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Chemoresistance is Not a Cause of the Apparent Failure of Adjuvant Chemotherapy in Postmenopausal Women

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Abstract—*Chemotherapeutic regimens used for the adjuvant treatment of breast carcinoma are less effective when applied to postmenopausal women than when applied to premenopausal women. Differences in growth fraction or altered chemosensitivity of tumors are potential causes of the differential effects of chemotherapy in younger and older patients. We have attempted to identify the presence of these putative causes by measuring size of clonogenic cell fraction and drug sensitivity of progenitor cells on the tumors from pre- and postmenopausal women. We found that the chemosensitivity of tumors was similar for patients of all ages. We further observed that the clonogenic cell fraction of tumors from women older than 65 years tended to be smaller compared to those of all other patients, while the hormone-sensitivity of tumors from these patients was higher. Our observations thus suggest that drug resistance related to inherent metabolic characteristics of tumor cells may not be a major contributing cause of failure of adjuvant chemotherapy in the treatment of postmenopausal women.*

INTRODUCTION

BREAST CARCINOMA is primarily a disease of the elderly woman and affects postmenopausal women more often than premenopausal women [1]. The disease remains difficult to cure. Since in a large proportion of patients the tumor is disseminated from the outset, systemic treatment has recently been tried to improve upon the effects of local treatment. Hormones and cytotoxic drugs that effectively control advanced disease have been used for this purpose. The impact on the disease course of these adjunctive treatments has recently been evaluated [2]. The result of the analysis revealed that chemotherapy had increased the cure rate for premenopausal women, but was of uncertain efficacy for postmenopausal women, particularly for those women who had more than three axillary nodes involved with disease. Based on these findings, adjuvant chemotherapy is no longer recommended for postmenopausal women unless the tumor is estrogen receptor-negative, and hormones are recommended in its place [2].

It is not clear what causes the difference in efficacy of chemotherapy, if used for pre- or postmenopausal women; and in the case of postmenopausal women, if used for treatment of visible (advanced) and non-visible (early) disease. Refractoriness of tumors towards chemotherapy often results from inherent metabolic properties of tumor cells or from the kinetic behavior of these cells [3]. The metabolic type of drug resistance refers to the availability to cells of biochemical pathways through which they can either avoid lethal drug–target interactions, or repair their effects. This type of resistance *increases* with the evolution of tumors. Conversely, the *kinetic type of drug resistance* relates to the proliferative activity of tumor cells and refers to the refractoriness of quiescent cells towards those cytotoxic drugs that target metabolites formed only during the active stages of the cell cycle. This second type of drug resistance *decreases* as the disease evolves. Different strategies are necessary to overcome these two types of drug resistance. A change of cytotoxic drugs is necessary to overcome the type of inherent drug resistance, and a change in the hormonal environment may be necessary to overcome the type of cytokinetically related drug resistance. Since both modifications are potentially available, proper identification of factors that cause the relative refractoriness to chemotherapy of postmenopausal women is important.

We have investigated this important question *in*

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vitro, by determining the size of the clonogenic fraction of tumors (as an estimate of the tumor's growth fraction), the chemosensitivity of tumors, and the hormone sensitivity of tumors. We have shown in the past that tumor growth stimulatory hormones can overcome the *in vitro* drug resistance related to cell quiescence, by increasing the proportion of actively proliferating cells [4]. Based on this observation, we assayed the chemosensitivity of tumors under conditions supplemented with growth factor and hormones.

MATERIALS AND METHODS

Patient characteristics

The tumors from 99 patients with breast carcinoma were studied during a 3 1/2 year period. The median age of these patients at the time of study was 51 years. Patients less than 50 years old at the time of study were considered premenopausal, and patients 50 years or older were considered postmenopausal. Thirty-eight patients were premenopausal (median age 41, range 30–49), and 61 patients were postmenopausal (median age 60, range 50–80). Twenty-six per cent of premenopausal women and 36% of postmenopausal women had not received chemotherapy prior to testing. All other patients had been treated with one or more chemotherapeutic regimens. For some aspects of the analyses, patients were divided into four age categories: < 45, 45–55, 55–65, > 65 years of age. Forty-three per cent of tumors from patients less than 45 years old were ER-negative, but only 15% of tumors from patients over 65 years old were ER-negative.

Sample acquisition

One hundred and four metastatic tumor lesions were assayed from 99 patients. Thirty-nine per cent of specimens were malignant fluids and 61% were solid tumors. Samples were collected in the Departments of Surgery or Medical Oncology of the U.T.M.D. Anderson Hospital. Solid tumors were collected into 10 ml of Ham's nutrient mixture F12 (F12) (Gibco, Grand Island, NY), to which 15% fetal bovine serum (FBS) (KC Biological, Lenexa, KS) and 1000 units of preservative-free heparin (Fisher Scientific, Houston, TX) has been added. Ten units of heparin were also added to each milliliter of aspirated fluid. Samples of normal bone marrow were aspirated from the posterior iliac crest in 38 patients with newly diagnosed solid tumors, whose bone marrow was not involved with disease, but was examined for diagnostic purposes. All patients agreed in writing for the experimental use of their tumor or bone marrow.

Measurement of clonogenicity

Colony-formation in agar cell cultures was used to estimate the size of the clonogenic cell fraction of tumors. Solid tumors were debrided and diced into 1 mm cubes with scalpels. Single cells were teased into suspension with 25-gauge needles. Cells were then suspended for 20 h in a mixture of enzymes which consisted of 1.0 type III collagenase, 0.6% elastase (Worthington Biochemical Corporation, Freehold, NJ) and 0.005% deoxyribonuclease (Sigma Chemical Corporation, St. Louis, MO), at 37°C, under continuous agitation. No elastase was used for the treatment of cells obtained from malignant effusions. Following enzymatic dissociation, cells were washed in calcium and magnesium-free Hank's balanced salt solution (GIBCO, Grand Island, NY) and resuspended in F12 with 10% FBS. Remaining aggregates of cells were removed by passing the suspension sequentially through 18-, 22- and 25-gauge needles. A Coulter counter (Coulter Electronics, Hialeah, FL) was used and the viability of cells was determined by their ability to exclude trypan blue dye.

Cells were then set into agar cultures. Underlayers consisted of 1 ml volumes of 70% F12, 20% conditioned medium, and 10% horse serum (KC Biological, Lenexa, KS) in 0.5% agar (Difco, Bactoagar, American Scientific Products, Houston, TX). Conditioned medium was obtained from the supernatants of three established human breast tumor cell lines (MDA-468, MDA-435 and MDA-231) [4]. 10 μg/ml bovine crystalline insulin, 2.5 μg/ml hydrocortisone, 5×10^{-7} M 17-*b*-estradiol, and 50 ng/ml epidermal growth factor were also added to underlayers. Estradiol and hydrocortisone were obtained from Sigma Chemical Corporation, St. Louis, MO and insulin and epidermal growth factor from Collaborative Research Inc., Waltham, MA. Upper layers consisted of 1 ml volumes of 85% alpha-modified essential medium (GIBCO, Grand Island, NY) and 15% FBS, in 0.3% agar. 5×10^5 cells were added to each culture dish. Three to six replicate cultures were obtained, and one additional plate was fixed with glutaraldehyde and stored at 40°C.

All other cultures were incubated in a fully humidified atmosphere of 5% CO_2 and 12% O_2 at 37°C. After a 2-week period they were scored for colony growth. Aggregates of 50 or more cells, or with a minimal diameter of 75 μm and of uniform morphology, were considered the progeny of clonogenic cells. The glutaraldehyde-fixed plate was scored similarly. Clumps so enumerated were subtracted from the score of the cultured plates to arrive at the final colony count of cultures.

Measurement of chemosensitivity

We defined the chemosensitivity of breast tumors by their responsiveness to the following drugs: 5-fluorouracil, adriamycin, cyclophosphamide and vinblastine. These drugs have well-estabished activity in the treatment of breast carcinoma. Doxorubicin and 5-fluorouracil were obtained from Farmitalia, Carlo Erba, SA, Italy; vinblastine from Eli Lilly, Indianapolis, ID and 4-hydroxyperoxycyclophosphamide from Dr. Michael Colvin, Johns Hopkins University School of Medicine, Baltimore, MD. 4-Hydroxyperoxycyclophosphamide is a metabolite of cyclophosphamide with *in vitro* cytotoxic activity.

We had measured previously the sensitivities of normal bone marrow progenitor cells to these four cytotoxic drugs. Eleven to 35 sensitivity assays per drug were performed on the marrows of 38 volunteers [5, 6]. From these data we constructed linear regression lines and used their slope to define the equitoxic dose ranges for drug testing. They were the following: 1–3 μg/ml for 5-fluorouracil, 0.01–0.04 μg/ml for adriamycin, 0.7–4.0 μg/ml for cyclophosphamide, and 0.0025–0.01 μg/ml for vinblastine. For our experiments, we evaluated drug effects on tumors up to the dose that inhibited approximately 99% of GM-CFU.

Tumor cells were dispersed and cultured as described above. Cytotoxic drugs were admixed to the upper layers (as had been done for bone marrow cultures), and the effects of at least three graded concentrations were measured. The dose–response curve over the entire exposure dose range and the tumor cell kill at the approximate LD_{50} for GM-CFU were used for the comparisons.

Measure of hormone sensitivity

Forty-five tumors were cultured in the absence and presence of hormone supplements. The hormone sensitivity of these tumors was then calculated as the ratio of colony formation under hormone-supplemented and regular conditions, and was expressed on a $\log_{10}$ basis.

RESULTS

Forty-seven per cent of all specimens cultured for various purposes during the 3.5 year time period could be utilized to measure the chemosensitivity of clonogenic cells. These were the tumor samples that formed 30 or more colonies and that were large enough in size for chemosensitivity testing in at least three graded concentrations. One hundred and seventy-four drug assays were altogether performed on the 104 tumor specimens.

The viability of cultured cells was in the average 90%, and 1% of colony growth represented in general clump contamination. Tumors formed between 0 and 477 colonies per 10^5 cells, if cultured under regular conditions and between 30 and 1574 colonies, if cultured under hormone-enriched conditions.

Under regular conditions tumors from premenopausal women formed an average of 82 colonies while tumors from postmenopausal women formed 89 colonies. But tumors from patients over 65 years old formed only 30 colonies. This decline of tumor clonogenicity in patients over 65 years was however not significant ($P = 0.2$). Conversely, the hormone sensitivity of tumors increased after the age of 65 years. Thus, the hormone sensitivity was 2.3 for tumors from patients less than 65 years old and 7.2 for patients older than 65 years. The difference was again not statistically significant. It is also of note that the proportion of ER-positive tumors increased with age (29% for premenopausal women and 55% for postmenopausal women), and that ER-positive tumors were more hormone sensitive than ER-negative tumors (the hormone sensitivity was 4.1 for ER-positive tumors and 1.7 for ER-negative tumors).

We used standard agents for treatment of breast carcinoma to evaluate the chemosensitivity of breast tumors, and we determined the chemosensitivity by measuring the inhibitory effects on the colony forming ability of tumor progenitors of 5-fluorouracil, adriamycin, 4-hydroxyperoxycyclophosphamide and vinblastine. All drugs were tested within the dose range that was equitoxic to GM-CFU.

We found that exposure to chemotherapy prior to the assay did not significantly change the chemosensitivity of tumors to 5-fluorouracil, adriamycin, hydroxyperoxycyclophosphamide and vinblastine ($P > 0.1$ for drug-induced tumor cell kill at the LD_{50} for GM-CFU of untreated and treated tumors). Only the sensitivity of tumors to hydroxyperoxycyclophosphamide decreased somewhat following their exposure to cytoxan-containing treatments. Similarly, the estrogen receptor status of tumors did not significantly influence the chemosensitivities of clonogenic cells, but ER-negative tumors tended to be more chemosensitive.

Figures 1–3 illustrate the dose-responses of tumors to 5-fluorouracil, adriamycin and 4-hydroxyperoxycyclophosphamide for premenopausal and postmenopausal women. The heavy-printed lines reflect the regression lines for the dose responses of bone marrow cells. Tumors obtained from premenopausal and tumors obtained from postmenopausal women displayed similar sensitivities towards these three drugs, with a similar distribution towards higher and lower sensitivities relative to that of the bone marrow. Thus no age-related difference of the chemosensitivity of tumors became apparent in this analysis. A comparison of chemosensitivities measured at the LD_{50} for GM-

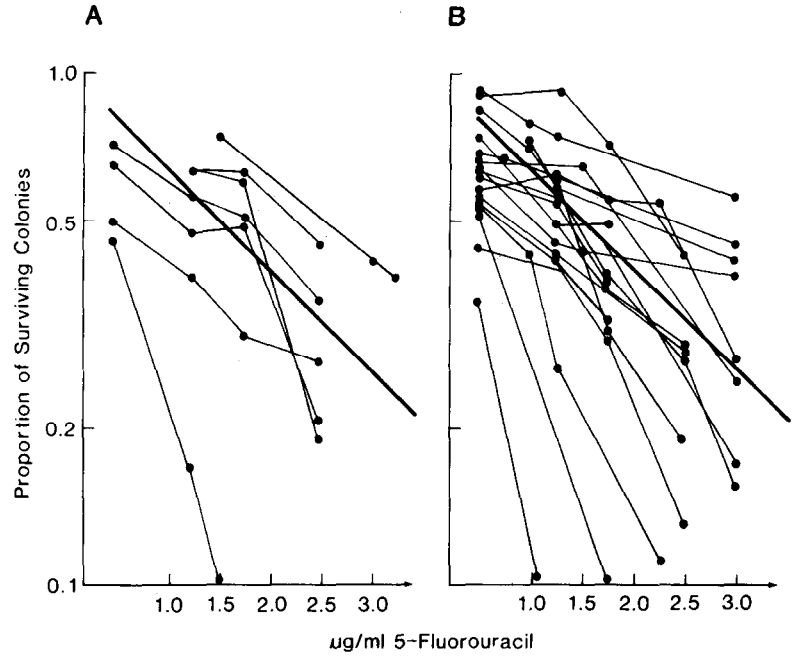

Fig. 1. Dose responses of 26 breast tumors towards 5-fluorouracil. Seven tumors from premenopausal women (Graph A) were assayed, and 19 tumors from postmenopausal women (Graph B). The log-linear line represents the standard of reference for the effects of 5-fluorouracil on normal bone marrow progenitors.

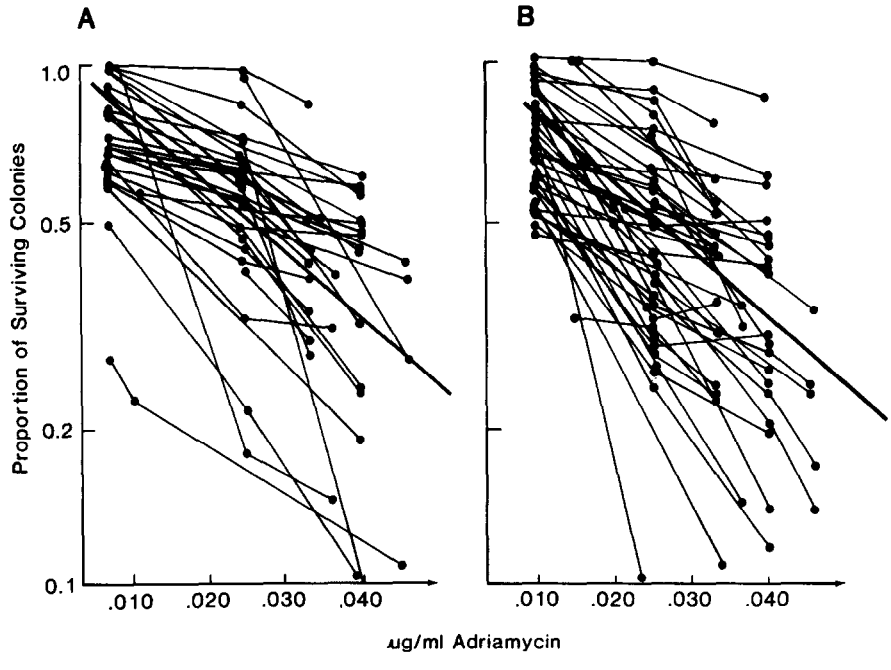

Fig. 2. Dose responses of 78 breast tumors towards adriamycin. Thirty-one tumors from premenopausal women (Graph A) were assayed, and 47 tumors from postmenopausal women (Graph B). The log-linear line represents the standard of references for the effects of adriamycin on normal bone marrow progenitor cells.

CFU and evaluated for four different age groups (< 45, 45–55, 55–65, > 65) did not reveal age-related changes in the chemosensitivities of clonogenic tumor cells (Table 1).

DISCUSSION

According to a recent analysis designed to define the impact of adjuvant chemotherapy on duration of disease-free survival and on survival of patients with breast carcinoma, elderly patients benefit less from these treatments [2]. The reason for this age-related difference in the efficacy of adjuvant chemotherapy is not known, nor is it known why this same treatment is effective in elderly women for treatment of advanced disease but not of early disease [7–9]. Since drug resistance related to the metabolic properties of cells and drug resistance related to the proliferative activity of cells (influenced also by the

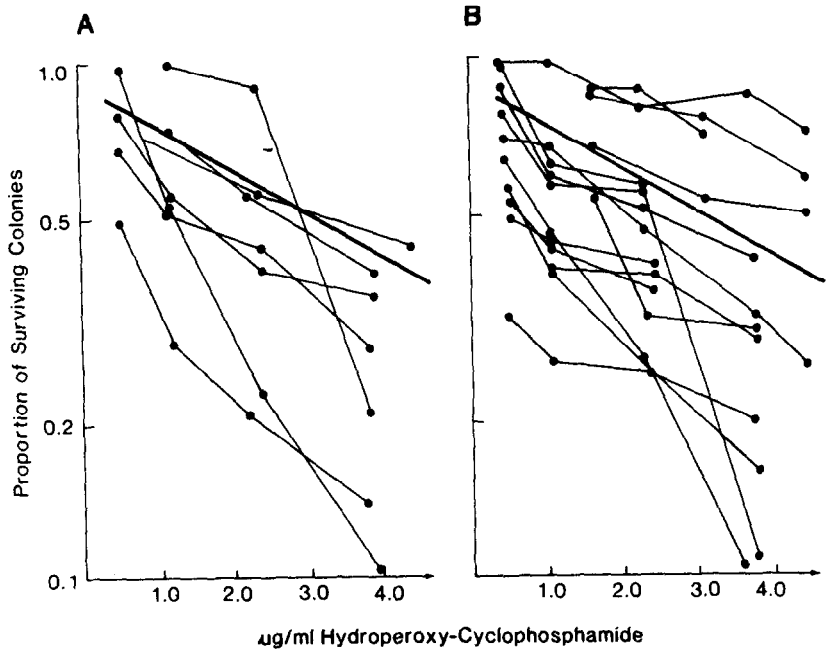

Fig. 3. Dose responses of 22 breast tumors towards 4-hydroxyperoxycyclophosphamide (the active in vitro *metabolite of cyclophosphamide). Seven tumors from premenopausal women (Graph A) were assayed, and 15 tumors from postmenopausal women (Graph B). The log-linear line represents the standard of reference for the effects of 4-hydroxyperoxycyclophosphamide on normal bone marrow progenitors.*

Table 1. Age of patients and chemosensitivity of tumors

	Percentage of surviving tumor progenitor cells following exposure* to							
	Adriamycin		5-Fluorouracil		Cytoxan		Vinblastine	
Age group	Mean (± SD)	Median (range)	Mean (± SD)	Median (range)	Mean (± SD)	Median (range)	Mean (± SD)	Median (range)
< 45	53 (± 22)	55 (18–95)	49 (± 19)	57 (17–71)	56 (± 21)	50 (44–86)	45 (± 17)	41 (24–77)
45–54	51 (± 15)	52 (16–72)	58 (± 7)	61 (48–63)	47 (± 17)	55 (21–57)	46 (± 24)	46 (9–78)
55–64	48 (± 29)	45 (9–83)	41 (± 18)	42 (5–63)	52 (± 20)	50 (26–80)	42 (± 23)	37 (1–73)
> 65	56 (± 9)	56 (49–62)	62 (± 14)	59 (45–88)	53 (± 25)	50 (25–94)	43 (± 21)	42 (14–70)

*LD_{50} for GM-CFU.

microenvironment) are considered frequent causes of chemotherapy failure [3], we examined the clonogenicity, chemosensitivity and hormone sensitivity of tumors from pre- and postmenopausal women.

Our analysis revealed that chemosensitivity, measured under hormone-enriched conditions, remained unchanged for the tumors of all age groups. Because we tested tumors from various sources and from different disease stages, as well as untreated and treated tumors, we cannot conclude with certainty that this is the case also for the primary operable tumors. We can say, however, that tumors from premenopausal women do not develop into metastatic clones that are different in their chemosensitivity from the metastatic clones that develop from tumors of postmenopausal women.

One could argue that our test system was not sensitive enough to discern small differences in the chemosensitivities of tumors. However, we were mainly concerned with drug resistance, and the ability of clonogenic assays to identify drugs that have no cytotoxicity is well-founded. Thus, it appears from our data that drug resistance related to the inherent metabolic properties of tumor cells is not a contributing factor of treatment failure. And it is therefore quite possible that the reduced dose schedules used initially for treatment of elderly patients are, in part, responsible for the apparent ineffectiveness of adjuvant chemotherapy.

In this context, we find our observation that tumors of patients older than 65 years tended to be less clonogenic and more hormone-sensitive (albeit not significantly) than those of all other patients of interest. We have observed previously that low-clonogenic tumors were associated with high degrees of histopathologic differentiation [10], frequent responses to *endocrine treatment* [11] and long survivals of patients [12], while conversely high-clonogenic tumors were associated with low degrees of histopathologic differentiation, frequent responses to *chemotherapy* and short survivals of patients. It is therefore possible that fewer progenitor cells in tumors of patients > 65 years are in active cell cycle, and that as a result these tumors are more resistant towards drugs with cell cycle-dependent activity (which is the case for most drugs in clinical use). Since these low-clonogenic tumors were highly hormone-sensitive, hormones might readily recruit quiescent progenitor cells into the cell cycle. Thus, it is possible that endogenous hormonal changes that occur with the declining endocrine functions of ovarian and adrenal gland may, by reducing the proliferative activity of cells, increase the kinetic type of drug resistance, and thereby contribute to the failure of adjuvant chemotherapy in postmenopausal women.

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