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In vitro sensitivity testing of human breast cancer cells to hormones and chemotherapeutic agents

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Summary. The sensitivities of human breast cancer cells to hormones and chemotherapeutic agents were measured using a new in vitro assay. Tumor cells from individual patients were cultured on collagen-coated dishes in medium containing the patient's serum. The rationale for use of the patient's serum is that the components of this serum interact with the cells and therapeutic agents in vivo. Cells were incubated for the length of the assay in the presence or absence of estrogen (E_2) with or without tamoxifen (TAM) or in the presence or absence of cortisol (F). At 1 day after cell seeding, cells were exposed to a chemotherapeutic agent, Adriamycin, melphalan, or 5-fluorouracil, for 24 h. After a 48-h recovery period, [^{3}H]-thymidine ([^{3}H]-TdR) was added to the cultures for 24 h. Depending on the concentration, E_2 generally stimulated or inhibited incorporation of [^{3}H]-TdR into the DNA of cells from estrogen-receptor (ER)-positive tumors. TAM eliminated the effects of E_2. F generally stimulated or inhibited incorporation in cells with no correlation to ER status. Stimulation of [^{3}H]-TdR incorporation by hormones increased cell sensitivity to Adriamycin. In contrast, hormone inhibition of [^{3}H]-TdR incorporation decreased cell responsiveness to this drug. This rapid assay, which can measure the sensitivities of breast carcinoma cells to hormones and drugs and identify effective combinations of therapeutic agents, should lead to a rational selection of treatment for the individual patient.

Introduction

Currently, the selection of hormones and drugs used in breast cancer therapy is empirically derived. However, tumors that are clinically similar vary in their response to drugs and the cure rate is low [17]. It would be of obvious clinical value to develop an in vitro assay that could identify appropriate therapies for the individual patient. Although clinical correlations indicate that the assays developed to date are useful for prediction of drug resistance, they cannot be used with confidence to predict the sensitivities of cells to therapeutic agents [13].

We have previously demonstrated that the growth and sensitivities to chemotherapeutic agents of human mammary epithelial cells cultured in medium containing the patient's serum differs from those of cells maintained in other media [15]. We suggest that a cell-culture system that uses the patient's serum has potential application for predictive sensitivity testing since the cells and therapeutic agents interact with the components of this serum in vivo. Furthermore, the high plating efficiency resulting from our cell-culture protocol provides us with sufficient cells to study hormone and drug interactions.

In the clinical setting and in cell lines in vitro, it has been demonstrated that inhibition of tumor growth by endocrine means, such as tamoxifen (TAM), may make cells refractory to the cytotoxic effects of chemotherapy [3, 7, 20–22, 25]. Alternatively, the therapeutic efficacy of chemotherapeutic agents may be enhanced by the use of growth-stimulating hormones such as estrogen [5, 9, 10, 16, 19, 21, 22, 28]. This interaction between hormones and drugs is not limited to hormone-receptor-positive tumors. TAM plus chemotherapy can decrease the efficacy of the drugs in estrogen-receptor-negative (ER–) tumors [33]. However, uncontrolled tumor growth resulting from the use of growth-stimulating hormones and ineffective chemotherapeutic agents could be hazardous to the patient. It is necessary to identify patients who will benefit from combination therapy. This research was designed to determine whether the exposure of cells cultured in the patient's serum to different concentrations and combinations of hormones and chemotherapeutic agents can identify effective antineoplastic agents as well as appropriate combination therapies for the individual patient.

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Materials and methods

Cell culture. Our culture procedure has previously been described [15]. Briefly, mammary carcinomas (infiltrating ductal carcinomas) from biopsies or mastectomies were minced and dissociated in a 1 : 1 mixture of Ham's F12 : Dulbecco's modified Eagle's medium (DME; Terry Fox Laboratory, Vancouver, B. C.) containing 10 m*M* HEPES buffer, 2% bovine serum albumin, 5 μg/ml insulin, 300 IU/ml collagenase, and 100 IU/ml hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) at 37° C for approximately 18 h. The epithelial cell pellet was collected by centrifuging the cell suspension at 80 *g* for 4 min. The pellet was washed twice with DME, then passed through 150-μm Nitex (Tetko, Inc., Elmsford, N. Y.) to collect small cell aggregates and single cells. Viable cells were determined by trypan blue exclusion and counted on a hemacytometer. The cells were resuspended in culture medium and seeded at 4×10^4 cells/5-mm well onto collagen-coated 96-well tissue-culture plates (Falcon). Cultures were incubated at 37° C in 95% air/5% CO_2.

The collagen was prepared by dissolving 1 g rat-tail collagen in 200 ml sterile acetic acid in water (1 : 1,000), centrifuging at 100 *g* for 5 min, and collecting the supernatant as previously described [14]. A drop of collagen solution was placed in each 5-mm well, spread evenly to coat the well, and allowed to dry. The culture medium consisted of Ham's F12 : DME (1 : 1) supplemented with 10 m*M* HEPES buffer, 5 μg/ml insulin, and 5% dextran-charcoal (DC)-treated or untreated serum from the patient, depending on the experiment. Some medium contained cortisol (F; 10^{-5} *M*; Sigma). Some medium contained 17-β-estradiol (E_2; 10^{-9}–10^{-6} *M*; Sigma) with or without the antiestrogen TAM (10^{-6} *M*; ICI Pharma Canada, Mississauga, Ontario).

Serum samples were collected in the mornings from patients who had fasted over the previous 8–12 h. Blood was received in non-heparinized tubes, incubated for 30 min at 37° C, and centrifuged at 100 *g*, and the serum was then collected. If not used immediately, the serum was stored at –20° C.

Drug protocol. The chemotherapeutic agents used in these experiments included Adriamycin (AD; Adria Laboratories Ltd. of Canada, Mississauga, Ontario), at 10^{-6} and 10^{-7} *M*; melphalan (MEL; Burroughs Wellcome Inc., Kerkland, Quebec), at 10^{-6} and 10^{-7} *M*; and 5-fluorouracil (5-FU); Adria Laboratories), at 5×10^{-4} and 5×10^{-5} *M*. The higher concentration of drug corresponds to the peak plasma level obtainable for each drug. Drugs were dissolved in distilled water and added to 1-day-old cultures for 24 h as previously described [15]. Cultures were washed twice with DME, then allowed to recover in drug-free medium for 48 h before incubation in 1 μCi/ml [^{3}H]-thymidine ([^{3}H]-TdR; New England Nuclear, DuPont Canada, Inc., Markham, Ontario) for 24 h. [^{3}H]-TdR incorporation into DNA was measured by treating cultures with 0.8% Triton-X 100 for 2 h and collecting cell fractions on Whatman GF/C filters in a cell harvester. The filters were submerged in Aquasol (New England Nuclear) and counted in a Philips liquid scintillation counter. Cytotoxicity was determined by comparing [^{3}H]-TdR incorporation into DNA in drug-treated and control cultures.

Results

Cell response to hormones

We have previously shown by light and electron microscopy that approximately 85% of the cells obtained from infiltrating ductal carcinomas were epithelial cells and that cell growth as determined by [^{3}H]-TdR incorporation into DNA occurred in these cells. Cultures not exposed to drugs and terminated on day 4 were in the exponential phase of growth [15].

Cultures from eight ER+ tumors and four ER– tumors were incubated with E_2. At 10^{-9} and 10^{-8} *M*, E_2 stimulated [^{3}H]-TdR incorporation into DNA in six of the cultures from ER+ tumors and inhibited incorporation in one (Table 1). At 10^{-7} and 10^{-6} *M*, E_2 inhibited four and stimulated one of the eight ER+ tumors. TAM generally cancelled both the stimulatory and inhibitory effects of E_2. E_2 and TAM had no effect on the growth of the four ER– tumors. Cultures from five tumors were incubated in F: F stimulated growth in two, an ER+ and an ER– tumor, and inhibited growth in two, an ER+ and an ER– tumor (data not shown). Therefore, there was no correlation between the effects of F on cell growth and ER status.

Cell response to drugs.

The effects of drugs on [^{3}H]-TdR incorporation varied with the drug, the concentration of drug, and the tumor specimen (Fig. 1). To determine whether the drug protocol measured cytotoxic rather than cytostatic effects of the drugs, cultures were terminated 2, 5, and 8 days following drug treatment. At 24 h prior to termination, cultures were incubated with [^{3}H]-TdR. Figure 2 illustrates that as late as 8 days following drug treatment, cells from one tumor did not regain a capacity to incorporate [^{3}H]-TdR comparable with that of controls. Similar results were obtained with three other tumor specimens.

Table 1. Effects of 17-β–estradiol (E_2) and tamoxifen (TAM) on the growth of estrogen-receptor-positive human breast cancer cells

Patient	Age (years)	E_2 concentration (*M*)							
		10^{-9}	+ TAM	10^{-8}	+ TAM	10^{-7}	+ TAM	10^{-6}	+ TAM
		(% of control)							
1	32	158	109	120	100	50	96	33	103
2	37	224	112	220	101	105	105	100	100
3	76	209	98	182	107	100	101	50	94
4	55	60	102	75	99	100	100	100	100
5	38	137	118	131	113	77	92	46	74
6	73	131	95	140	101	100	100	60	72
7	57	200	200	233	233	182	118	100	100

Tumors from 7 patients were cultured in triplicate in medium containing 5% dextran-charcoal (DC)-treated patient's serum in the presence and absence of different concentrations of E_2 with or without TAM (10^{-6} *M*). Cultures were exposed to [^{3}H]-TdR on day 4 for 24 h and were then terminated. Cell growth was determined by measuring [^{3}H]-TdR incorporation into DNA. Cultures maintained in the absence of E_2 served as controls. The SEM was <5% for all samples

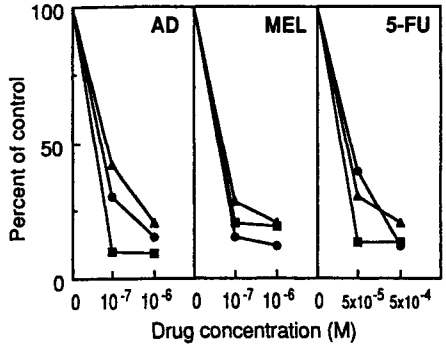

Fig. 1. Chemosensitivities of cells from 3 human breast carcinomas (—●—, —▲—, —■—). Cultures were maintained in medium containing 5% patient's serum. Cells were exposed to Adriamycin (*AD*), melphalan (*MEL*) and 5-fluorouracil (*5-FU*) for 24 h on day 1. Cultures were incubated with [^{3}H]-TdR for 24 h on day 4. Results were obtained from triplicate determinations; the SEM was <5% for all samples

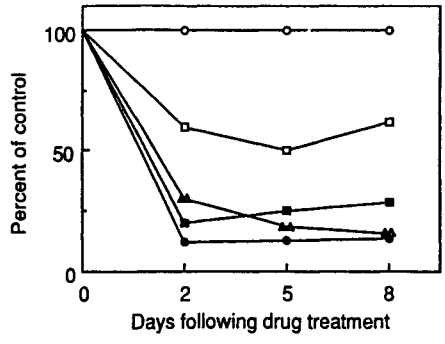

Fig. 2. Time course of effects of cytotoxic agents on the growth of cells from a breast carcinoma. Cultures were exposed to drugs for 24 h on day 1. At 24 h prior to termination, cultures were incubated with [^{3}H]-TdR. AD: 10^{-7} *M*, —○—; 10^{-6} *M*, —●—. MEL: 10^{-7} *M*, —□—; 10^{-6} *M*, —■—. 5-FU: 5×10^{-5} *M*, —△—; 5×10^{-4} *M*, —▲—. Each point represents the mean of triplicate cultures; the SEM was <5% for all samples

Cell response to hormone and drug combinations

Cultures were incubated with E_2 or F for the length of the assay and exposed to AD for 24 h on day 1. In the cases studied to date, stimulation of cell growth by E_2 (Fig. 3; one of four different experiments) or cortisol (Fig. 4; one of two different experiments) increased cell sensitivity to the cytotoxic effects of AD. In contrast, the inhibition of cell growth by these hormones decreased responsiveness of the cells to AD.

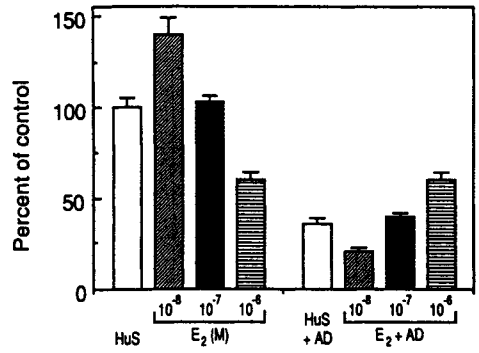

Fig. 3. Effects of E_2 on AD cytotoxicity. Breast carcinoma cells were cultured in triplicate in medium containing 5% DC-treated patient's serum in the absence (*HuS*) or presence of different concentrations of E_2 and were exposed to AD (10^{-7} *M*) for 24 h on day 1. Cultures were incubated in [^{3}H]-TdR for 24 h before termination on day 5

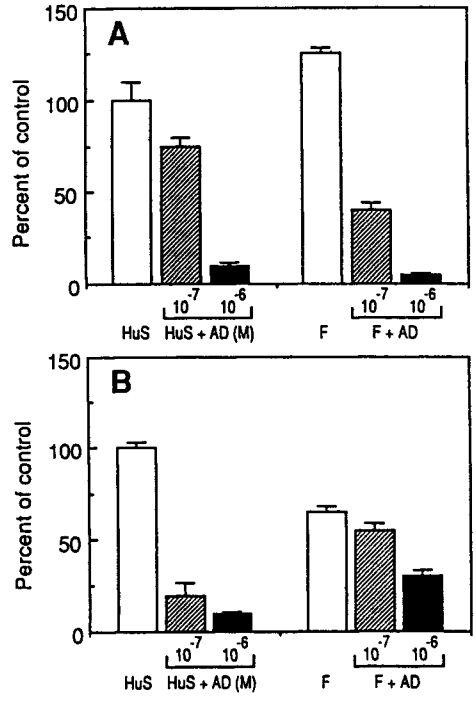

Fig. 4 A, B. Effects of cortisol (*F*) on AD cytotoxicity. Breast carcinoma cells from 2 patients (**A**, **B**) were cultured in medium containing 5% DC-treated patient's serum in the absence (*HuS*) or presence of F (10^{-5} *M*) and were exposed to 2 concentrations of AD for 24 h on day 1. Cultures were incubated in [^{3}H]-TdR for 24 h on day 4 and then terminated

Discussion

The purpose of this research was to determine whether human breast tumor cells cultured in the patient's serum were sensitive to hormones and drugs used either alone or in combination. The clinical relevance of such determinations requires the identification of effective hormone-drug combinations for cells obtained from the tumor in question. Previously, experiments designed to determine hormone-drug interactions in human breast cancer have been conducted on cell lines. A cell-culture system used for predictive sensitivity testing should simulate in vivo conditions as closely as possible. The patient's serum provides factors to which the cells and therapeutic agents are exposed in vivo.

At physiological concentrations (10^{-9}–10^{-8} *M*) E_2 acts as a mitogen, and at high doses it decreases proliferation in some ER+ and ER– tumors from patients and in some ER+ and ER– human breast-cancer cell lines [2, 8, 11, 22, 23, 27, 32]. In the present study, E_2 generally stimulated growth at physiological concentrations and inhibited

growth at pharmacological concentrations in cells from ER+ tumors. It had no growth effects on cells from ER– tumors. TAM was used at 10^{-6} *M*, the concentration achievable in patients. At 10^{-6} *M*, TAM also inhibits E_2 stimulation of growth of MCF-7 cells (a human breast-cancer cell line) [6, 26]. We have demonstrated that TAM negates the effects of E_2 on the growth of cells in primary culture. F has been shown to influence the growth of human mammary epithelial cells in culture [18, 24, 29]. In the present study, F either stimulated, inhibited, or had no effect on cells from both ER+ and ER– tumors. The data indicate that the effects of these factors on cell growth can be detected in this culture system.

Cells maintained in this culture system were also sensitive to the cytotoxic effects of chemotherapeutic agents. The concentrations of drugs used were the peak plasma levels achievable for each drug and 1/10 of this level. Current data indicate that clinically relevant concentrations in vitro appear to be approximately 1/10 of the peak plasma concentration [1, 4]. Although higher concentrations may produce false-positive responses, their use may identify tumors that are sensitive only to high concentrations. This could lead to the selection of high-dose drug treatment or regional administration of drugs [12, 31].

The present study supports previous reports [5, 9, 10, 16, 19, 21, 22, 28] demonstrating that the growth-promoting effects of hormones may enhance cell kill by chemotherapeutic agents. A positive interaction between E_2 and AD in particular has been demonstrated in some human breast-cancer cell lines [5, 21, 28] and, to a limited degree, in patients [9, 10, 19]. Likewise, this data is consistent with those from other studies [3, 7, 20–22, 25] showing that growth-inhibiting hormones may make cells refractory to chemotherapy.

It is obvious that there are important clinical implications concerning interactions between hormones and cytotoxic agents for breast cancer. Since drug and growth-inhibiting hormone combinations generally are not beneficial in the treatment of breast cancer, new approaches involving stimulation of tumor cell growth by hormones to improve the therapeutic efficacy of drugs is a possibility. A problem is that breast cancers vary in their sensitivities to different hormones and drugs, both in their response to growth-stimulating hormones and drugs and in the optimal timing for drug administration. The doses of hormones and drugs and the interval of administration of these agents are critical for true synergism [30]. Furthermore, if not conducted properly, these protocols could have adverse effects on patients due to stimulation of tumor growth. An in vitro assay designed to reflect the in vivo situation of the individual patient could streamline therapy for that patient. The use of the patient's serum should optimize conditions in culture for predictive sensitivity tests, since the cells and therapeutic agents are exposed to the components of this serum in vivo. This culture system could be used to eliminate ineffective agents, test additive or synergistic effects of hormones and drugs, and test different treatment schedules. We are currently evaluating the validity of this assay by correlating results with clinical response.

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