSION

General comment

As an *in vivo* method the SRCA has been criticised concerning the effect of graft rejection reactions on evaluation of the implant size at termination of the assay [11]. It has been shown, however, that xenograft size on day 6 is related to the mitotic activity of the donor tumor and host cell infiltration is not artifactual to a tumor size parameter [12–15].

Although the 6-day SRCA is technically demanding as a routine laboratory procedure, it has many advantages. It uses little tumor tissue and is rapid, results being obtained after only 6 days. The evaluable assay rate is greater than 85% and can be improved if examinations are repeated. Half of uninterpretable tests are those of soft tissues, sarcomas or lymphomas. In those two cases tissues are very soft and friable. Cells spontaneously separate from each other, giving almost an aspect of liquid tumor. This is one of the first technical limitations of the assay for tissues which are not dense enough. On the other hand, in some other cases it is fibrosis which is responsible for an implant's lack of growth. The latter occurs more rarely because the surgeon chooses which tumor fragment to biopsy out of very fibrous areas and the pathologist also chooses which implant is going to be grafted.

Assay/clinical correlations

It is easier to group the results according to the analysis to be made. In the retrospective study it is better to group results according to clinical responses (Table 3) which exactly follows clinical status. At the beginning of the test patients already are divided into two groups: poor and good clinical responders. For the prospective study, on the other hand, it is better to group results according to assay data: active and non-active drugs (Table 6).

Table 5. Fifty prospective correlations (SRCA then patient chemotherapy)

	Breast	Cervix	Digestive	Head and Neck	Lymphomas	Ovarian	Osteo-sarcomas	Soft tissue sarcomas	Testicular	Uterin	Metastasis	Total	
True negative −/−	9	1	3	7	1	1	1	0	1	2	0	26	30 Poor clinical responders
False positive +/−	2	0	0	1	1	0	0	0	0	0	0	4	
True positive +/+	2	0	1	6	5	3	0	2	0	0	0	19	20 Good clinical responders
False negative −/+	0	0	0	1	0	0	0	0	0	0	0	1	
Total	13	1	4	15	7	4	1	2	1	2	0	50	

Table 6. Summary of 50 prospective correlations

	Test	Clinic	Number	
True negative	−	−	26	96% Of good negative correlations
False negative	−	+	1	
True positive	+	+	19	82% Of good positive correlations
False positive	+	−	4	

Table 7. Summary of 106 correlations

	Test	Clinic	Number	
True negative	−	−	71	98% Of good negative correlations
False negative	−	+	1	
True positive	+	+	27	79% Of good positive correlations
False positive	+	−	7	

Retrospective correlations (Table 3)

These are very useful for test validation. It must be emphasized that tests indicating resistance occur more frequently and that good results in negative correlations (−/−) are to be expected. In every case, for patients indicated to be resistant, chemotherapy was ineffective with progressive tumor growth most of the time.

As for the three false positives (+/−) the question is whether chemotherapy was ineffective in quality (genuine false positive) or in quantity. A larger dose or a better treatment regimen might have induced tumor regression. It is not possible to extrapolate dosage directly from the test to clinical application as we use maximum tolerated doses in mice. Therefore, one of the test limitations could be the use of two low drug doses for patient chemotherapy as compared to experimental doses; as some prospective results indicated. Whenever possible, we used an optimum dose extrapolation according to pharmacokinetics data. For example, in the case of high dose methotrexate or L disine [16, 17], SRCA provided us with qualitative information in the choice of anticancer drugs which was then completed by quantitative pharmacokinetic information.

Positive retrospective correlations (+/+) were not as numerous, only eight were observed and chemotherapy was effective. In these cases (three lymphomas, three soft tissue sarcomas, one ovarian cancer and one head and neck cancer), a second biopsy was still possible though at least 50% tumor regression had already occurred during the first 2 months. Thus, the second test often permitted making a better choice among the anticancer drugs and to obtain a more significant regression leading to seven true prospective positives. The 8th case in this group, a lung metastasis of a soft tissue sarcoma, is not evaluable at this time because there is no measurable or visible tumor. There were no retrospective false negatives (−/+).

Prospective correlations (Table 6)

Positive correlations are very important for the selection of effective chemotherapeutic agents with which to treat. Prospective negative correlations, on the other hand, provide useful information as to the inactive drugs with which not to treat.

A greater number of prospective negative correlations (−/−) were recorded, but we will observe less of them in the future. We now take into account negative test results, which was not always done at the beginning of our studies. Drugs indicated to be inactive in the assay are almost never used. The possibility of finding no drug sensitivity is not a rare occurrence for patients having already been treated with several anticancer drugs. In such cases, it is logical to use new or untested drugs. For these multitreated patients, drug resistance in the assay indicates a very poor prognosis.

The test may need further refinement of activity criterion as a prospective false negative (−/+) was observed. It was a squamous cell carcinoma of the tongue which showed significant difference (growth inhibition) from the control (Student's *t*-test) for VDS, ADR and Cis DDP. These drugs were classified as inactive having induced no implant regressions, i.e., a $\Delta TS < 1.0$ omu among the treated mice groups. ΔTS was + 0.2 omu for VDS, + 1.0 omu for ADR, and + 1.2 omu for *cis* DDP. Control ΔTS was very high: + 4.69 omu corresponding to one of the largest tumor growth we have observed. This may be an example of under evaluating a positive test by using an activity criterion not suitable for high growth fraction tumors. In this case, rare in our study, the difference of growth between control and experimental groups could be the most important parameter rather than the need to show a $-\Delta TS$. Generally this difference between control and drug treated groups expresses itself by tumor regression with a $\Delta TS < -1.0$ omu and control tumor growth more often with a $+\Delta TS$.

More false negatives will need to be observed to obtain a more precise idea about the limits of our activity criterion. Meanwhile it seems advisable not to systematically reject some anticancer drugs when there is a big discrepancy of this type, or with border line results, i.e., a tumor regression with ΔTS inferior but very near to − 1.0 omu.

As for the four false positives (+/−), our remarks are similar to those concerning the retrospective

study. Chemotherapy may have been under dosed or employed following inadequate modalities (i.e., flash instead of infusion). In these four cases, tumor stabilization or a minor response (regression) less than 50%) was quite clear, for example, in the head and neck cancer; followed by tumor progression after 5 or 6 weeks. Chemotherapy may not have been quantitatively adequate during the period of therapeutic sensitivity or response time. But above all, we observed acquired chemoresistance. It may be that one or several resistant clones may have emerged and become dominant as a result of the destruction of one or several sensitive clones. At the time of the test, tumor heterogeneity is such that sensitivity is observed, and in the treated patient the sensitive tumor portion is relatively quickly destroyed. According to which tumor proportion it represents, one observes either a poor clinical response (stabilization or minor response) or a good clinical response (partial or complete regression).

We were able to perform a second test for two head and neck cancers at the time of tumor regrowth the results of which support this hypothesis. The first corresponds to the false positive with minor response for a nose terebrant squamous cell carcinoma. A first ineffective treatment with MTX, VDS, BLEO and Cis DDP was replaced by ADR alone, the first SRCA having shown sensitivity to this drug. Regression being less than 50% did not permit classifying this patient as a good responder, especially since relapse occurred before the end of ADR treatment in the third month. The second test made at the time showed no anticancer drug sensitivity; drug resistance was thus confirmed.

The second case is even more illustrative, a patient was treated without any effectiveness by VCR, ADR and CTX for an oropharynx spindle cell carcinoma. A first SRCA confirmed clinical inactivity of these drugs. In contrast, PEPLO and Cis DDP, which tested sensitive, induced a partial clinical response with more than 50% tumor regression, for 3 months. Then, tumor regrowth began even while the patient was undergoing the same chemotherapy. The second test confirmed acquired chemoresistance through insensitivity to all tested anticancer drugs including PEPLO and *cis* DDP. The problem of tumor heterogeneity with the risk of acquired chemoresistance remains.

Considering the 19 positive prospective correlations (+/+), discussion is different according to tumor site. One of the three ovarian cancers belongs to true positive groups, in both prospective and retrospective studies. For the other two, a second SRCA allowed us to change an ineffective chemotherapy and to obtain a good therapeutic response. Soft tissue sarcomas also belong to true positive groups both retrospectively and prospectively.

Patients in the lymphoma group were not multitreated. Generally, they only had one or two chemotherapy regimens and no radiation therapy. Three of the five belong to the group of true positive in both retrospective and prospective studies. For the two others, the test permitted a shift from ineffective chemotherapy to an active one.

Only two patients among 13 prospective cases with breast cancers had a good clinical response. Before SRCA these two patients had been treated only by surgery and/or radiation, no chemotherapy. The patient with digestive cancer is in the same situation as he also did not have any chemotherapy before SRCA.

The six true prospective positive head and neck cancer patients present various cases. One of them corresponds with the last case of true positive group both retrospectively and prospectively. Two patients did not have chemotherapy before SRCA. For the three others, the test permitted a shift from an ineffective chemotherapy to an effective one.

The 19 cases of true positive prospective patients can be divided into two groups. The first includes 12 patients without chemoresistance (five not having had chemotherapy before the test, seven already being good responders at test time). The second group includes seven patients treated with ineffective chemotherapy at test time (three head and neck cancers, two ovarian cancers and two lymphomas). For these seven patients, chemotherapy given after the test gave excellent results for the two lymphomas (long duration remission still going on) and medium results for the others (several months remission, then relapse). The change in chemotherapy sensitivity took place between 3 and 6 months after the beginning of the first treatment regimen. For true prospective negative, i.e., breast cancers, there often was more than 6 months between the first chemotherapy and the test.

Survival duration of responder patients is obviously longer than that of nonresponders. For true prospective negative (−/−) the mean is 4.5 months and median 3 months beginning on the day of SRCA. For true prospective positive (+/+), the mean is 10 months and median 8 months. The number of patients in discrepant groups, (+/−) and (−/+), is too small to be reported.

Detailed analysis of the results illustrates the great potential of the SRCA for routine use. It assists the Oncologist in designing optimum chemotherapy of advanced cancers by permitting selection of an effective treatment when the tumor is SRCA sensitive to one or more anticancer drugs. For multitreated patients we frequently do not have any drugs sensitive in the assay. Pleiotrophic

cross-resistance is a clinical reality and the SRCA allows us to avoid ineffective chemotherapies and to use, more quickly, new or untested drugs.

This analysis also emphasizes the question of the influence of prior chemotherapy on subsequent treatments, especially on the problem of acquired chemoresistance. A similar systematic study on previously untreated cancer patients would be of much interest.

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