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In Vitro Adriamycin Sensitivity Test and Hormonal Receptors in Primary Breast Cancer

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Abstract—*Cytoplasmatic estrogen (ER) and progesterone (PgR) receptors were determined by the dextran coated charcoal method in 90 primary breast cancer tissues. In parallel common intrinsic chemoresistance to cytotoxic drugs was examined, using a simple standardized* in vitro *short term predictive test (^{3}H-uridine incorporation adriamycin inhibition test).*

The in vitro *response rate to adriamycin is negatively correlated to steroid receptor content, which was classified receptor-negative, -poor, and -rich.*

Estrogen receptor-negative cancers demonstrate an increased incidence of in vitro *responses to adriamycin (23/38) compared to receptor-rich cancers (7/41). The same hold true for the relationship progesterone receptor content/*in vitro *sensitivity to chemotherapy. Results are most clearcut in cases of identical (ER, PgR) receptor content, where response to adriamycin was observed in 17/26 receptor negative cancers against 1/12 with receptor positive specimens.*

These tools may predict prognosis of early recurrence following mastectomy, and may be useful in stratifying patients for individual adjuvant hormone and/or chemotherapy regimens.

INTRODUCTION

STEROID hormone receptors have proved to predict hormonal responsiveness in breast cancer. This is of relevance for strategy in clinical breast cancer treatment [1, 2]. Patients with estrogen-positive tumours show response to hormonal therapy in about 40–85% according to different studies, whereas estrogen-negative tumours respond to hormonal manipulations in about 15% or less. Progesterone receptors have been found in 30–60%.

The question whether positive steroid receptor content is associated with enhanced or decreased sensitivity to cytotoxic chemotherapy is still controversial [3–6]. To get more information on this subject we determined simultaneously the receptor content and the sensitivity to cytotoxic drugs *in vitro* in primary breast cancer. For prediction of tumour response to chemotherapy a short term incubation method has been used, which according to preliminary studies is predicting satisfactorily *in vitro* chemoresistance to cytotoxic treatment [7–9]. Preliminary results indicated trends without obtaining statistical significance [10]. This paper presents more extensive data on a larger population.

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MATERIALS AND METHODS

Patients

Tumour tissues of primary breast cancer from 90 patients (ages 27–84) treated at our Department were examined in the same laboratory. All the patients had surgical modified radical mastectomies, and were without distant metastases. 26 of the women were pre-, 64 postmenopausal.

Methods

Hormone receptor assay. Tumour tissue specimen were transported to the laboratory immediately after excision and kept at storage at −70°C. The assays were performed within 2 weeks.

The tumour tissue was homogenized by cooling with liquid nitrogen. Cytosol was obtained by ultracentrifugation (105,000 *g*, 60 min). Determination of ER and PgR was done by incubation of the cytosol with ^{3}H-17β-estradiol and ^{3}H-promegestone (^{3}H-R5020), respectively. Nonspecific binding was determined by parallel incubations with non-labelled steroids (100-fold excess). Separation of bound and free steroid was done with dextran coated charcoal [16]. Depending on the amount of cytosol, a five point Scatchard plot or only one saturating concentration of labelled steroids was used. The results were related to the total protein content of the cytosol and expressed as fmole/mg protein. Classification of the receptor content was: ER (PgR) -negative: <5 (<20), -poor: 5–10 (20–50) and receptor rich: >10 (>50).

In-vitro *adriamycin test for chemosensitivity* (*chemoresistance predictive test*). To study the *in vitro* chemosensitivity against cytotoxic drugs, we used a simple standardized short term test system, suitable for clinical routine work [11–13].

Most current antineoplastic substances show effects on the biosynthesis of nucleic acids on the cellular level [14]. Thus instead of miscellaneous drugs we used adriamycin (ADM) as a specific inhibitor of nucleic acid synthesis [15] to determine a common intrinsic chemosensitivity or chemoresistance to cytotoxic drugs.

Immediately after surgical tumour sample extirpation, tumour cells were mechanically isolated without trypsinizing procedures. Cell suspensions (500,000 cells/ml) were incubated for 180 min. Effects of adriamycin (10^{-1}–10^{-4} mg/ml TCM 199) were studied on the ^{3}H-uridine incorporation into acid (TCA)-precipitable material. As evident inhibition effects compared with the control values were seen with 10^{-2} mg/ml adriamycin as the lowest test concentration, we used this concentration for definition of *in vitro* chemosensitivity, respectively chemoresistance. In this test system adriamycin inhibits ^{3}H-uridine incorporation more than ^{3}H-thymidine incorporation. Thus we used uridine as index for adriamycin sensitivity.

Viability of the isolated cell suspensions was proved by the trypan blue exclusion test. Viable cell suspensions were reached in 30–85%. Because of the varying degree of the tumour cell damage during their isolation it is not possible to compare the control values of the different tumours. Therefore in each experiment a control set without drug exposure was used, and test results with drug exposure were specified as percentage inhibition of the control. Furthermore, incorporation kinetic studies were done during the last hour of incubation to determine the viability of the cells. Here test results with negative kinetics were not evaluable in 15% of all the cases.

Definition of chemoresistance: reduction of ^{3}H-uridine incorporation less than 25% compared with the control values without addition of ADM.

Statistical analyses. The difference of chemoresistance in receptor-negative and receptor-rich groups were performed by the contingency χ^2 test (with Yates correction in the case of small numbers).

RESULTS

In all cases ER-receptor content and simultaneous chemoresistance predictive tests were performed; in 62 cases the PgR-receptor content was also determined.

The ER- and PgR-receptor content is shown together with the results of the predictive test in pre- and post-menopausal women in Table 1. The total rate of cases with ER (PgR)-negative receptors clearcut is 42% (63%), and with clearcut ER (PgR)-positive receptors is 46% (24%). There are more postmenopausal women with ER- and PgR-receptors compared with pre-menopausal women. The total rate of *in vitro* chemoresistant tumours is 61%. Post-menopausal patients showed more chemoresistant tumours (66%) as compared to pre-menopausal women (50%).

An analysis of the results of the predictive test and the hormone receptor contents is demonstrated in Fig. 1. Out of the ER-negative cases the short term prediction test determined only 39% (15/38) as chemoresistant. This relationship was 83% (33/40) for the ER rich tumours. Only 46% (18/39) of the PgR-negative cases were chemoresistant, but 80% (12/15) of the PgR rich tumours.

Out of all cases examined, in 39 patients ER and PgR-receptor content was identical, either negative, poor or rich. The results in these cases are shown in Fig. 2. Steroid receptor-negative were 67%, receptor-poor only one, and receptor-rich 31% of the tumours. Out of the 26 negative cases, 9 were chemoresistant (35%), in comparison to 11/12 (92%) of the receptor-rich tumours.

Lack of these hormone receptors is therefore associated with an increased response *in vitro* to adriamycin in primary breast carcinomas.

Table 1. *Estrogen (ER), progesterone (PgR) content and common chemoresistance [effect of adriamycin* (10^{-2} mg ml) *on* ^{3}H-*uridine incorporation] in primary invasive breast cancer (± axillary modes) of pre- and post-menopausal women*

	Receptor content ER n(%)			PgR n(%)			Predictive test chemoresistant *in vitro* $n=90$
	negative	poor	rich	negative	poor	rich	
pre-menopausal ER $n=26$ PgR $n=19$	18 (69)	3 (12)	5 (19)	16 (84)	1 (5)	2 (11)	13 (50%)
post-menopausal ER $n=64$ PgR $n=43$	20 (31)	8 (13)	36 (56)	23 (54)	7 (16)	13 (30)	42 (66%)
total ER $n=90$ PgR $n=62$	38 (42)	11 (12)	41 (46)	39 (63)	8 (13)	15 (24)	55 (61%)

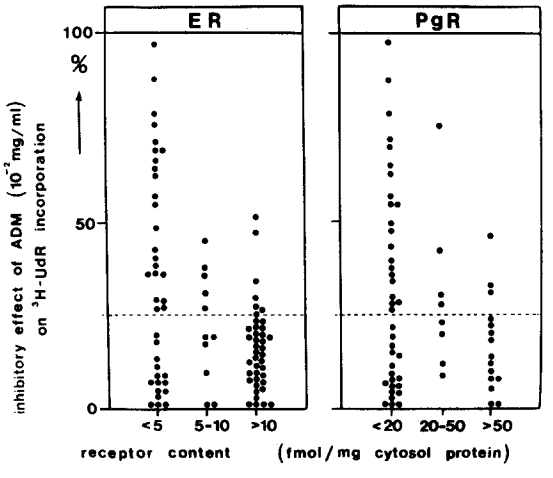

Fig. 1. *Influence of steroid hormone receptor content (ER = estrogen, PgR = progesterone) on response to adriamycin* in vitro (*ordinate*) *in primary invasive breast cancer without distant metastases.* ADM = adriamycin, UdR = uridine, ER: $P<0.001$ ($\chi^2=17.9$), PgR: $P<0.05$ ($\chi^2=5.0$).

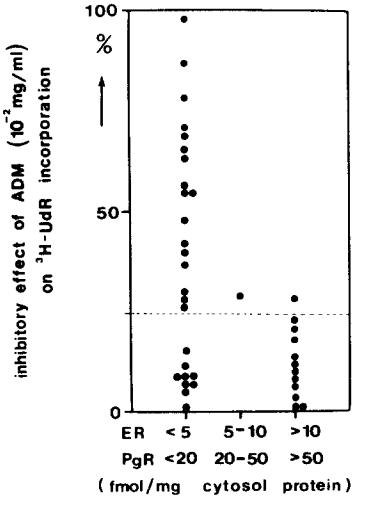

Fig. 2. *Influence of estrogen and progesterone receptor with corresponding, either negative or positive hormone content (definition see Materials and Methods) on response to adriamycin* in vitro *as shown in Fig.* 1. ER, Pgr: $P<0.005$ ($\chi^2=8.55$).

DISCUSSION

Knight *et al.* [18] found ER as an independent prognostic factor for early recurrence in breast cancer. The absence of ER in tumours at primary mastectomy was associated with increased risk of recurrence independent of other known prognostic factors such as age, primary tumour size, location of the tumour, or number of involved axillary lymph nodes. Presence of ER in invasive primary carcinomas of the breast is associated with a low growth fraction (represented by thymidine-labelling index) as demonstrated by Meyer *et al.* [19].

Recent studies reported by Allegra *et al.* [3] and Lippman *et al.* (6) found in retrospective analyses a correlation between ER, PgR and response to chemotherapy in advanced breast cancer, tumours with negative receptor content responding better to chemotherapy than receptor rich tumours. We found a correlation of the steroid hormone receptor (ER, PgR) amount and common chemoresistance of primary breast tumour cells to cytostatic drugs.

These results obtained *in vitro* are comparable to the findings of Allegra *et al.* and Lippman *et al.* in chemotherapeutically treated patients, but are contradictory to the results of Kiang *et al.* [5].

Our hormone receptor assay was done with the dextran-coated charcoal method, which is relatively simple, fast and sensitive and at present seems best suited for clinical work. This method was compared with the more specific, but more time consuming agar-gel electrophoresis method, and a good correlation was found ($r=0.9$) [17]. We determined a common intrinsic chemosensitivity respectively chemoresistance *in vitro* with an *in vitro* short term incubation test, involving native tumour cell suspensions. This method was proved by Mattern *et al.* [8], Kaufmann *et al.* [7], and Volm *et al.* [9], and a good correlation was observed between the effects of ADM on tumour cells *in vitro* in the predictive test and the clinical outcome in patients with lung and ovarian carcinomas, treated with conventional chemotherapy regimens. Adriamycin was found out as a general test drug, which allows the prediction of a common chemoresistance in human tumours with 96% accuracy. Preliminary results of a co-operative study (Co-operative Study Group for Sensitivity Testing of Tumours) in West Germany confirm these data, in such as *in vivo* resistance to cytotoxic drugs is predictable with such high reliability (to be published).

The concentration of the drugs usable in this short term test (3 hr) is relatively high, because of the short incubation time, compared to other test systems with incubation times of about 2–3 or more weeks [20]. Therefore, 3-hr exposure is not comparable to the 1-hr exposure of the drugs in the semi-solid agar assay. In both test systems, however, results were seen without efficacy even after exposure to very high doses of the test agents.

Both tools have in common, that they have to determine the behaviour of the critical cells representative for the total tumour cell population. Because of heterogenity of solid tumours, this is a great problem in studying solid tumours. Thus, homogenized tissue for the receptor assay, respectively, cell suspensions of the tumour for the chemoresistance test are investigated.

Results from the predictive test on chemoresistance are more reliable than results on chemosensitivity, because any possible predicted positive response can be affected by physiological differences between patients, such as different metabolism and pharmacodynamics of single drugs, blood supply to the tumour etc.

Lack of ER- and/or PgR-receptor is associated with an increased response *in vitro* to antineoplastic medicaments in tumours of patients with invasive primary breast cancer but the relation to clinical outcome in terms of survival, recurrence and effect of adjuvant treatment have to be awaited before drawing firm conclusions.

Further work, however, is needed to establish the validity in clinical routine management of both tools in prospective studies to get better therapy results in breast cancer patients.

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