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Subrenal Capsule Assay in Human Breast Cancer. Response to Cytostatic Drug Combinations and Correlation with Receptor Status*

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Abstract—*The subrenal capsule assay (SRCA) was used to study the sensitivity of breast cancer to cytostatic drug combinations. The results were compared with steroid receptor status. Forty-five of 46 SRCAs (98%) were macroscopically evaluable. However, a histological study implied that the transplants should also be evaluated histologically, because in only 14/21 (67%) of the control SRCAs examined were histologically viable tumor cells seen. An inflammatory cell reaction was noticed in half of the cases. In the groups treated with cytostatics only 3/21 (14%) had vital tumor cells, whereas inflammation was evident in 4/21 (19%) of the cases. The rate of resistance to A + CTX was 30%. By testing several drug combinations against each tumor the rate of chemoresistance was reduced to 10%. The differences between A + CTX and the best combination were statistically significant ($P < 0.05$). Of the tumors 79% were ER-positive and 67% PR-positive. Receptor-negative tumors tended to be more sensitive to cytostatics than receptor-positive tumors (100 vs 85%). The difference was not, however, statistically significant. It can be concluded that the SRCA under standardized conditions is a good method for studying the response of individual breast cancers to chemotherapy.*

INTRODUCTION

IN PATIENTS with advanced breast cancer it could be of utmost importance to know the sensitivity of the tumor cells to chemotherapeutic agents or other biological factors after radical surgery. The subrenal capsule assay (SRCA) of Bogden *et al.* [1] exploits normal, immunocompetent mice and offers a practical, new possibility to test the responsiveness of tumor cells to chemotherapeutic agents in biological circumstances.

In stage III breast cancer, and sometimes also in stage II, there is an obvious need of adjuvant therapy, because the 5 yr survival rate after surgery, if axillary metastases are present, is only 54% [2] and in stage III only 29–48% [3, 4].

Combination chemotherapy appears promising in the treatment of advanced breast cancer, e.g. CMF treatment has produced significantly superior results when compared to a control group [5]. Because controversial reports with poor 5-yr survival rates have also been published [6], further investigation is needed of the role of combination chemotherapy in stages II and III breast cancer.

We have studied the sensitivity of breast cancer to hormonal and cytotoxic agents with the SRCA. In this paper we present the growth of the breast tumors in subrenal capsule, their sensitivity to cytostatic drugs, the correlation to estrogen and progesterone receptors, and the results of histological screening of the transplants.

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MATERIALS AND METHODS

Patients

Participants consisted of 43 patients with mammary cancer. Of these, 31 patients underwent

operations at the Department of Surgery, University Central Hospital of Turku and 12 at the Department of Surgery, Municipal Hospital of Turku, Finland. The mean age of the 13 premenopausal patients was 43 yr (range 26-50 yr) and that of the 30 postmenopausal patients 67 yr (range 50-81 yr). Thirteen premenopausal patients were distributed evenly in clinical stages I-III; only one patient was in stage IV. In the postmenopausal group 24 patients (80%) had a disease of stages I-II. All tumors were invasive ductogenic cancers. The histological grade is presented in Table 1 and the steroid receptor status of the tumors in Table 2. The receptor status of one patient was not determined. Thirty-three tumors were ER+ (79%) and nine ER-. The corresponding figures for PR were 28 (67%) and 14, respectively. Receptors were distributed evenly with regard to histological grading in premenopausal patients. The same held true for PR in postmenopausal patients whereas all (14) postmenopausal ER- patients had poorly differentiated tumors (grade III). There were more ER+ patients in the postmenopausal than in the premenopausal group ($P < 0.05$).

Table 1. *Histological grade of the tumors as related to menopausal status*

		Histological grade		
	n	I	II	III
Premenopausal patients	13	-	7	6
Postmenopausal patients	30	2	21	7
Total (%)	43	2 (5)	28 (65)	13 (30)

Table 2. *Distribution of estrogen (ER) and progesterone (PR) receptors in the tumors as related to menopausal status*

		ER		PR	
	n	+	-	+	-
Premenopausal patients	12	7	5	6	6
Postmenopausal patients	30	26	4	22	8
Total	42	33	9	28	14

All but one of the patients had not been previously treated; one patient had received radiation therapy 2 yr earlier. Of the samples 41 were of primary tumors and five of axillary lymph node metastases. There were three patients from whom samples were taken from both the primary tumor and a lymph node metastasis. The samples for histology, SRCA and steroid receptor determination were taken during the operation from adjacent areas of the tumors.

SRCA

The samples for the SRCA were transferred immediately after excision to sterile tubes containing medium 199 (KC Biological, Lenexa, KS, U.S.A.) in the operating theatre. The technique of the SRCA has been described in detail by Bogden and co-workers [1]. In short, a preselected fresh piece of about 1 mm^3 of tumor tissue is implanted under the renal capsule of mice; the exact size of the fragment is measured by a stereomicroscope fitted with an ocular micrometer; on the sixth day the animals are killed and the final tumor size is measured and the change in tumor size is calculated. The tumor sizes (length + width/2) are expressed in terms of ocular

Table 3. *Cytotoxic drugs tested, their abbreviations, the dosages and routes of administration in the SRCA*

Drug	Abbreviation	Manufacturer	Daily dosage in mg/kg	Route of administration
1. Aclarubicin A	Acl	All-Union Research Institute of Antibiotics, Moscow, USSR	3	s.c. or i.p.
2. Adriamycin	A	Farmitalia Carlo Erba, Milan, Italy	3	s.c. or i.p.
3. *cis*-Platinum	DDP	Lääkefarmos, Turku, Finland	1*	s.c. or i.p.
4. Cyclophosphamide	CTX	Lääkefarmos, Turku, Finland	30	s.c. or i.p.
5. Dibromodulcitol	DBD	Chinoin, Budapest, Hungary	150	p.o.
6. Etoposide	VP-16	Lääkefarmos, Turku, Finland	20	s.c. or i.p.
7. 5-Fluorouracil	5-FU	Hoffman La Roche, Basel, Switzerland	30	s.c. or i.p.
8. Ftorafur	Ft	Lääkefarmos, Turku, Finland	250	p.o.
9. Methotrexate	MTX	Lääkefarmos, Turku, Finland	3	s.c. or i.p.
10. Vincristine	V	Lilly, Indianapolis, U.S.A.	0.4*	s.c. or i.p.

*1 mg/kg in combinations, 2 mg/kg alone.

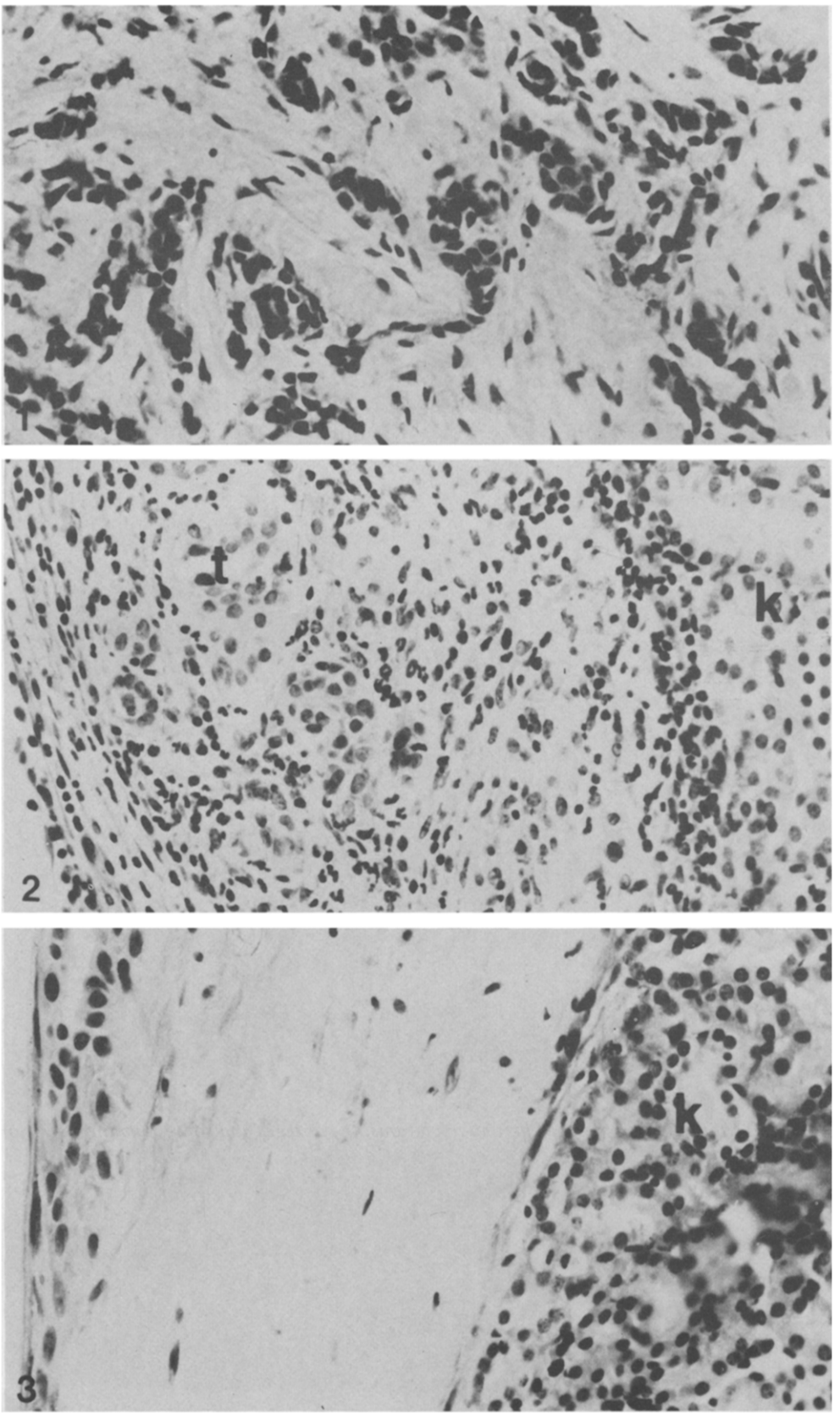

Fig. 1. Histology of a primary breast cancer. The tumor cells are growing infiltratively in a fibrous stroma. sometimes the tumor cells form glandular structures. Hematoxylin-eosin. Magnification × 450.

Fig. 2. Histology of a control transplant. The transplant is seen on the surface of the kidney (k). In the transplant glandular structures formed by tumor cells (t) are seen. Between the tumor cells there are inflammatory cells, most of which are polymorphonuclear leukocytes. Hematoxylin-eosin. Magnification × 450.

Fig. 3. Histology of a transplant from a mouse treated with the combination of cytostatics. The transplant is seen on the surface of the kidney (k). Some surviving tumor cells are seen but most of the transplant consists of fibrin with a few fibroblasts. Only some inflammatory cells are seen on the borderline between the kidney and the transplant. Hematoxylin-eosin. Magnification × 450.

micrometer units (omu), where 10 omu equals 1 mm.

The initial and final body weights of the mice are recorded. Toxicity is considered tolerable when the relative initial/final weight ratio is ≥ 0.80 [1].

Normal immunocompetent BDF_1 and CDF_1 female hybrid mice weighing 20-25 g were used as recipients. They were provided by Bomholtgaard, Copenhagen, Denmark and by the Laboratory Animal Center, Kuopio, Finland, respectively.

For each tumor the test groups (five animals per group) were treated by drugs on a daily schedule for 5 days; the five control animals received physiologic saline. Drugs were administered subcutaneously, intraperitoneally or perorally. Dosage followed the schedule of Bogden *et al.* [1] but was modified by taking into account the toxicity of the drug combinations. The cytotoxic drugs tested and their abbreviations appear in Table 3.

An individual SRCA was considered evaluable if the tumor grafts in the control group did not decrease in size. For interpretation of the results, the response of tumors to drugs was evaluated by a 3-grade scale. A tumor was considered to be sensitive (S) if the mean decrease in tumor size (ΔTS) was >0.5 omu, intermediately sensitive (I) if the decrease was $\leq$0.5 omu or increase <50% of that of the control group, and resistant (R) if the increase was $\geq$50% of that of the control group (Fig. 4).

Receptors

The samples for steroid receptor determination were immediately frozen after excision. The tissues were homogenized in ice cold TEDG buffer (0.04 M Tris-HCl, 0.002 M EDTA, 0.001 M dithiothreitol and 10% w/v glycerol, pH 7.4) with an Ultra-Turrax 18/10 shaft 10 N. The cytosols were obtained after centrifugation at 40,000 *g* at 0-4°C for 1 hr. Aliquots of cytosol (0.1 ml) were incubated for 16-20 hr at 0°C with six different concentrations (0.16-5 nM) of [^{3}H]2,4,6,7-estradiol (115.0 Ci/mmol, New England Nuclear) or 0.32-10 nM [17-methyl-^{3}H]promegestone (R-5020; 87.0 Ci/mmol, New England Nuclear) in 0.1 ml of TEDG buffer with or without a 200-fold excess of unlabeled diethylstilbestrol or R-5020. Bound and free hormones were separated by incubation with 0.5 ml of a mixture of 0.5% charcoal and 0.05% dextran in TEDG buffer for 30 min. After centrifugation 0.5 ml of the supernatant was used for measurement of the radioactivity. The number of binding sites and dissociation constants was determined for each sample according to Scatchard [7]. The protein content of the cytosols was assayed according to the method of Bradford [8]. ER was regarded as positive if the amount was $\geq$10 fmol/mg of cytosol protein and PR if it was $\geq$20 fmol/mg.

Statistics

The statistical analyses of the frequency tables were performed using the χ^2 test and Fisher's four-fold test.

Histology of the transplants

Of the 46 tumors studied with the SRCA, 21 were available for histological control study. In these cases the mouse kidney was fixed in 10% neutral buffered formalin after tumor size measurement. The transplanted tumors were then dissected free from the surrounding renal tissue, dehydrated, embedded in paraffin, cut in 5-μm-thick sections and stained with hematoxylin and eosin.

RESULTS

Evaluability

Samples from breast cancer tissue grew well under the renal capsule as 45/46 of the assays (98%) were evaluable. The one non-evaluable sample was taken from a lymph node metastasis.

Response to the cytotoxic agents used (Table 4)

The drug combination used most was A + CTX, where the resistance rate was 30%. By adding DDP or Ft, the number of resistant tumors decreased to 12.5-25%. Also, the combinations A + DBD, Acl + CTX and DDP + VP-16, and DDP as a single agent were effective whereas nearly half of the tumors were resistant to MTX + V, and nearly all tumors were resistant to MTX + 5-FU or MTX + Ft in spite of varying treatment schedules.

As noted, 30% of the tumors were resistant to A + CTX (Table 4). However, there were seven tumors which were resistant to A + CTX while being, at the same time, at least intermediately sensitive to some other drug combination. In total 10% of the tumors were resistant to all cytostatic treatments (Table 5), which is a significantly smaller rate of resistance than that to A + CTX ($P < 0.05$, χ^2 test).

Histology

The amounts of tumor cells, fibrous stroma and inflammatory cells in the subrenal capsule implants derived from 21 tumors were estaimated histologically.

Tumor cells could be recognized in the control grafts in 14 cases (67%). In half of these grafts there was also infiltration of inflammatory cells (lymphocytes, histiocytes and some polymorphonuclear leukocytes; Fig. 2). In the transplants treated with cytostatic drugs, living tumor cells

Table 4. Response to cytostatics in the SRCA

Treatment	n	Sensitive	Intermediately sensitive	Resistant
A + CTX	44	5	26	13 (30%)
A + CTX + DDP	16	1	13	2 (12.5%)
A + CTX + Ft	16	4	8	4 (25%)
A + DBD	15	2	9	4 (27%)
Acl + CTX	16	2	8	6 (37.5%)
DDP + VP-16	19	3	10	6 (31.5%)
DDP	16	1	11	4 (25%)
MTX + V	16	1	8	7 (44%)
MTX + 5-FU daily	7		1	6 (86%)
MTX + 5-FU seq + CaFol	7			7 (100%)
MTX + 5-FU + CaFol	7		1	6 (86%)
MTX + Ft + CaFol	7			7 (100%)

For abbreviations of drugs see Table 3.

Table 5. Chemosensitivity of the tumors; evaluation based on most effective drug combinations

S*	10 tumors (25%)
I	26 tumors (65%)
R	4 tumors (10%)
Total	40 tumors (100%)

*S = sensitive, I = intermediately sensitive, R = resistant.

Table 6. Chemosensitivity of the tumors as related to estrogen (ER) and progesterone (PR) receptor status

	n	ER +	ER -	PR +	PR -
S + I*	37	28	9	25	12
R *	5	5	0	3	2
Total	42	33	9	28	14

*S = sensitive, I = intermediately sensitive, R = resistant.

were found only in three cases (14%). Most transplants consisted of histiocytes, fibroblasts and fibrin. A slight infiltration of inflammatory cells was seen in four cases (19%; Fig. 3).

Receptors

The correlation of receptor status with the cytotoxic effect of the best drug combination among the individual assays is presented in Table 6. Tumors were sensitive (S + I) in 28 (85%) of the ER+ group. None of the nine ER- tumors was resistant. No such clear correlation was seen in regard to PR- status. The difference between the sensitivity of ER+ and ER- tumors was not statistically significant ($P > 0.05$, Fischer's four-fold test) either.

DISCUSSION

The SRCA is a rather new test of sensitivity to chemotherapy and the interpretation of the results is still partially unresolved. In Bogden *et al.*'s original method three different criteria for drug response have been suggested: decrease in xenograft size (1) by more than 0.5 omu [9] or (2) by more than 1 omu [9], and (3) a xenograft regression of ⩾25% [10]. Based on the experiments with rat tumors, a 3-grade interpretation of drug responses (sensitive, intermediately sensitive and resistant) has been developed [11]. For these rather chemosensitive and rapidly growing animal tumors, the limit of sensitivity was defined at the level of ΔTS of -1 omu. For the more slowly growing human breast cancer a ΔTS of -0.5 omu was considered appropriate. This scale takes into account the individual growth properties of each tumor, in that the ΔTSs of the treated groups are compared to those of the controls.

A rate of evaluability in the SRCA (more than 90%) in this study is comparable to the results of Bogden *et al.* [9], who reported 130/141 tumors evaluable (92%). Bodgen *et al.* [9] state that it is sufficient to measure the growth of the control grafts by a stereomicroscope for purposes of quality control. However, our histological control screening showed that in the case of breast cancer it is necessary to test histologically the quality of tissue growth in the subrenal space because cancer cells were not seen in all of our control grafts. Fiebig *et al.* [12] and Edelstein *et al.* [13, 14] have noted that the inflammatory cell reaction of the host makes interpretation of the SRCA-results more difficult, especially when CTX has been used as a test drug. In the present study the inflammatory cell response was often diminished in drug treated grafts. On the other hand, it is possible that the inflammatory response is more marked in the control group because the growth of the foreign tissue stimulates

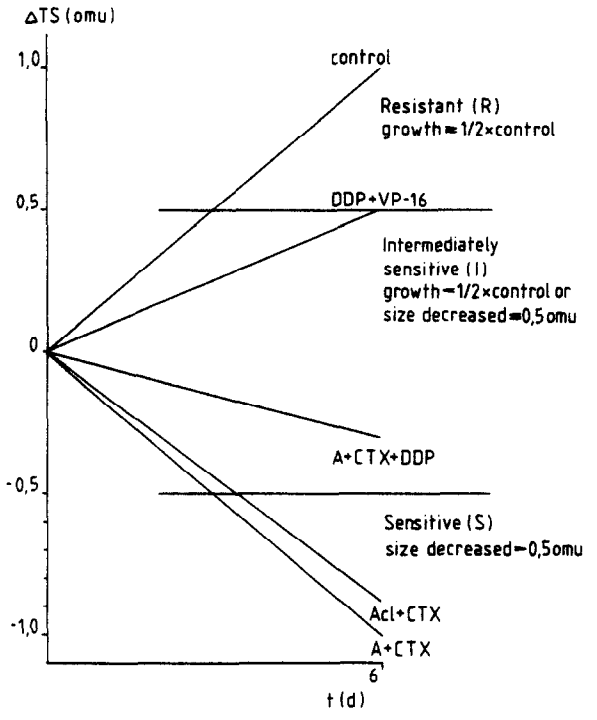

Fig. 4. Sensitivity of a breast cancer (pat. No. 35) to A + CTX, Acl + CTX, A + CTX + DDP and DDP + VP-16. For abbreviations see Table 3.

the host's defense system, whereas the transplants, being drug-treated and consequently degenerated, do not provoke an inflammatory reaction. According to Reale *et al.* [15], host cell infiltration in the control implants does not significantly affect tumor size for up to 6 days.

In contrast to the reports of Bogden's group, who used single agents, we have used almost exclusively drug combinations because chemotherapy of mammary cancer is nowadays mainly combination therapy. The role of the SRCA in predicting the response to combination therapy has been studied with rat tumors, where sensitivity to combined drug therapy could reliably be predicted in 11/12 assays (92%) [11].

The combination of A + CTX was selected as the reference combination because it is widely used in clinical practice and because these drugs may have a synergistic antitumor effect [16]. The combination A + CTX had a good antitumor effect in our study (70% response; Table 4). This is in agreement with clinical studies, e.g. of Jones *et al.* [17], who reported a response of 70% to A + CTX in breast cancer. We also found other effective combinations. By testing several combinations against each tumor the rate of chemoresistance decreased to 10% (Table 5).

MTX and 5-FU in combination have been found to be schedule-dependent in their effect [18, 19]. Therefore, we investigated the significance of different treatment schedules (Table 4). We found the combination to be ineffective in all schedules used.

There was a notable sensitivity of the PR- and ER- tumors to cytostatic drug combinations in the present study. The high mitotic activity often seen in anaplastic tumors might be an explanation of the chemosensitivity found also in the receptor-negative tumors. Kaufmann *et al.* [20] found similar results *in vitro*. However, there are contradictory clinical reports [21]. There were only a few estrogen- and progesterone-receptor-negative tumors in our material. Therefore great care is necessary in interpreting these findings.

This study indicates that receptor-negative patients, in whom hormonal treatment is generally considered to be less effective, can be offered an alternative for treatment: an SRCA-pretested drug combination may improve final results after surgery. This statement must, of course, be resolved by a controlled follow-up study.

We conclude that the SRCA under controlled circumstances offers the opportunity to study the growth behavior of breast cancer and the responsiveness of individual tumors to cytostatic drugs.

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